Simultaneous Detection of High-Sensitivity Cardiac Troponin I and Myoglobin by Modified Sandwich Lateral Flow Immunoassay: Proof of Principle

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BACKGROUND: Although numerous lateral flow immunoassays (LFIsAs) have been developed and widely used, inadequate analytical sensitivity and the lack of multiple protein detection applications have limited their clinical utility. We developed a new LFIA device for the simultaneous detection of high-sensitivity cardiac troponin I (hs-cTnI) and myoglobin (Myo).

METHODS: We used a gold nanoparticle (AuNP) doubly labeled complex, in which biotinylated single-stranded DNA was used as a linkage to integrate 2 AuNPs and streptavidin-labeled AuNP, as an amplifier to magnify extremely low signals.

RESULTS: The detection limit of 1 ng/L achieved for hs-cTnI was 1000 times lower than that obtained in a conventional LFIA. The detection limit for simultaneously measured Myo was 1 μg/L. The linear measurement ranges for hs-cTnI and Myo were 1–10 000 ng/L and 1–10 000 μg/L, respectively. We observed concordant results between the LFIA and clinical assays in sera from 12 patients with acute myocardial infarction (hs-cTnI r = 0.96; Myo r = 0.98). Assay imprecision was <11% for both hs-TnI and myo.

CONCLUSIONS: The described proof-of-principle LFIA method could be used as a point-of-care device in multiple protein quantification and semiquantitative analysis.

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Acute myocardial infarction (AMI)3 is a major cause of death and disability worldwide. The early exclusion or diagnosis of AMI allows efficient and cost-effective triage as well as the successful management of patients with AMI. Serum high-sensitivity cardiac troponin I (hs-cTnI) and myoglobin (Myo) have been shown to be biomarkers for early detection of AMI (1–3). The simultaneous detection of these 2 biomarkers may help facilitate a diagnosis of AMI within the first 90 min after presentation. However, the rapid detection of protein markers, such as hs-cTnI, remains a challenge.

The simultaneous detection of multiple proteins present in the circulation in widely different concentrations is desirable (4–6). Although Western blotting (7), protein microarray (8), ELISA (9), flow cytometry (10, 11), and quantitative PCR (12, 13) provide relatively specific and sensitive detection, these techniques require sophisticated laboratory facilities (14), highly trained medical technologists (15, 16), and time-consuming procedures (17, 18). These problems can hamper the adoption of proteins analysis in point-of-care (POC) settings.

Lateral flow immunoassay (LFIA), known for its application to the commercially available pregnancy test (19), has the advantage of circumventing these difficulties and inconveniences. LFIA not only provides a means for performing the assay without the extensive handling of specimens, but also accelerates the analytical process to 15 min in most cases. Thus, numerous LFIsAs have been developed and used in clinical medicine (20–22). Two main drawbacks that limit the practical application of LFIsAs in POC settings: (a) limited applications of LFIA to multiple proteins at widely different concentrations and (b) the microgram-per-liter detection limit range (18). For example, the nanogram-per-liter hs-cTnI concentration (23) necessary for the diagnosis of AMI cannot be detected in a conventional LFIA.

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3 Nonstandard abbreviations: AMI, acute myocardial infarction; hs-cTnI, high-sensitivity cardiac troponin I; Myo, myoglobin; POC, point-of-care; LFIA, lateral flow immunoassay; AuNP, gold nanoparticle; ssDNA, single-stranded DNA; mAb, monoclonal antibody; CRP, C-reactive protein; BNP, brain natriuretic peptide; PEG, polyethylene glycol; UV-Vis, ultraviolet/visible.
To overcome these 2 disadvantages of the conventional LFIA, we developed and evaluated a modified sandwich LFIA designed for simultaneous detection of 2 proteins present in circulation at widely different concentrations. Our approach was to combine the detection of hs-cTnI and Myo on a single LFIA device.

