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Application of Nanogold Probe Coupled with Silver Enhancement in Rapid cTnI Colorimetric Immunoassay

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Abstract: A rapid, inexpensive, reliable, and flexible quantitative immunoassay for cardiac troponin I (cTnI) based on the concepts of one-step dual monoclonal antibody “sandwich” principle. The low density protein array, the nanogold probe, and the silver enhancement on the gold particle were provided. The whole detection procedure of the assay could be fulfilled within 40 min with the pretreated colloidal gold-labeled detection antibody and supporting substrate. The assay showed good specific response to cTnI with very low cross-reactivity ratio to the skeletal isoforms of troponin I (sTnI), cardiac troponin T (cTnT), and myoglobin. 588 serum samples were assayed simultaneously by enzyme-linked immuno sorbent assay (ELISA) and this colloidal gold method to test the validity of the method and the data were analyzed using the statistical package SPSS version 11.0 (SPSS Inc.). There was no significant difference between these two assays ($P=0.66>0.05$). The agreement between this method ($\geq$ or <0.3 ng/mL) and ELISA was 86%.

Keywords: Cardiac troponin I, protein array, nanogold particles, silver enhancement, rapid quantitative immunoassay.

Cardiac troponin I (cTnI) in the serum of patients has been established as a principal biochemical marker of myocardial damage[1-4]. Fast detection of cTnI will be helpful for the diagnosis of acute myocardial infarction (AMI), the identification of myocardial injury (MI) and the stratification of cardiac risk. A number of serological tests have been developed for the detection of cTnI. As known, among many methods, enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA) were most commonly used, but antibodies labeled with horseradish peroxidase (HRP) or ^{125}I were needed in these approaches[5-8]. These tests are quite sensitive and specific, and have been proved very useful in the diagnosis of AMI. However, they are all time-consuming and require proper instruments and trained personnel. In this study we provide a rapid, inexpensive, reliable, and flexible quantitative immunoassay for cTnI. The test is based on the concepts of one-step dual monoclonal antibody “sandwich” principle. The low density protein array, the nanogold probe, and the silver

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enhancement on the gold particle were provided.

A pair of antibodies that recognize different epitopes of cTnI was used to capture and detected certain antigen. These two monoclonal anti-human cTnI antibodies and cardiac troponin I from human myocardium muscle used in the assay were obtained from Research Institute of Cardiovascular Disease of First Affiliated Hospital of Nanjing Medical University. Colloidal gold was purchased from Sino-American Biotechnology Company. Other reagents were commercially available and were all of analytical reagent grade.

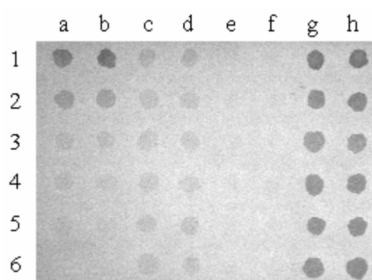
To fulfill the fast detection of cTnI in serum, two main substrates, namely the colloidal gold-labeled detection antibody (cAu-IgG_2) and the pretreated supporting membrane, should be prepared in advance. The cAu-IgG_2 was prepared as follows. An appropriate amount of IgG_2 and colloidal gold (pH 8.2) were mixed up at room temperature. After 15 min incubation, a bovine serum albumin (BSA) solution was added into this stirred mixture to a final concentration of 1%, and 15 min more incubation was required. Then the mixture was centrifuged in the cold at 300 ×g for 20 min and the pellet was discarded. The supernatant was centrifuged in the cold at 10000 ×g for 1 h and the pellet of the cAu-IgG_2 complex was resuspended in 5 mL of 0.05 mol/L tris(hydroxymethyl)aminomethane (Tris)-HC1 buffer solution (TB, pH 8.2) containing 0.1% BSA. Finally, the mixture was centrifuged in the cold at 300 ×g for 20 min, the supernatant was harvested and stored under 4℃ for further use. TEM images showed good monodisperse suspension with average particle diameter of 15 nm. Another substrate, the supporting membrane was pretreated according to the following procedure. A suitable size (*e.g.*, 4 cm×4 cm) of cellulose nitrate membrane (Bio-Rad) was cut out and soaked in a 30% glycerol solution for 1 min. After the aspiration of the water, 2 μL of the individual capture antibody (IgG_1) at a concentration of 0.60 mg/mL was spotted onto the membrane and deposited in a humidity chamber for 2 h to prepare the array. Unbounded IgG_1s were washed away with 0.05 mol/L TBST (a TB solution containing 0.5% Tween 80 and 10% NaCl). Arrays were then blocked with a 2% gelatin solution at room temperature, washed with TB and aspirated. Both of the cAu-IgG_2 conjugates and the pretreated supporting membrane could be preserved for further use at least for 3 months in a 4℃ refrigerator.

