Highly sensitive and selective lateral flow immunoassay based on magnetic nanoparticles for quantitative detection of carcinoembryonic antigen

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Abbreviation: CEA, carcinoembryonic antigen; LFIA, lateral-flow immunoassay; MNPs, magnetic nanoparticles; ssDNA, single-stranded DNA; ECLIA, electrochemiluminescence immunoassay; GMBN, gold magnetic biofunctional nanobeads; MRI, magnetic resonance imaging; AuNPs, gold nanoparticles; LOD, limit of detection; NC membrane, nitrocellulose membrane; cTnI, cardiac troponin I; HFABP, heart fatty acid binding protein; CRP, C-reactive protein; SNR, signal to noise ratio.

Abstract: Carcinoembryonic antigen (CEA) is an important biomarker in cancer diagnosis. Here, we present an efficient, selective lateral-flow immunoassay (LFIA) based on magnetic nanoparticles (MNPs) for in situ sensitive and accurate point-of-care detection of CEA. Signal amplification mechanism involved linking of detection MNPs with signal MNPs through biotin-modified single-stranded DNA (ssDNA) and streptavidin. To verify the effectiveness of this modified LFIA system, the sensitivity and specificity were evaluated. Sensitivity evaluation showed a broad detection range of 0.25 ng/ml–1000 ng/ml for CEA protein by the modified LFIA, and the limit of detection (LOD) of the modified LFIA was 0.25 ng/ml, thus producing significant increase in detection threshold compared with the traditional LFIA. The modified LFIA could selectively recognize CEA in presence of several interfering proteins. In addition, this newly developed assay was applied for quantitative detection of CEA in human serum specimens collected from 10 randomly selected patients. The modified LFIA system detected minimum 0.27 ng/ml of CEA concentration in serum samples. The results were consistent with the clinical data obtained using commercial electrochemiluminescence immunoassay (ECLIA) ($p < 0.01$). In conclusion, the MNPs based LFIA system not only demonstrated enhanced signal to noise ratio, it also detected CEA with higher sensitivity and selectivity, and thus has great potential to be commercially applied as a sensitive tumor marker filtration system.

Keywords: lateral-flow immunoassay; magnetic nanoparticles; carcinoembryonic antigen; diagnosis.

Introduction

Carcinoembryonic antigen (CEA), a type of oncofetal glycoprotein present in cell surfaces, may be crucial in metastatic dissemination of cancer cells [1, 2]. Since CEA is produced in gastrointestinal tissues during embryonic development and stopped after birth, a low level of CEA is found in the blood of healthy adults. CEA has already been validated as a crucial tumor biomarker and ideal diagnostic adjuvant [3] for diagnosis of several types of cancer such as lung cancer [4], colorectal cancer [2], breast cancer [5], and bladder cancer [6], etc. Thus, development of a rapid, efficient method for detecting CEA is in great demand for clinical diagnosis and patient condition evaluation.

The lateral-flow immunoassay (LFIA) or immunochromatography, a rapid and convenient immunodiagnosics tool, has become a hotspot in the past decade. LFIA is based on a solid-phase thin layer that combines the principles of capillary phenomenon and immune recognition reaction [7]. Antibody-labeled nanoparticles, such as colloidal gold [8], superparamagnetic nanoparticles [9], silica nanoparticles [10], quantum dots [11] or other composite materials like gold magnetic biofunctional nanobeads (GMBN) [12], have been used as color constituent. Among the nanoparticles mentioned above, colloidal gold has been widely adopted because of its excellent optical and chemical stability. However, colloidal gold is easily affected by circumstantial factors such as pH value of electrolyte solution. During labeling process of antibody or oligonucleotide probe, non-optimal conditions always cause irreversible coagulation of colloidal gold. In addition, the detection limit of nanogold-based LFIA is yet to be improved. Therefore, other alternatives have been developed overcoming such disadvantages.

To develop stable and sensitive diagnosis method with LFIA, magnetic nanoparticles (MNPs) have been explored as a new nano-biolabel material. MNPs have widely employed in different biomedical purposes such as protein and DNA separation [13], biosensing [14], bacterial detection [15], magnetic resonance imaging (MRI) [16], and drug delivery. The stable physical properties of MNPs make the label process independent of experimental factors such as pH value and salt ions strength.

