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PDMS gold nanoparticle composite film-based silver enhanced colorimetric detection of cardiac troponin I

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ABSTRACT

This paper described poly(dimethylsiloxane) (PDMS)-gold nanoparticles (AuNPs) composite film as basis with silver enhancement for colorimetric detection of cardiac troponin I (cTnI). Stable PDMS-AuNPs composite film was simply prepared with dropping $HAuCl_4$ solution in PDMS microchip, gold nanoparticles were both used as nuclei for silver enhancement and as basis for adsorption of antibody, immunoassay was implemented after BSA blocking. Because of the inhibition ability difference for silver enhancement between BSA and antibody-antigen complex, darkness of silver deposition was relative to the amount of antigen. The proposed colorimetric method was applied for clinical sample cTnI detection, results were consistent with that from enzyme-linked immunosorbent assay (ELISA). The composite film could be coupled with digital transmission of images for remote monitoring system in diagnosis, food control, and environmental analysis.

1. Introduction

Among various types of sensors, the development of biosensor is a significant technology breakthrough. The concept of biosensors firstly came from description of enzyme electrode given by Clark [1], and a biosensor makes good use of natural biological selectivity to recognize one component in a complex mixture, such as unique interaction between biological macromolecules (e.g. antibodies, enzymes, receptors and ion channel proteins, nucleic acids, aptamers and peptide nucleic acids) [2,3]. Making use of diversified signals produced by bio-reactions (photo effect, pyrometric effect, field effect as well as change in quality), scientists can make various precise device for cell biology, clinical diagnostics, drug delivery, environmental monitoring.

There is widespread interest in the development of cost-effective, practical diagnostic tools that are amenable to rapid screening of specific target analytes in the health sector, food industry, and the environment [4,5]. A number of materials have already been used for colorimetry, such as silicon chip [6], glass surfaces [7], gold electrodes [8]. Paper, being relatively cheap and abundant, sustainable, disposable, and easy to use, store, transport, and modify, has been the focus of significant attention as a platform for the development of paper-based analytical devices [9–13]. Poly(dimethylsiloxane) (PDMS), is one of the most utilized polymeric material employed in fabricating microfluidic devices, the surface of PDMS can be chemically modified or physically masked by adsorption. Anionic, neutral, and cationic surface can be generated.

It is known to all that myocardial infarction (MI) is a leading cause of death. Serum cardiac troponin I (cTnI), being called gold mark, is the most specific marker of cardiac injury currently. When myocytes die, their cellular contents are dispersed in the blood, and cTnI has a molecular mass of approximately 24 kDa which can release rapidly from the injured myocardium [14]. Compared to other biomarkers, for example creatine kinase (CK), cTnI is cardiac-specific that permit discrimination of cardiac damage from damage to skeletal muscle or other organs [15].

Previously many methods have been used for cTnI detection and quantification, such as immunoenzymometric assays (ELISA) [16], chemiluminescent immunoassays [17], fluoro-immunoassays [18], electrical detections [19], surface plasmon resonance detection (SPR) [20], colorimetric protein array [21] and so on. In order to meet the increasing demand of quick diagnosis and clinical therapeutics, we are in dire need of a certain device that has small size and weight, fast response time, high sensitivity, stable characteristics and more importantly ease of operation and fabrication.

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We developed a PDMS-gold nanoparticles composite film-based biosensor, which is convenient to fabricate [22], and without layer-to-layer assembly on the surface. Also, this PDMS-AuNPs composite film is stable which has long shelf-life of several months if stored at 4 °C. Furthermore, the reagent used in each well is only 15 μL or less, which is much more less than usual ELISA. To combine the advantages mentioned above, this small device can be put into commercial process and extensively used in clinical diagnosis. Then we take advantage of this simple device to form a label-free immunosensor coupled with silver enhancement to colorimetrically detect cTnI which is a specific cardiac marker in patients' blood serum who are suffering from coronary artery disease (CAD) like myocardial infraction (MI). The results showed a high sensitivity and low detection limit, while the process costs less time than ELISA and other assays. We believe that, this optical detection coupled with simple and stable material, and going through less reagent and time-consuming process, may have great potential acting as a start point for a diagnostic system.