The analytical procedure was as follows. 10 μL of serum sample and 5 μL of cAu-IgG_2 were spotted, followed by incubation for 20 min in TBST. After a stringent wash with deionized water, the arrays were immersed in the silver amplification solution for 10 min and washed with deionized water again. Finally, the array image was recorded with a flatbed scanner or judged with the naked eye (**Figure 1**). The whole process was finished in about 40 min. Actually, it was a rapid detection for cTnI.

The optimal experiment conditions were obtained by single factor transformation, as follows. A Tris-HCl buffer solution of pH 8.2 was used throughout the experiment. 0.60 mg/mL spotted capture antibody was employed. Incubation for 2 h at 37℃ was selected to bind the IgG_1 to the membrane. A 2% gelatin solution was selected as the blocking reagent and 2 h at room temperature or, alternatively, 12 h at 4 ℃ was used to block the unconjugated element on the membrane. Immunoreaction environment has a significant influence on the assay performance. Different immunologic reaction

medium

Figure 1 The gold-silver detection image of different concentration of cTnI



1a, 1b: 4.8 ng/mL; 2a, 2b: 2.4 ng/mL; 3a, 3b: 1.2 ng/mL; 4a, 4b: 0.8 ng/mL; 5a, 5b: 0.4 ng/mL; 6a, 6b: 0 ng/mL; column c, d: 1st positive control (2.0 ng/mL); column e, f: negative control; column g, h: 2nd positive control (5.0 ng/mL).

conditions were examined, including TB, TB containing Tween 80, TB containing NaCl, and TB containing both Tween 80 and NaCl. The immunologic reaction under an ambient medium containing 0.5% Tween 80 and 10% NaCl showed the best results. It could be interpreted by the fact that the coexistence of an appropriate concentration of the surfactant and the salt prevented most of the nonspecific binding during the incubation, while it had little effect on the specific immunologic reaction. The most suitable immunologic reaction time was investigated as well. Considering both sensitivity and rapidness, an immunologic reaction time of 20 min was selected. The silver enhancement time had been optimized to obtain the highest detectable sensitivity and a good signal/noise ratio. In our experiment, it was found that the optimal silver enhancement time was from 10 to 15 min.

The sensitivity and specificity of the method have been examined by the analysis of serum samples of patients, using the detection results obtained from the enzyme-linked immunoadsorbent assay (ELISA) as a reference standard. The assay showed good specific response to cTnI. No cross-reactivity was observed with the skeletal isoforms of troponin I (sTnI), cardiac troponin T (cTnT), and myoglobin up to the concentration of 30 μg/mL, 0.2 μg/mL, and 100 μg/mL, respectively. No hook effect was observed when the concentration of cTnI was up to 168 ng/mL in the serum. It means false negative results could be avoided at high cTnI concentration level. A reference control group (200 normal serum samples) was used to determine the cut-off value of the method according to the ESC/ACC documents[1] and a cut-off level of cTnI in the serum of 0.3 ng/mL was obtained. To ensure the performance of this method the tests were performed on a double blind basis. Serum samples were judged with this cut-off value using ELISA as a comparison analysis method. Totally 588 serum samples were assayed simultaneously by ELISA and this colloidal gold method to test the validity of the method. There was no significant difference between these two assays ($P= 0.66>0.05$). The agreement between this method (≥ or <0.3 ng/mL) and ELISA in 588 serum samples was 86%.

From all the above results, we come to the conclusion that a rapid, economical, quantitative immunoassay for cTnI has been provided by this method. The assay needs only small amounts of serum samples of patients. The whole detection procedure of the assay could be fulfilled within 40 min with the pretreated colloidal gold-labeled detection antibody and supporting substrate (much faster than the traditional ELISA which takes usually at least 3 hours for a turnaround test). It can be expected to have good prospect in real clinical applications. The assay would be more timesaving with direct plasma sample. The detailed investigation of this method and its application will be reported elsewhere.

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