Additionally, it's harder for labeled MNPs to react with other substances, thus improving reproducibility. All of these factors together have widened applicability of MNPs in the biomedical domain. Apart from good stability and reproducibility, a sensing element should also possess high sensitivity. A few efforts have been made to enhance sensitivity of MNPs-based LFIA. Liu et al [17] prepared magnetic Fe_3O_4 particle aggregates by cross-linking carbonyl mediated Fe_3O_4 nanoparticles with poly-L-lysine for the detection of paraoxon methyl and achieved an optimized detection limit of almost 40-fold lower than that of the mother Fe_3O_4 particles. For more efficient detection in LFIA, other agents, such as composite of MNPs with gold nanoparticles have also been tested to develop a novel immunochromatographic test strip [12]. However, little has been done in the direction of signal enhancement for MNPs-based LFIA.

Our group has already contributed in improving the detection limit of LFIA system. In our previous study [18], we proposed a signal enhancement mechanism for gold nanoparticles (AuNPs) based LFIA, where biotinylated single-stranded DNA (ssDNA) was used to link two AuNPs and streptavidin-labeled AuNPs were used as amplifier. As a result, we not only reached ultrasensitive detection limit but also achieved simultaneous detection of high-sensitivity cardiac troponin I (cTnI) and myoglobin.

In this paper, we present a highly sensitive MNPs based LFIA, combining the amplification strategy described above with the advantages of MNPs. To achieve signal enhancement, we linked secondary MNPs (carboxy group modified) with detection MNPs through biotin-streptavidin interaction. We evaluated the specificity of the new LFIA as well as demonstrated its efficacy by testing clinical samples (human serum samples). In addition, we compared the detection results of serum samples obtained from our newly developed LFIA with commercial electrochemiluminescence immunoassay (ECLIA).

Materials and methods

Reagents

Carboxy group modified MNPs (average size 200 nm) were purchased from Xintong Biotechnology Co., Ltd (Shanghai, China). Anti-CEA mAb₁ and Anti-CEA mAb₂ were purchased from Medix Biochemica (Kauniainen, Finland), and CEA protein was purchased from Fitzgerald (Luminairs, UK). Tumor Marker Plus Control was purchased from Bio-Rad (California, USA). Anti-mouse IgG, bovine serum albumin (BSA), trehalose and 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide (EDC) were purchased from Sigma-Aldrich (St. Louis, USA). Streptavidin was purchased from Invitrogen (Camarillo, USA). The biotin-modified oligonucleotide probes were synthesized by Takara Bio Inc. (Shiga, Japan). The sample pad, conjugate pad, absorbant pad, nitrocellulose (NC) membrane and baseboard were all provided by JieYi Biotechnology Co., Ltd. (Shanghai, China). Tween-20, sodium chloride (NaCl) and sodium tetraborate (Na₂B₄O₇·10H₂O) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Hydrochloric acid and boric acid were bought from Ling Feng Chemical Reagent Co., Ltd (Shanghai, China). Sucrose was purchased from DingGuo Biotechnology Co., Ltd. (Shanghai, China). In addition, Tris-EDTA (TE) buffer was purchased from Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China).

Assay principles of modified LFIA

To enhance the signal intensity of MNPs based LFIA assay, the following approach was adopted. The secondary MNPs were linked to the primary detection MNPs of same sizes through biotin-modified ssDNA. Fig. 1 illustrates a schematic of point-of-care detection of CEA protein. Firstly, the carboxy modified MNPs were labeled with anti-CEA mAb₁ through covalent binding with amino group. Embracing a similar approach, the oligonucleotide probe modified with biotin at 5' end was bound with MNPs through amino group at 3' end. Signal MNPs were coated with streptavidin. These diversely modified MNPs were then coated on two pieces of conjugate pad, respectively (Fig. 1A).

The sensing process is as follows: typically, a solution containing CEA protein is

dropped on the sample pad, and the solution rehydrates streptavidin/MNPs and biotin/ssDNA/MNPs/anti-CEA mAb₁ complexes in turn. Those complexes migrate along NC membrane by capillary action. When the solution reaches the test line containing immobilized anti-CEA mAb₂, CEA protein is finally identified and captured by anti-CEA mAb₁ and anti-CEA mAb₂ via immune responses, forming biotin/ssDNA/MNPs/anti-CEA mAb₁/CEA/anti-CEA mAb₂ compounds. With the addition of signal MNPs, MNPs/streptavidin/biotin/ssDNA/MNPs/anti-CEA mAb₁/CEA/anti-CEA mAb₂ compounds are finally generated and fixed on the test line (Fig. 1B). The rest of MNPs/streptavidin/biotin/ssDNA/MNPs/anti-CEA mAb₁ complexes reach control lines where coated anti-mouse IgG is captured via unspecific conjugation between monoantibody and anti-mouse IgG. Thus, two brown bands can be observed as a result of MNPs accumulation. In absence of antigen in sample solution, only control line will be visible.