2. Experimental

2.1. Reagents, materials and equipments

Rabbit anti-human IgG and human serum IgG was purchased from Beijing Biosynthesis Biotechnology Company (Beijing, China), bovine serum albumin (BSA) was purchased from Roche company, silver enhancement solutions A and B were both obtained from Sigma–Aldrich (St. Louis, MO, USA), and $HAuCl_4{\cdot}4H_2O$ was from Shanghai Chemical Reagent Company (Shanghai, China). cTnI, monoclonal antibody against cTnI and patients' serum samples were all provided by The First Affiliated Hospital of Nanjing Medical University. PDMS was obtained from Dow Corning (midland, MI, USA). Two-dimensional gel electrophoresis was purchased from Bio-Rad. All the other reagents were of analytical grade, and deionized water was used throughout.

2.2. Methods

2.2.1. Fabrication of PDMS-AuNPs composite film

PDMS-AuNPs composite film was prepared as the described procedure elsewhere [22]. Briefly, PDMS monomer and the curing agent were firstly mixed in a proportion of 1:0.06 and cured at 90 °C for 20 min. A 3-mm-thick PDMS film with a circular opening of 4-mm-diameter was formed from a predefined mold and was bound to the flat PDMS surface to form the antibody–antigen (Ab–Ag) reactor. $HAuCl_4$ 0.01 g/mL were dropped into each microreactor and incubated at 37 °C for 48 h. All resulting chips were washed with deionized water for three times, and stored at 4 °C when not in use.

2.2.2. Preparation of the immunosensor

For model protein of IgG, Rabbit anti-human IgG was immobilized on the PDMS-AuNPs composite film after they were rinsed and dried with N_2, as shown in Fig. 1(c). A 15-μL aliquot of 0.05 mg/mL anti-human IgG solution (10 mM PBS, pH 7.38) was dropped into the wells. The chips were incubated at 4 °C in a moisture chamber overnight (12 h). After incubation, they were rinsed with buffer (50 mM PBS, pH 7.38, 0.05% Tween 20) to remove physically adsorbed antibody. Then deionized water was used to rinse for three times. Finally the chip was dried with N_2. After the rinsing process, the chip was incubated with blocking solution (BSA or skimmed milk) at 37 °C for 1 h and rinsed with buffer, deionized water, at last dried with N_2. After the blocking step, the antibody-modified chip was incubated with 15 μL of detecting human IgG samples for 1 h at 37 °C.

For cTnI detection, the monoclonal antibody against cTnI was firstly immobilized on the PDMS-AuNPs composite film, followed by blocking solution and cTnI under the same procedure as the model protein.

2.2.3. Colorimetric detection

After rinsing process, 15 μL silver enhancement solution (mixture of two solutions from the Silver Enhancer Kit in a 1:1 volume ratio prepared just prior to use) was dropped into each well, the chip was incubated in dark for 20 min, and then we capture the live photos of the micro array using ordinary digital camera. In our experimental, camera and parameters are as follows: Olympus FE-210 digital camera with CCD sensor (Tokyo, Japan), maximum resolution 3072 × 2304 pixels, programmed auto exposure without flash light, the object distance is 30–40 cm.

These photos were imported into the computer and color differences were analyzed by image software Quantity One (Bio-Rad company). Darkness density of each well was picked to describe color difference among the volume data in Volume Analysis Report.

