A Washing-free Gold-nanoparticle-based Plasmonic Immunoassay for Point of Care Test of Cardiac Troponin I

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Abstract

Acute myocardial infarction (AMI) was a common fatal cardiovascular disease, causing millions of deaths worldwide every year. Cardiac troponin I (cTnI) was considered the 'gold standard' biomarker for AMI diagnosis. The nano-plasmonic immunoassay was a promising method for point of care test (POCT) application due to its high sensitivity and wide dynamic range. However, because of the requirement multiple liquid adding and washing steps, current nano-plasmonic immunoassays were limited to the professional laboratory. In this study, we report a washing-free gold-nanoparticle-based plasmonic immunoassay (WFPI) method based onthe immuno-reaction induced gold nanoparticles (AuNPs) aggregation used for cTnI POCT detection. The detection can be completed within 15 min, the linear detection range was 15.63 ng/mL-500 ng/mL and the limit of detection was 13.70 ng/mL. cTnI in patient serum samples were analyzed by the WFPI method, and their results were a good correlation with gold standard enzyme linked immunosorbent assay (ELISA). These results demonstrating the WFPI method provide a promising method for POCT diagnosis of AMI, especially in developing countries.

Keywords

Cardiac troponin I (cTnI), Gold nanoparticles (AuNPs), Washing-free.

1. Introduction

Acute myocardial infarction (AMI) was necrosis of myocardial tissue caused by acute and continuous ischemia of coronary arteries and hypoxia. It was the most severe manifestation of coronary artery disease. Acute myocardial infarction causes more than 2.4 million deaths in the USA, 4 million deaths in Europe and northern Asia, and a third of deaths in all developed nations annually [1, 2]. Most AMI patients die within the acute stage (30 days after onset), with 75% dying during the first 24 hours. AMI patients experience only mild symptoms initially, however they can deteriorate quickly. About 62% of AMI deaths occur before arriving at the hospital [3]. Therefore, point of care test (POCT) AMI diagnosis was critical for prevention and treatment [4-7].

Cardiac troponin I (cTnI) was considered the "gold standard" for the diagnosis of AMI due to its high specificity [8-13]. Common methods to detect cTnI include enzyme-linked immunosorbent assay (ELISA), Chemiluminescence immunoassay (CLIA) electro chemiluminescence immunoassay (ECLIA), time-resolved immunoassay (TRIA), electrochemical immunoassay and so on [14-17]. Even high sensitivity and high stability, these methods were restricted in concentrated laboratory or hospital due to the requirement of expensive types of equipment and maintenance, complex operation and professional operators [18-22]. Therefore, it was very important to develope a low cost, easy operation and expensive types of equipment free immunoassay for cTnI POCT.

In this work, we developed novel gold nanoparticles based on the washing-free plasmonic immunoassay for POCT of cTnI [23-25]. In the washing-free gold-nanoparticle-based plasmonic

immunoassay (WFPI), gold nanoparticles were aggregated mediated by immunologic reaction and the signal enhanced by growth Au on the surface of gold nanoparticles, which induces the color of the bulk solution changes to be blue and absorbance of gold nanoparticles have taken place significant changes [26-30]. Using PVP 2000 as crowding reagent can greatly reduce the time required for antigen-antibody binding, NH2OH reduction of HAuCl4 as reagent just needs 2 min, so the WFPI method was finished within 15 min and the sensitivity was comparable with enzyme-linked immunosorbent assay (ELISA). These results demonstrating this work provide the new method for early POCT diagnosis of cTnI, especially in developing countries.

2. Methods

2.1 Chemicals and materials.

Cardiac troponin I (cTnI) was obtained from HyTest (Finland). Antibody 1 and antibody 2 were a pair of monoclonal antibodies that specifically recognize cTnI and are produced by our laboratory. PVP2000 was came from Sigma. Creatine Kinase Isoenzyme (CK-MB) was purchased from Shanghai Leading Biotechnology. Myoglobin was purchased from Beijing lanbolide trading co. Skeletal muscle troponin was purchased from Shanghai Wuhao biotechnology.

UV-vis spectrophotometer (Beckman Coulter DU800, Brea, CA). Transmission electron microscopy (TEM) (Hitachi H600 TEM instrument).