**Materials and Methods**

**ASSAY PRINCIPLE**

Our new format used a gold nanoparticle (AuNP) doubly labeled complex, in which biotinylated single-stranded DNA (ssDNA) was used as a linkage to integrate 2 AuNPs, and streptavidin-labeled AuNP (41 nm) was used as an amplifier of small-sized AuNP (13 nm) to magnify extremely low signals. The slow migration speed of a larger-sized AuNP (41 nm) with attached streptavidin ensured its binding, after the initial sandwich of hs-cTnI antigen and antibody was fixed in place (Fig. 1), to the faster-moving AuNP (13 nm) that was coated with anti–hs-cTnI monoclonal antibody (mAb) and conjugated with biotinylated ssDNA.

**MATERIALS**

ssDNA 5’ (SH)-TTTTT TTTTT GGCTT TCAGT TATAT GGATG ATGTG GTATT TTTTT TTT (Biotin)-3’ was synthesized by TaKaRa. We purchased anti–hs-cTnI mAb and standard sample of hs-cTnI from HyTest; anti-Myo mAb and standard samples of Myo and C-reactive protein (CRP) from Fitzgerald; standard sample of brain natriuretic peptide (BNP) from Tocris; BSA, polyvinylpyrrolidone, and goat anti–mouse IgG antibody from Sigma-Aldrich; streptavidin from New England Biolabs; hydrogen tetrachloroaurate trihydrate (HAuCl₄·3H₂O) from Acros; nitrocellulose membrane (HiFlow Plus HFB135) from Millipore; glass fiber (SB06), polyester fiber (VL68), and polyvinyl chloride from Gold-bio; and polyethylene glycol (PEG) (M₉8000) and sucrose from Dingguo Biotechnology.

All other chemicals were of analytical-reagent grade and used without further purification. Deionized and autoclaved water (resistance >18 MΩ·cm) was used throughout the experiments.

Storage buffer solution 1 consisted of 50 mmol/L phosphate sodium buffer (pH 7.8) containing 5% polyvinylpyrrolidone (wt/vol), 1.25% sucrose (wt/vol), 0.05% PEG8000 (wt/vol), 0.2% BSA (wt/vol), and 0.05% Tween-20 (vol/vol). Storage buffer solution 2 consisted of 10 mmol/L sodium borate buffer (pH 7.8) containing 0.05% PEG8000 (wt/vol) and 0.05% Tween-20 (vol/vol).

**CHEMICAL SYNTHESIS OF AuNP**

We prepared AuNP (13 nm in diameter) by trisodium citrate reduction of HAuCl₄·3H₂O, a method pio-
neered by Frens (24) and Turkevich et al. (25). Briefly, all glassware was cleaned with aqua regia (3 parts HCl, 1 part HNO₃), thoroughly rinsed with distilled water, and dried in an air drier before use. Aqueous solution of 0.01% (wt/vol) chloroauric acid and 1% (wt/vol) sodium citrate were prepared. Using an oil-bath thermostat in a 2-neck round-bottomed flask with a magnetic stirrer, 0.01% chloroauric acid solution (100 mL) was heated to boiling, followed by an addition of 3 mL of the 1% sodium citrate solution to the flask, with constant stirring at a maximum speed to synthesize 13 nm AuNP. The reaction time lasted 10 min, during which the color of the solution changed to wine red. The AuNP solution was cooled to room temperature before storage at 4 °C for future use.

AuNP (41 nm in diameter) solution was also prepared by using the same method except that only 1 mL of the 1% sodium citrate solution was added to the 0.01% chloroauric acid solution (100 mL).

The successful formation of 13-nm and 41-nm AuNPs was qualitatively characterized by transmission electron microscopy and ultraviolet/visible (UV-Vis) spectroscopy (Fig. 2).

PREPARATION OF AuNP CONJUGATES
We prepared AuNP labeled with anti–hs-cTnI mAb according to our previously reported procedure (26), but with a few modifications. We adjusted the pH value of the AuNPs (13 nm) to 8.5 with 0.2 mol/L K₂CO₃ dropwise and added 9 μL anti–hs-cTnI mAb (1 g/L) to the 1 mL AuNPs. After a 30-min incubation at room temperature, the AuNPs modified with anti–hs-cTnI mAb reacted with ssDNA at a final concentration of 1 μmol/L for 16 h. We added PEG-8000 to a final concentration of 0.5% to stabilize the AuNPs. We brought the solution to a concentration of 0.1 mol/L NaCl by adding centrifugation at 8603g for 50 min at 4 °C, and 200 μL of storage buffer solution 1 was added to the AuNP conjugate to be resuspended. We repeated the centrifugation and suspension process twice. The precipitate was suspended in 200 μL storage buffer solution 1 and stored at 4 °C for future use.