3. Results and discussion

3.1. Mechanism of gold basis silver enhancement immunosensor

AuNPs could catalyze silver reduction and act as the nuclei for silver precipitation. Many researches were carried out using AuNPs labeled antibody as the detection sensor, then coupled with silver metal precipitation on the surface to enhance signals [23]. Although the mechanism of AuNPs-silver enhancement method has been already mature, disadvantages still existed during the experimental procedures. AuNPs in liquid system were extremely easy to aggregate, so do the proteins labeled with them. Therefore, aggregation, together with the complicated and time-consuming procedures of sandwich assay, has become the common bottleneck during the AuNPs-silver enhancement immune assay. However, Yang and Wang [24] had reported a label-free immunosensor without sandwich assay. They immobilized AuNPs firstly onto polycarbonate (PC) films and then covered with antibodies, after blocking with BSA and capturing antigens they dropped silver enhancement solution onto the film and took live photos. In our work, the core mechanism of our novel method is that AuNPs play a role of catalyst during reactions of silver reduction, and this catalytic ability could be inhibited when there were proteins covering the surface of AuNPs, which influenced the amount of reduction silver metal and led to the color difference of the reaction wells. Most important of all, this inhibition effect could be distinct due to different species, quality and/or quantity of covering proteins (see Fig. 2), after the dropping of silver enhancement solution and incubation in dark for 18 min, we took the live photos using an ordinary digital camera. Under illumination, well A appeared to be totally black because the AuNPs could greatly catalyze the reduction of the silver ions. On the contrary, in well B, BSA was introduced to block the active spot and the light intensity increased significantly. The reason was that BSA plays the role of both occupying the active spot and inhibiting the catalytic capability of the AuNPs. After immobilized with antibody and then blocked with BSA, well C become darker than well B but lighter than well A. The phenomena was caused by the different inhibition between BSA and IgG, in other words, BSA has a stronger ability than rabbit anti-human IgG for preventing AuNPs from catalyzing silver reduction. For well D, the color was a lot lighter than C due to the formation of the immune-complex after the immobilization of antigen. From the results of Fig. 2, we could draw a conclusion that different proteins have distinct inhibiting ability in this PDMS-AuNPs composite film-based silver enhancement system.