2.2 Preparation cTnI washing free nano-plasmonic immunoassays (WFPI) method.

First, we prepared colloidal gold. The specific procedure for synthesizing 16 nm of colloidal gold using the trisodium citrate reduction method of Frens et al.wasas follows [31]. Briefly, the 100 mL conical flask was immersed in aqua regia and then siliconized and dried. 50 mL ultrapure water was added into a conical flask, heated and stirred, and 1 mL of 1% chloroauric acid (HAuCl4) and 2 mL of 1% sodium citrate were added to the boiled water, heated, and stirred for 15 min. The solution changed from colorless to brown/purple and eventually to red. The heat was removed and the mixture was stirred at the same speed for 15 min. After cooling to room temperature, the volume was set to 50 mL and 200 μL was aliquoted for spectral scanning. Finally, AuNPs solution was stored at 4 ℃. After 24 h, the prepared colloidal gold particles were observed by scanning TEM.

For colloidal gold labeled antibodies (Ab-AuNPs): 6 μL/4 μL of 0.25 mM K2CO3 and 2 μL/5 μL of the antibody 1 / antibody 2 were added to 1 mL AuNPs, stirred at room temperature for 15 min, and left to stand for 15 min. 200 μL of 1% PVP2000 was added, reaction was stirred for 15 min, and thenleft to stand for 15 min.

We used this as a way to detect cTnI. 5 mL of PBST (include 1% PVP2000, which was used for crowding reagent) [32], and 1 mL of Ab-AuNPs labeled antibodies were mixed. 100 μL of mixed Ab-AuNPs was added to each microwell and then 100 μL of different concentrations of a cTnI standard was added (0, 15.63, 31.25, 62.5, 125, 250, 500, and 1000 ng/mL). After 10 min, The growth agent was added, and after 2 min, the UV-vis spectra were read using the microplate reader.

2.3 Preparation cTnI ELISA kit.

The ELISA for cTnI detection was performed using a double-antibody sandwich ELISA protocol format. Polystyrene microwells were coated with 100 μL per well of anti-human cTnI antibody1 in a 50 mM coating buffer and incubated overnight at room temperature. The coated microwells were then washed three times with PBST buffer and then 200 μL of 1% BSA was added to block the surface of each microwell. Plates were incubated at 37 °C for 2 h. The coated microwells were washed three times with PBST and then stored at − 20 °C for future use.

To construct the calibration curve for the cTnI ELISA, 100 μL of different concentrations of a cTnI standard (0, 19.50, 39.10, 78.10, 156.30, 312.50, 625, 1250, 2500, 5000, and 1000 ng/mL) were mixed and added to microwells. After 45 min, dissociated cTnI was removed by washing three times with PBST. Then, 100 μL of PBST buffer with a diluted anti-human cTnI antibody2-horseradish peroxidase conjugate (100 ng/mL) was added to each microwell and incubated at 37 °C for 30 min.

Each microwell was washed with PBST buffer five times, and then 100 μL of the TMB substrate was added and incubated for 10 min after which 50 μL of stop solution was added to each microwell to terminate the reactions. Transmitted absorbance of TMB substrate was measured by using the microplate reader.

2.4 Detect of clinical samples.

To evaluate the sensitivity and accuracy of the ELISA kit and the WFPI method for detection in patient samples, 100 μL of clinical serum samples diluted twice with PBST were tested. The ELISA protocols and the WFPI method protocols followed for the detection of this protein were similar to the one followed for cTnI detection.

3. Results

3.1 Principle experiment.

Fig. 1 shows a schematic illustration of the washing-free gold-nanoparticle-based plasmonic Immunoassay (WFPI). Ab-AuNPs solution was mixed with the sample. If the sample did not contain cTnI, no antigen-antibody specific binding reaction occurred, the particles would evenly distribute, and the distance between Ab-AuNPs was large. Once the growth agent was added containing HAuCl4 and NH2OH, NH2OH reduced HAuCl4 to produce an Au atom. Au atoms were adsorbed to the surface of Ab-AuNPs uniformly and increased particle size. Due to the large distance between Ab-AuNPs and their quasi-spherical shape, no aggregates formed, and the solution was red. If the sample contained cTnI, antigen-antibody specific binding formed a sandwich-like structure, decreasing the distance between the Ab-AuNPs. Au atoms cannot be uniformly adsorbed on the surface of particles, due to the uneven adsorption, the irregularity aggregation particles were formed, which turned the solution from red to blue and reduced the absorbance. During this process, the solution was diluted, making the distance between different Ab-AuNPs large. Therefore, the unbound Ab-AuNPs had little influence on the Ab-AuNPs who were bound with cTnI, making washing to remove unbound Ab-AuNPs unnecessary.