We prepared anti-Myo mAb–labeled AuNP following the reported method (26), with a few modifications. We adjusted the AuNP solution to pH 8.5 with 0.2 mol/L K₂CO₃ and added 8 μL anti-Myo mAb (1 g/L) to the 1 mL AuNP solution dropwise. The mixture was incubated for 30 min at room temperature, followed by addition of 100 μL of 10% BSA solution to block the residual surface of the AuNP particles. The obtained solution was centrifuged at 15 294g for 25 min at 4 °C, after which the supernatant was discarded and 1 mL of 1% BSA solution was added to the AuNP conjugate to be resuspended. We repeated the centrifugation and suspension process twice, with the precipitate resuspended in 200 μL storage buffer solution 1 and stored at 4 °C for future use.

We prepared streptavidin-labeled AuNP following the reported method (26), with a few modifications.
We used AuNP (41 nm) for the conjugation of streptavidin. We adjusted the AuNP solution to pH 9.3 with 0.2 mol/L K2CO3 and added 10 μL streptavidin (1 g/L) to the 1 mL AuNP solution dropwise. The mixture was incubated for 30 min at room temperature, followed by addition of 100 μL of 1% PEG-8000 solution to block the residual surface of the AuNP particles. The obtained solution was centrifuged at 15,294 g for 25 min at 4 °C, after which the supernatant was discarded and 1 mL storage buffer solution 2 was added to the AuNP conjugate to be resuspended. We repeated the centrifugation and resuspension process twice, with the precipitate resuspended in 200 μL storage buffer solution 2 and stored at 4 °C for future use.

** Pretreatment of Sample Pad, Conjugate Pad, and Nitrocellulose Membrane **

The sample pad, made from glass fiber, was saturated with a buffer (pH 7.4) containing 20 mmol/L sodium borate, 1% (wt/vol) sucrose, 1% (wt/vol) BSA, 0.5% (vol/vol) Tween-20, and 0.05% (wt/vol) NaN3 in water for 1 h before drying in an air drier at 50 °C and stored in a dry state for future use.

The conjugate pad, made from polyester fiber, was immersed in a solution containing 5% (wt/vol) sucrose and 0.05% (wt/vol) NaN3 in water for 1 h and then dried at 50 °C for 2 h. Anti–hs-cTnI mAb–AuNP complex was then mixed together with anti-Myo mAb–AuNP complex in a 1:1 ratio. When 3 μL/strip of AuNP-labeled antibody probe complex was placed onto the polyester fiber to be used as the first conjugate pad, 2 μL/strip of AuNP-labeled streptavidin probe was applied to another polyester fiber as the second conjugate pad. Both pads were dried for 30 min at 37 °C and stored in a dry state for future use.

The nitrocellulose membrane was treated with a buffer (pH 7.4) containing 10 mmol/L PBS solution, 3% (wt/vol) BSA, and 0.5% Tween-20 for 2 h, dried for 2 h at 50 °C, and stored in a dry state for future use.

** Assembly and Analysis of the LFIA **

The strip comprised 5 main elements: a sample pad, 2 conjugate pads, a nitrocellulose membrane, and an absorbent pad. The strip was positioned in such a way that the ends of the elements overlapped, ensuring a continuous flow by capillary action of the developing solution from the sample pad to the absorbent pad (Fig. 1A). Afterward, 1 g/L anti–hs-cTnI capture antibody (test line 1, a), anti-Myo capture antibody (test line 2, b), and goat anti–mouse IgG antibody (control line, c) were each immobilized on the nitrocellulose membrane by a dispenser system. The whole device was subsequently dried overnight at 37 °C and stored in a dry state until use.

We measured hs-cTnI and Myo in sera of 12 patients with AMI by use of the LFIA strips and compared the results to values obtained by a Hitachi Modular 7600 Chemistry Analyzer.