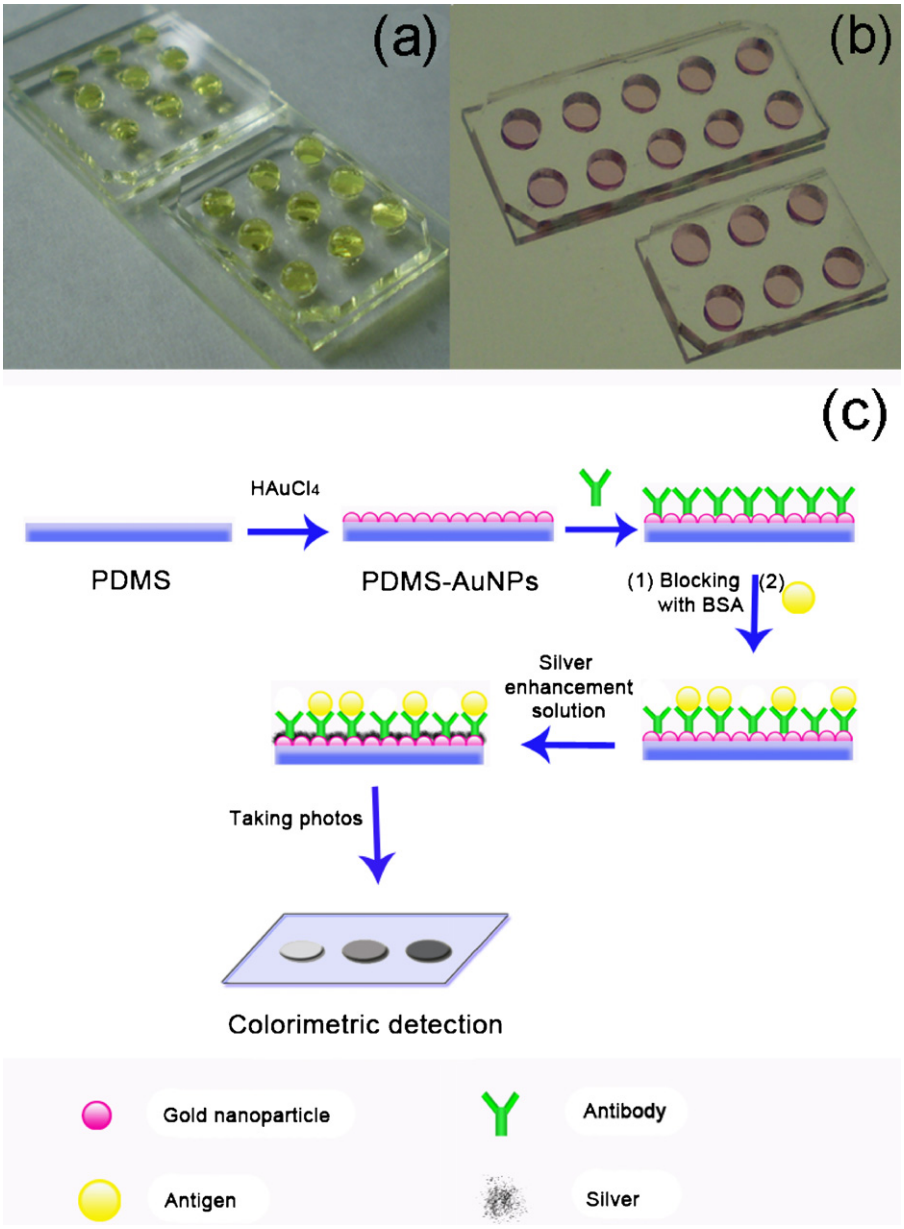

Fig. 1. Experimental procedure for silver enhancement colorimetric detection of cardiac troponin I; (a) is the PDMS chip with $HAuCl_4$ solution; (b) is the photo of PDMS-AuNPs composite film; (c) schematic diagram for colorimetric detection.

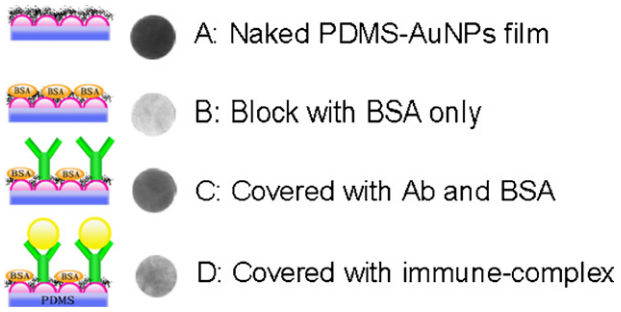

Fig. 2. Chromatic aberration of different covering components after silver enhancement for 18.5 min. Well A was the naked PDMS-AuNPs film, well B was immobilized with BSA only, well C was covered with antibody and then BSA blocking, well D is assembled with immune-complex (antibody, BSA and finally antigen of 1 ng/mL).

3.2. Stability of PDMS-AuNPs composite film

PDMS is one of the most widely used polymer materials for fabricating microfluidic chips because of its excellent transparency, outstanding elasticity, good thermal and oxidative stability, and ease of fabrication and sealed with various materials. Moreover, it is well known that gold nanoparticles could perform as a good substrate to be functionalized with antigen, enzymes and other biomolecules. Therefore, AuNPs patterned on PDMS film surely have the application potential in immunoassays. Zhang et al. [22] had reported a method of in situ synthesis of PDMS-AuNPs composite films, which used only $HAuCl_4$ and PDMS matrix to achieve a well-patterned film. This material has plenty of advantages. Firstly the polymer matrix of PDMS could protect AuNPs from aggregation, so this PDMS-AuNPs composite film could be well stored at 4 °C for several months which enhanced the stability and prolong shelf-life of the device. Besides, as the procedure of the film fabrication was very simple, this sensing device could be extensively produced on

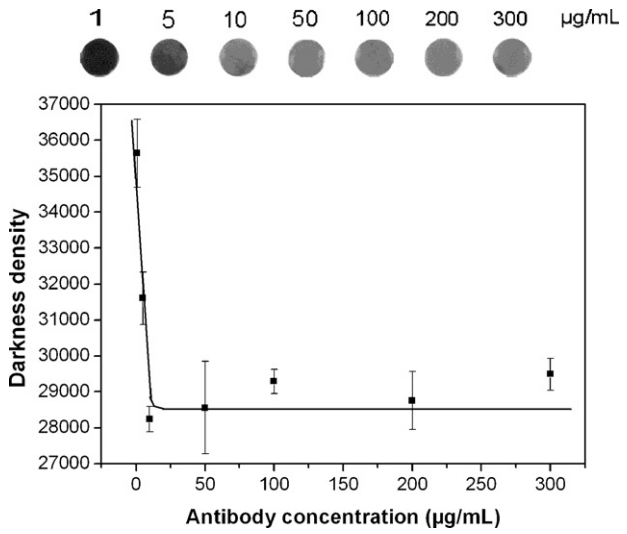

Fig. 3. Darkness density versus concentration of coating antibody for detection of 1 ng/mL antigen.