[Insert Fig. 1.]

Optimizing LFIA system

Preparation of CEA specific MNP probe

To prepare the detection probe, 0.13 mg magnetic beads were first washed with 0.01 M MES solution, and then the nanoparticles were mixed with 65 µg EDC, 3.9 µg anti-CEA mAb₁, and 13 µl ssDNA (1 µM). After gently mixing, this MNP mixture was incubated at room temperature for 2 h.

To prepare the signal probe, 50 µg EDC and 3 µg streptavidin were added into 0.1 mg magnetic beads, and then the solution was mixed at room temperature for 2 h.

Preparation of sample pad, conjugate pad, and the MNPs based LFIA strips

The conjugate pad was made with treated glass fiber. It was soaked in aqueous solution containing 0.003 M boric acid, 0.004 M sodium perborate, 10% (w/v) sucrose, 2% (w/v) trehalose and 0.05% (v/v) Tween-20 for 30 min. The sample pad, made from treated glass fiber, was obtained by steeping in aqueous solution

containing 0.003 M boric acid, 0.004 M sodium perborate, 0.5% (w/v) sodium chloride, 0.5% (w/v) BSA and 2% (v/v) Tween-20 for 30 min. Both of the pads, conjugate and sample, were dried at a constant temperature of 37 °C for 3 h and stored in a dryer.

The main components of the test strip was consisted of five parts including sample pad, conjugate pad, NC membrane, absorbant pad, and support baseboard. These parts was stacked in a certain sequence (as shown in Fig. 1A) so that ends of the parts were overlapped, thus ensuring smooth liquid flow.

Patients' characteristics

Serum samples of 10 randomly selected individuals, who visited Zhongshan Hospital of Fudan University (either for medical examination or inpatient service) during June 2011 to August 2013, were collected. All specimens were subjected to initial tests by conventional ECLIA. All the samples were obtained with informed consent. This research work was approved by institutional review board of Shanghai Zhongshan Hospital.

Evaluation procedure

The modified LFIA detections were performed for CEA standard antigen, standard serum samples (Tumor Marker Plus Control), and clinical serum specimens. Optical density signals of test line and control line were measured 15 min after the lines developed using Image-Pro Plus 6.0 software. The correlation between CEA concentration and optical density was determined by linear or nonlinear regression, and was adopted as a standard protocol for sample detection appraisalment. Relationships between modified LFIA test and ECLIA were assessed by Sigma Plot 12.0 software (SPSS Inc.).

Detection of CEA with standard antigen sample for modified and traditional LFIA

Standard CEA antigen sample was diluted to obtain various concentrations of

1000, 500, 100, 50, 10, 5, 1, 0.5, 0.25, and 0.05 ng/ml. In order to verify the sensitivity of signal-enhanced system, CEA detection was performed by both enhanced and traditional LFIA. The traditional LFIA was prepared by anti-CEA mAb₁ labeled 200 nm MNPs but without adding signal MNPs. A volume of 60 μ L antigen suspension was dropped upon the sample pad. After 15 min, signals of test lines and control lines were tested by the signal analysis system. Same procedure was performed for newly developed LFIA.

Specificity evaluation

Cross-reactivity would generate nonspecific visible color signals at test lines, contributing to poor judgement of target protein. Thus, it is essential to distinguish biomarkers accurately from human specimens containing complex serum protein composition component. In order to verify the specificity, 5 ng/ml CEA antigen and other various proteins, such as CYFRA21-1, cTnI, myoglobin, heart fatty acid binding protein (HFABP) and C-reactive protein (CRP), were detected by enhanced LFIA system.

Detection of CEA in clinical serum specimens

CEA concentrations in clinical serum specimens were evaluated using our modified method. A standard curve needs to be constructed as a reference for quantitative assessment. To obtain a standard curve, a Tumor Marker Plus Control, with CEA content approximately of 90 ng/ml, was diluted serially and CEA concentrations were measured. Based on these data, accurate CEA concentration present in patients' serum could be calculated. For experimentation, Tumor Marker Plus Control was subjected to 10-fold dilution at least to assure good liquidity.

Comparison of the modified LFIA and ECLIA

Detection results of serum samples by modified LFIA were compared with ECLIA (the traditional clinical analysis routinely performed in Zhongshan Hospital of Fudan University). To evaluate similarity between these two methods, Pearson's

Correlation Coefficient was calculated; and $p < 0.05$ is statistically significant.