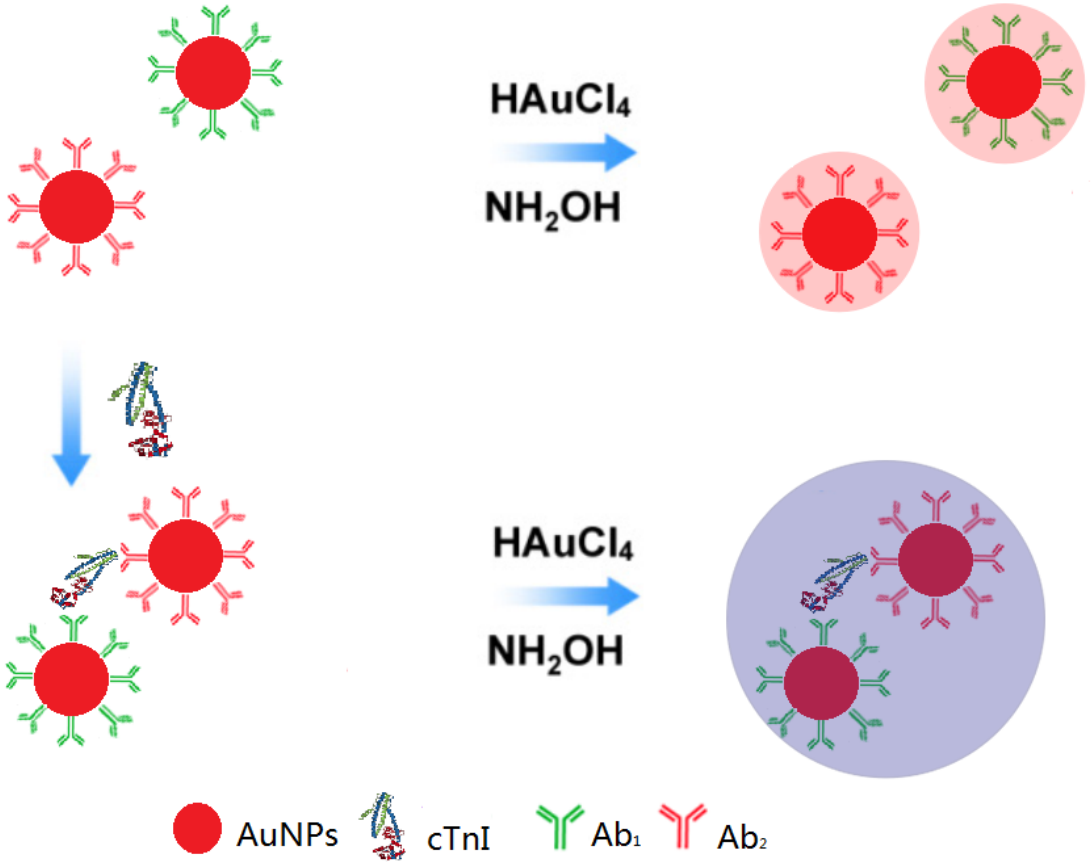

Fig. 1: Schematic illustration of the washing-free gold-nanoparticle-based nano-plasmonic immunoassays.

3.2 Principle characterization.

In order to prove the of synthesized Ab-AuNPs was successful and Ab-AuNPs can aggregate due to antigen bind to it, the size and morphology of the particles were observed under the transmission electron microscopy (TEM) and are shown in Fig. 2a-d. In Fig. 2a, the 16 nm AuNPs diluted with PBST are small, uniform in size, well dispersed, quasi-spherical, and nonaggregated particles. As shown in Fig. 2b, the Ab-AuNPs diluted with PBST are also of uniform size, well dispersed, quasi-spherical, and nonaggregated particles, but they are larger than a. In Fig. 2c Ab-AuNPs were growth without cTnI, the Au atoms were adsorbed to the surface of Ab-AuNPs uniformly, we can see the uniform size, well dispersed, quasi-spherical, nonaggregated particles, much larger than b. In Fig. 2d Ab-AuNPs were growth with cTnI (100 ng/mL), the particles are different sizes, irregularly shaped, aggregated particles, with a size similar to Fig. 2c. Fig. 2e shown the color of the above four solutions, and Fig. 2f was the UV-vis spectra of the solutions. The characteristic absorption peaks of the solutions are 520 nm,522 nm,532 nm, and 532 nm respectively, as we can see from the TEM result this red-shifts was caused by the increased of the particle.