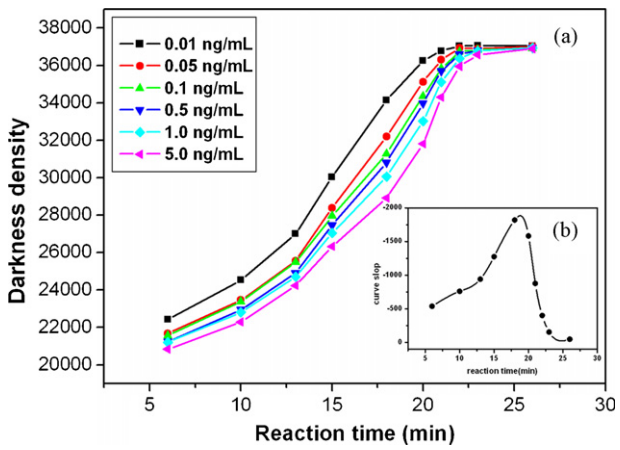

Fig. 4. (a) Darkness density versus silver enhancement time for different Human IgG concentrations; (b) optimization of silver enhancement time.

production line. Moreover, we have fabricated a microarray chip using this in situ method, which is small in volume, convenient to carry away and portable for immediate usage for quick diagnosis and point of care.

In order to investigate the stability of the film, the films were stored in fridge at 4 °C for 2 months, which were coated with 0.05 mg/mL antibody and then 1 ng/mL antigen. We found the PDMS-AuNPs composite film could retain activity all the same.

3.3. Antibody concentration in coating buffer

Antibody immobilization capacity of this composite film should be considered. The concentration of antibody in coating buffer affected the sensitivity of the detection greatly. For the detection of 1 ng/mL IgG, coating antibody concentration ranging from 10^{-3} to 0.3 mg/mL was investigated. The result (see Fig. 3) showed that when antibody concentration increased from 10^{-3} to 10^{-2} mg/mL, optical darkness density (DD) decreased correspondingly, yet DD did not change as further increasing of the antibody concentration from 0.05 to 0.3 mg/mL. The results showed that higher concentration of antibodies led to more immobilization onto the AuNPs surface, then forming a better "compact" film to inhibit silver reduction from catalysis of AuNPs. When the surface of AuNPs was saturated with antibodies (at a concentration of 0.01 mg/mL), the amount of the immune-complex stayed unchanging and the DD was a constant. As a result, we select 10 μg/mL antibody for the further research, compared to other methods such as ELISA which usually need the reagent volume of 100–200 μL for each reactor, and 0.1–0.3 mg/mL for the concentration of antibody [25,26]. This biocompatible composite film could largely save the reagent of antibody for only 15 μL in volume and 0.01 mg/mL in concentration for each well.

3.4. Optimal silver enhancement time

Once the silver enhancement solution was dropped into each well, silver metal was reduced and deposited on the surface of the composite film. At the beginning, little silver metal was reduced, so the DD value was low. As time went on, more silver metal precipitated on the surface of the film which made the color turn darker (see Fig. 4(a)). The result indicated that the DD increased along with silver enhancement time, each curve represented a certain concentration of Human antigen (the antigen concentration for each well: from 10^{-5} to 5×10^{-3} μg/mL). As a result of interwork between AuNPs acceleration mechanism and the immune-complex inhibition effect, we could achieve the highest sensitivity at a certain point of time during the whole silver enhancement period. After that point, due to the factors of both self-reaction and catalysis mechanism, silver enhancement reaction will be gradually completed, which made all the wells reach the same DD value finally (after 22.5 min). We took a series of photos during the silver-reduction period, and got the slop values of calibration curve for each time point. According to Fig. 4(b), 18.5 min was selected as the detection point.