Results and discussion

Efficacy of the modified LFIA over the traditional LFIA

To check the efficacy of the newly developed LFIA over the traditional one, we prepared a series of CEA standard antigen solutions of different concentrations (0.25 ng/ml–1000 ng/ml) and compared the detection efficacy of both assays. Fig. 2A shows decrease in the intensity of test line with decreased concentration of CEA while using modified LFIA, implying a change in the quantity of MNPs/streptavidin/biotin/ssDNA/MNPs/anti-CEA mAb₁/CEA/ anti-CEA mAb₂ compounds. In contrast, the color of the control line deepens with decreasing CEA content, indicating a proper coating concentration of MNPs on the conjugate pad. We could not detect antigen concentration beyond 1000 ng/ml accurately, because all the binding sites of mAb₂ immobilized on the membrane were occupied. We attained a naked-eye observation of 0.25 ng/ml with the modified LFIA. Fig. 2B depicts the visual detection limit of traditional LFIA, showing a limit of 5 ng/ml. Therefore, we can infer that the modified LFIA demonstrates much lower detection limit (observed by naked eyes) when compared with the traditional LFIA, confirming remarkable efficiency of biotin-streptavidin system in signal enrichment.

[Insert Fig. 2.]

Establishment of quantitative detection system

We obtained the quantitative detection of the CEA sample by correlating optical density values with concentration. We observed that the optical density values of control lines increased with decreasing CEA concentration, while that of test lines decreased, which was consistent with naked-eye observation. We constructed a standard curve by plotting ratios of optical density values of test and control lines (T/C) against CEA concentration (using CEA standard antigen) and obtained a univariate cubic equation with a significant correlation coefficient as 0.9994 (Fig. 3), indicating feasibility of quantitative assessment.

To judge the limit of detection (LOD) value detected by modified LFIA, we first calculated a critical value based on the following formula:

$$\text{Critical value} = \bar{X} + kS \quad (1)$$

Where $\bar{X}$ refers to average T/C value of blank control, here we use phosphate buffer as negative control. k is a numerical factor ($k=3$) and S is variance of T/C values of the blank control. As a result, the critical value was worked out as 0.71. T/C ratio above 0.71 was defined as positive. From Figs. 2 and 3, concentrations of 0.25 ng/ml or higher could be detected by improved LFIA, and their T/C values were higher than 0.71. Thus LOD for standard CEA antigen detection was 0.25 ng/ml.

[Insert Fig. 3.]

The stable physical and chemical properties of MNPs not only contribute to the improved quantitative assessment of the modified LFIA, the particles, post labeling, barely aggregate or react with other reagents, thus remain homogeneously dispersed over the course of experiment. These properties ensure reproducibility as well as specificity of the amplified detection system. It offers better stability of antibody-MNPs conjugates, and test strips based on MNPs consume less antibody than traditional AuNPs based chromatography [19]. Moreover, it is easier to link amino DNA probes with carboxylic MNPs, and the rate of combination is also higher. In contrast, attaching DNA to AuNPs through Au-S bonding often takes several days using salt-aging protocol. Although a breakthrough functionalization method of AuNPs with thiolated DNA by a pH-assisted process [20] has been proposed, the method is not applicable for the systems dealing with antibody because of the destructive impact of strong acid.

Specificity of the modified LFIA system

In order to verify the specificity of our modified MNPs detection system, we tested and evaluated various biomarkers including CYFRA21-1, cTnI, myoglobin, HFABP, and CRP. For the specificity study, a fixed concentration of 5 ng/ml was used. Fig. 4 shows markedly lower optical density values of CYFRA21-1, cTnI, myoglobin,

HFABP and CRP when compared with CEA protein. This suggests that the test lines hardly capture any amount of interfere protein molecules. Therefore, we can infer that the modified LFIA demonstrates higher specificity.

[Insert Fig. 4.]

Quantitative detection of the standard serum samples

We first constructed a standard curve by plotting T/C optical density and CEA concentration in Tumor Marker Plus Control. Fig. 5 shows a significant mathematical relationship between T/C optical density results and antigen concentration with a correlation coefficient of 0.9991. CEA concentration of 0.27 ng/ml or higher in the Tumor Marker Plus Control could be detected by our improved LFIA system. For the T/C values of 0.27 ng/ml were higher than 0.71, thus we obtained a LOD of 0.27 ng/ml for standard serum samples, slightly higher than that of standard antigen. Utilizing this standard curve, CEA content in clinical serum specimens can be determined.

[Insert Fig. 5.]