To obtain the quantitative results, after 10 min of color development, the optical images of the dipsticks were captured using a digital scanner (Epson 1640SU) and imported into Quantity One software to be converted automatically to grayscale. The intensities of each signal were then quantified using the Peak Density option in Band Attributes.

** Results and Discussion **

** Assay Procedure **

In this study, AuNPs of 2 different diameters in connection with biotin–streptavidin linkage were used together for the development of an LFIA device to detect 2 model protein analytes, hs-cTnI and Myo, through a modified sandwich strategy (Fig. 1). In the second conjugate pad, part of the AuNPs (13 nm) was coated with anti–hs-cTnI mAb and then with biotinylated ssDNA, whereas another part of the AuNPs (13 nm) was treated with anti-Myo mAb only. In addition, the streptavidin-coated AuNPs (41 nm) were immobilized on the first conjugate pad as an intensifier, designed to bind specifically with the AuNPs (13 nm) labeled with anti–hs-cTnI mAb in the second conjugate pad to enlarge extremely low signals (down to 1 ng/L).

First, we added the solution containing hs-cTnI and Myo onto the sample pad. Next, the solution migrated via capillary action and rehydrated the AuNP–anti–hs-cTnI mAb and AuNP–anti-Myo mAb conjugates, and hence the binding between hs-cTnI and AuNP–anti–hs-cTnI mAb and between Myo and AuNP–anti-Myo mAb occurred via interaction between the antigen and corresponding detection antibody. When the complexes reached test lines 1 and 2, they were captured, respectively, with the capture anti–hs-cTnI mAb and capture anti-Myo mAb immobilized on the distinct test line via the interaction between the antigen and corresponding capture mAb. Meanwhile, the streptavidin-coated AuNP continued to migrate along the strip at a relatively slow speed because of its larger size. When streptavidin-coated AuNP reached test line 1, the binding between biotinylated ssDNA–coated AuNP (13 nm) and streptavidin-coated AuNP (41 nm) occurred via the interaction between biotin and streptavidin. Thus, 2 characteristic red bands were observed for the accumulation of AuNPs in the 2 test lines (Fig. 1B, a and b). The capillary action caused the liquid sample to migrate further. Once the solution passed through the control line, the excess conjugates were captured on the control line via the conjugation between the goat antimouse IgG and either of the 2 superfluous AuNP-coated mAbs, producing a third red
band in the control line, which confirmed the exact performance of the strip (Fig. 1B, c). In the absence of Myo and hs-cTnI, only the red band was observed in the control line rather than in the 2 test lines (Fig. 1B, a and b).

OPTIMIZATION OF THE STREPTAVIDIN/ANTIBODY-AuNP CONJUGATES

The gold aggregation test, which was designed to detect salt-induced AuNP aggregation and determine the optimal streptavidin concentration and pH value for the coupling of streptavidin with AuNP, was carried out by mixing 50 μL AuNP with a series of volumes of K2CO3 and then with 10 μL streptavidin solutions at different concentrations. After incubation for 15 min, 5 μL of 10% NaCl solution was added. Subsequently, the color change and UV-Vis absorption spectrum of the AuNP suspension were analyzed with a UV-Vis spectrophotometer.

The AuNPs showed no aggregation in the streptavidin at a concentration of 8 mg/L (Fig. 3). To stabilize the AuNP, we chose approximately 120% of the minimum unaggregated amount of streptavidin as its working concentration. Moreover, the AuNPs indicated no aggregation in the streptavidin with pH 9.0–9.3 (data not shown). Therefore, in our modified sandwich LFIA test, the final optimal streptavidin concentration was chosen to be 10 mg/L, which was higher than the critical concentration of AuNP aggregation. Meanwhile, the pH value for the conjugation of streptavidin to AuNP was chosen to be 9.0. Anti-Myo mAb–labeled AuNP and anti–hs-cTnI mAb–labeled AuNP were also tested using the same method to validate the optimal concentration and pH value (data not shown). In our approach, the final optimal anti-Myo mAb concentration for the conjugation of anti–hs-cTnI mAb to AuNP (pH 8.5) was chosen to be 8 mg/L, and the final optimal anti–hs-cTnI mAb concentration for the conjugation of anti–hs-cTnI mAb to AuNP (pH 8.5) was chosen to be 9 mg/L.