3.5. Relationship between darkness and antigen concentration

As the relationship between the concentration of antigen and the DD value was established, we took real time photos using the ordinary digital camera at 18.5 min after the silver enhancement solution was added. After analyzed the live photos by image software, we could get clear results shown in Fig. 5. Fig. 5(a) showed that the DD value changed along with antigen concentration from 10^{-5} to 10^{2} μg/mL. The DD value of the well in the absence of IgG was higher than that in the presence of IgG, and it decreased gradually with the increasing concentrations of IgG from 10^{-5} to 10^{-1} μg/mL. The reason was that the higher concentration of IgG was in the well, the more compact immune-complex was formed on the AuNPs surface. Because of the inhibition effect of immune-complex, the less contact between the AuNPs and silver enhancement solution was, the lower silver-reduction speed was. When concentration of IgG kept increasing from 10^{-2} μg/mL, we found an apparent increase of the DD value. The reason is that in the presence of excessive antigen, binding rate of antigen and antibody was decreased and led to less immuno-complex formation consequently [27], the binding efficiency of antigen and antibody was strictly confined by the concentration difference, a much higher binding rate could be attained when the concentrations of antigen and antibody were relative consistent. The plot in Fig. 5(b) showed that DD level of the wells is linear to the logarithm of the antigen concentrations in a range from 10^{-5} to 10^{-2} μg/mL and the detection limit is 10^{-5} μg/mL. The result indicated the colorimetric detection method has a large linear detection range (about five orders of magnitude) for the components in the solution system, as well as a low detection limit of 10^{-5} μg/mL. Therefore, it counted much for practical application in quantitative analysis.

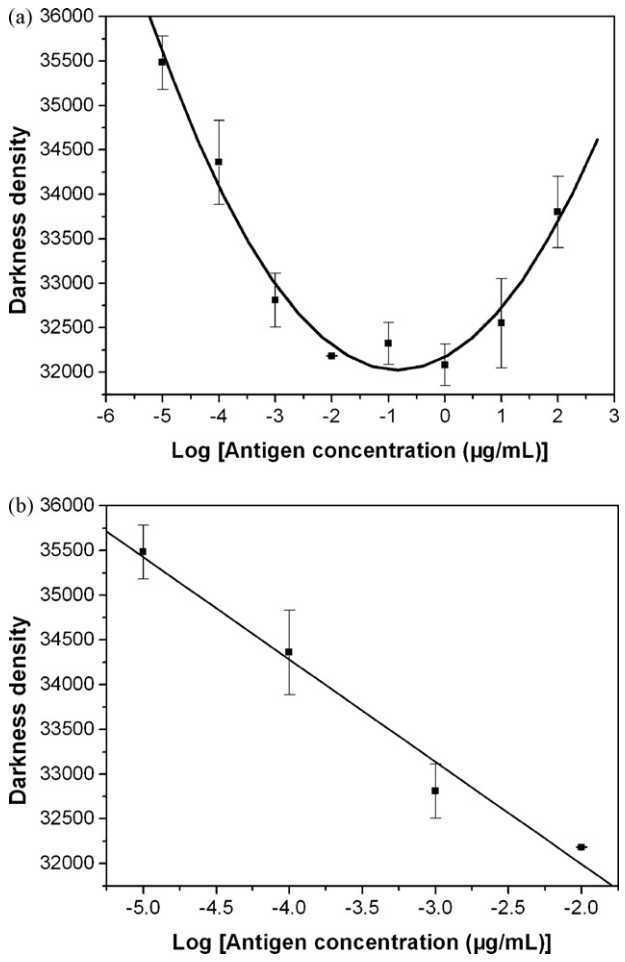

Fig. 5. Relationship between darkness and antigen concentration: (a) darkness density versus antigen concentration from 10^{-5} to 100 μg/mL; (b) Darkness density as function of the logarithm of antigen concentration from 10^{-5} to 10^{-2} μg/mL.