Comparison of the modified LFIA and ECLIA

To check the efficacy of the modified LFIA over the standard ECLIA routinely used in hospitals, we tested 10 serum samples collected from randomly selected patients at Zhongshan Hospital of Fudan University. Color development was completed within 15 min, and the test lines showed color promptly for the serum samples containing CEA. We determined the concentration of CEA in the serum samples from patients based on the standard curve established using standard serum samples. Next we compared the values obtained for 10 samples using LFIA with the clinical data obtained using ECLIA. As evident from Fig. 6, the modified LFIA could efficiently detect CEA protein present in human serum, indicating that the newly developed LFIA is sensitive enough to meet clinical detection standards. We calculated Pearson's Correlation Coefficient and found good consistency between LFIA detection system and conventional clinical detection ($p < 0.01$). Therefore, we

can infer that the modified LFIA detection system is capable to meet the requirements of clinical diagnosis.

[Insert Fig. 6.]

We observed that certain serum samples, which were defined as negative in ECLI assay, were detected as weakly positive in LFIA detection. Therefore, we claim that despite the slight deviation between the two methods, a constant error may still assure the reliability of the modified LFIA method. We obtained a cut off value of 5 ng/ml for CEA by ECLI assay and thus, CEA concentration higher than 5 ng/ml is considered excess or above normal, which is often used as diagnostic criteria. CEA concentrations of the serum samples, detected in LFIA as weakly positive, were lower than 5 ng/ml; therefore, they can also be considered normal or healthy. Since the diagnostic results were consistent with the results obtained from ECLI, the slight deviation between the two methods would not interfere with clinical judgment.

Although the newly developed MNPs based LFIA demonstrated high specificity and capability of detecting CEA present in real samples, this detection system showed few limitations such as slight lack of sensitivity and higher background in clinical serum samples. In addition, there are some experimental factors (e.g., viscosity, pH, presence of multiple salt ions, etc.) that limit the applicability of this detection method. Viscosity of serum samples lowers the speed of chromatography, affecting the rehydration of nanoparticles coated on the conjugate pad. Presence of multiple salt ions and fluctuation in pH level could also negatively impact sample detection and lower signal to noise ratio (SNR). Overall, we obtained a LOD of 0.27 ng/ml for human serum samples, satisfying the clinical needs completely.

Conclusions

This article presents an improved LFIA detection system based on signal-enhanced MNPs lateral flow immunoassay. The modified LFIA exhibited better efficacy and sensitivity over the traditional LFIA and achieved a visual LOD of 0.25 ng/ml for standard CEA antigen. Importantly, the detection process could be

completed within 15min. The modified LFIA demonstrated excellent selectivity for CEA protein in presence of various interfering proteins such as CYFRA21-1, cTnI, myoglobin, HFABP, and CRP. Based on the standard curve obtained from plotting optical density values and CEA concentration, we could accurately quantitate CEA present in the clinical serum samples. Moreover, we found a high correlation between amplified LFIA and ECLIA. Our strategy of modifying LFIA using MNPs offered good stability to the detection process. This signal-amplified method not only applies to CEA evaluation, but also to other various disease biomarkers. Considering the conveniences as well as low cost of the newly developed LFIA compared with ECLIA, the former will find more use in biomedicine. Therefore, we can conclude that the newly developed MNPs based LFIA is a credible, rapid and convenient quantitative detection model that might have potential application in clinical diagnosis.

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Figure captions

Fig. 1. Schematic diagram of the newly developed MNPs based lateral flow immunoassay. The detection MNPs are labeled with anti-CEA mAb1 and biotin-modified ssDNA, while the signal MNPs are coated with streptavidin. MNPs/streptavidin/biotin/ssDNA/MNPs/anti-CEA mAb1/CEA/anti-CEA mAb2 compounds are fixed on test lines in the presence of CEA protein.

Fig. 2. CEA sensing with the modified LFIA (A) and traditional LFIA (B). Optical density values decrease on test line with decreasing protein concentration, while optical density values on control line exhibit just the opposite. The developed LFIA demonstrates higher sensitivity than traditional LFIA.

Fig. 3. A linear relationship for quantitative assessment established with R square value of 0.9994 using CEA standard antigen.

Fig. 4. Specificity of the modified LFIA. 5 ng/ml of CEA and interfere proteins were used.

Fig. 5. Color images and signal analysis of standard serum (A); regressive relation curve between T/C optical density values and concentration of CEA antigen (B).

Fig. 6. Serum detection using modified LFIA system and conventional clinical detection ECLIA.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6