VISUAL ASSESSMENT OF ANALYTICAL SENSITIVITY OF LFIA

It is essential for each analyte from the solution to accurately conjugate only with the specific capture antibody, which is immobilized on the corresponding test line of the strip. Cross-reactivity, if present, would generate nonspecific signals at various test lines, leading to false-positive results. To investigate whether there was any cross-reactivity of the immobilized capture antibody, 3 standard solutions, 2 containing each of the tested analytes and the third containing combined CRP and BNP solutions, were subjected to the testing. The results demonstrated the specificity of the assay (Fig. 4A), suggesting that hs-cTnI (Fig. 4A-1) and Myo (Fig. 4A-2) were captured only by the corresponding test line and not the other, and that 50 μg/L CRP and 50 μg/L BNP (Fig. 4A-3) produced no observable binding in either test line. Thus, it is reasonable to expect that there is no detectable cross-reactivity at the position that corresponds to the missing analytes.

To investigate whether the LFIA could provide simultaneous and semiquantitative detection of Myo and hs-cTnI, the assay was performed using the standard samples with a series of known concentrations of hs-cTnI and Myo. In these experiments, 1 of the analyte concentrations was kept constant and the other analyte concentration was adjusted to validate the dynamic range for each analyte when measured in our modified sandwich format. The hs-cTnI assay performance was carried out with the Myo concentration held constant at 10 μg/L (Fig. 4B). Consequently, a distinct red line was observed in test line 1, and the color intensity of the hs-cTnI line increased gradually, corresponding to increasing hs-cTnI concentrations of 1, 10, 100, 1000, and 10 000 ng/L hs-cTnI. In this study, the detection of hs-cTnI in the modified sandwich LFIA was about 1000-fold higher compared with that of the conventional LFIA format, which was mainly attributed to the application of biotin-streptavidin linkage and the aggregation of 2 different diameters of AuNPs in the test line. The conventional format detecting only hs-cTnI without any signal amplification seems to show lower detectability. The dose–response curve spanned orders of magnitude beyond the suggested clinically relevant ranges (Fig. 4D). The error bars (Fig. 4D) represent SDs, calculated from triplicate experiments performed on different LFIA. In comparison with the conventional format, our new approach produced no differences in Myo detectability, in which Myo produced gradual concentrations of 1, 10, 100, 1000, and 10 000...
μg/L, each maintaining a constant hs-cTnI concentration of 10 ng/L (Fig. 4E).

To document whether the LFIA can work using serum specimens, hs-TnI and Myo were assessed in serum samples from 12 patients with AMI. In addition, the 2 analytes were measured using a standard laboratory instrument (Hitachi Modular 7600 chemistry analyzer). The serum samples had hs-cTnI and Myo concentrations in the range of 9.2–13 900 ng/L and 5.9–2771 μg/L, respectively. The results showed that the modified LFIA had concordant trends reflecting the concentrations of hs-cTnI ($r = 0.96$) and Myo ($r = 0.98$). Finally, we tested 2 different serum samples with high (1038 ng/L) and low (16 ng/L) concentrations of hs-cTnI 10 times to evaluate imprecision. The concentrations of Myo in these 2 samples were 25.6 μg/L and 132 μg/L. The imprecision was <11% for the 2 analytes at both concentrations.

In summary, in this proof of principle, our modified sandwich LFIA method may be an attractive approach for rapid POC detection of multiple protein analytes. In addition, our approach can provide a higher detectability for the analytes, at extremely low concentrations, that are difficult to detect via the conventional LFIA.

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Fig. 4. (A), Specificity of the capture lines of the LFIA. (B), LFIA assessing the detection of varying hs-cTnI concentrations with constant Myo. (C), LFIA assessing the detection of varying Myo concentrations with constant hs-cTnI. (D), Dose–response curves of varying hs-cTnI concentrations with constant Myo. (E), Dose–response curves of varying Myo concentrations with constant hs-cTnI. AU, arbitrary units.
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