3.6. Application of the colorimetric approach in human serum for cTnI detection

It is of great importance to the development of sensing devices for point-of-care applications that the sensor is able to quick and accurately detect biomarkers in human serum. Devices for the detection of cTnI in human blood serum were therefore largely fabricated and fast developed. The cTnI concentration in healthy people's blood is less than 0.3 ng/mL [28], when myocardial cells were broken due to lack of oxygen or blood, the dissociative cTnI molecules quickly went through the epicyte and spread in the circulation of blood. Along with the cardiac injury getting worse, the bound cTnI molecules were decomposed and continuously went into blood leading directly to the sustainable growth of cTnI content [29]. For our novel sensor device, the possibility of using this composite material and method for clinical applications was investigated by detecting not only cTnI in buffer solution, but also cTnI in human serum.

To develop a PDMS-AuNPs composite film-based biosensor array and make it feasible for specific detection of cTnI in real serum samples, purified cTnI was applied as calibration. Fig. 6 (*X* axis represents logarithm of concentrations from 0.01 to 5 ng/mL: 0.01, 0.05, 0.1, 0.5, 1, 5 ng/mL, and *Y* axis represents the darkness density) showed that the relationship between DD value and the concentration of cTnI was established. In order to make the serums under the same environmental conditions as standard samples, we diluted serums (from the centrifuged raw human blood) with

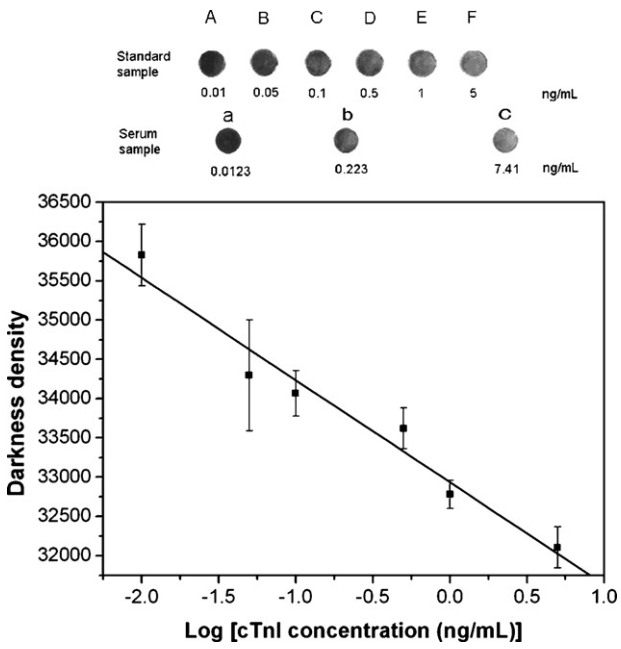

Fig. 6. Detection of cTnI in human serum. Serums were diluted one-half with 10 mM PBS buffer (pH 7.38). The error bars show standard deviations ($N = 3$).

Table 1
Comparison of cTnI detection on human serum samples by colorimetric method and ELISA.

	Proposed method (ng/mL)	ELISA (ng/mL)
Sample a	0.0246	<0.1
Sample b	0.446	0.46
Sample c	14.8	>2.0

10 mM PBS buffer (pH 7.38) to half of their original concentrations. We detected these serum samples by internal standard method, and photos were taken by the digital camera and DD values were analyzed by Bio-Rad software. The results were compared with those of ELISA method and shown in Table 1.

It can be seen that there is no significant difference between the data obtained by the proposed colorimetric analysis and ELISA method, moreover the proposed method could detect the serum samples much more quickly and accurately than traditional ELISA due to its wide detection range and low detection limit. So the results indicated that the developed novel composite film-based colorimetric immunoassay method could be practically used for the determination of cTnI in human serum during clinical diagnosis.

4. Conclusions

We have demonstrated a PDMS-AuNPs composite film-based biosensor coupled with silver enhancement colorimetric detection for cTnI in less than 20 min, the detection limit is 0.01 ng/mL. Moreover, this simple composite film is low cost, convenient to carry with, as well as its ease of fabrication and operation. This simple device coupled with a digital camera eliminates the need for expensive, highly specialized equipment in diagnostic procedure. As a result, the detection of cardiac biomarkers and other biomolecules using PDMS-AuNPs composite film as a portable strip is currently underway.

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