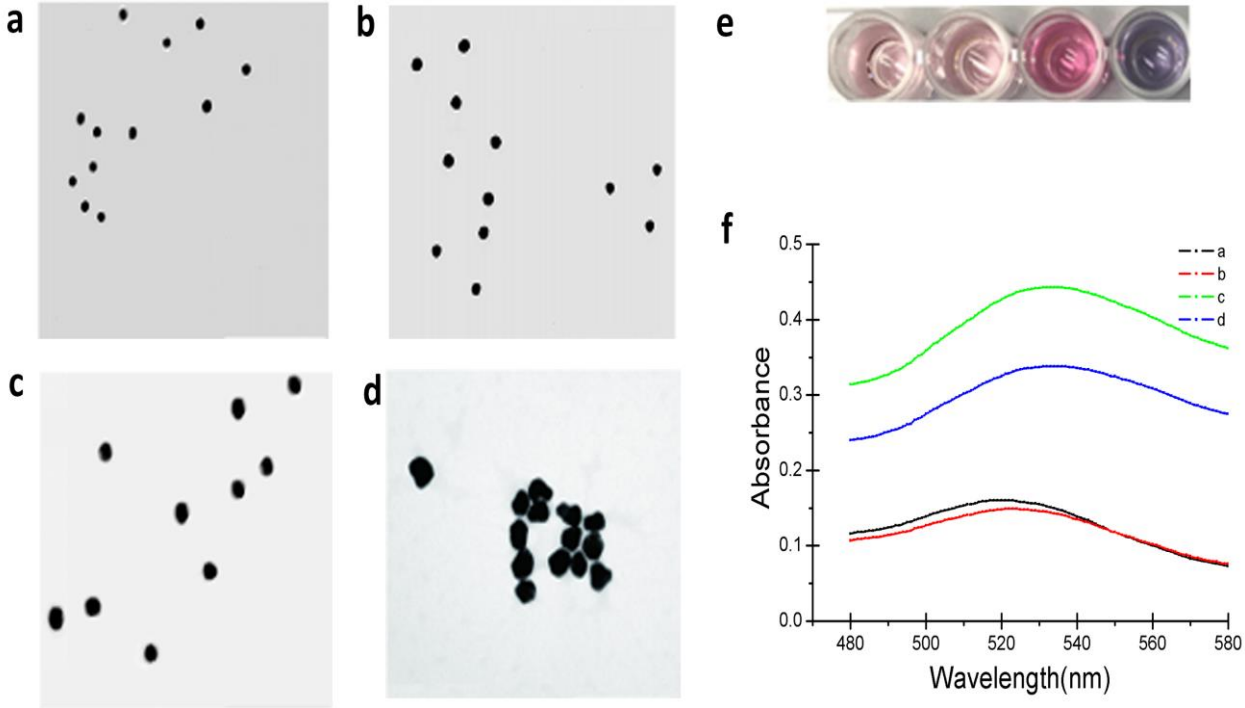

Fig. 2: TEM images of the samples. (a) 16 nm AuNPs. (b) Ab-AuNPs. (c) Negative sample after growth. (d) Positive samples after growth. (e) Color results of the a-d solutions. (f) UV-vis spectra of a-d solutions.

3.3 Optimization AuNPs growth condition.

AuNPs growth requires HAuCl4 (to provide Au+) and NH2OH to reduce Au+ to Au. In this experiment, we used 1% HAuCl4 to provide Au+ and 10 mM NH2OH as the reducing agent (called 'growth agent'). The contents of these two solutions were key to this reaction. Ab-AuNPs and different volumes of 1% HAuCl4 (0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, and 1.4 µL) were added to the wells respectively, followed by 1 µL of 10 mM NH2OH. A microplate reader was used to detect the UV-vis spectra of the mixture after 2 min. The results were shown in Fig. 3a. Added too much HAuCl4 made the size of Ab-AuNPs too large and very prone to self-aggregation, so we chose 0.6 µL as the optimal amount of HAuCl4.

100 µL diluted Ab-AuNPs solution and 0.6 µL of 1% HAuCl4 were added to each well, and different volumes (0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, and 3.5 µL) of 10 mM NH2OH was added into the mixture.

After 2 min, plates were read and the UV-vis spectra determined. The results are shown in Fig. 3b. When the amount of NH2OH was 1 μL, the absorption value of the peak was the largest, indicating it was the optimal amount.

100 μL of diluted Ab-AuNPs and 100 μL of different concentrations of a cTnI standard (0, 250, and 1000 ng/mL) were added to each well, after 10 min followed by the gold growth agent. A microplate reader was used to detect reaction kinetics. The results are shown in Fig. 3c. Within the first 40 seconds, the absorbance increased rapidly, indicating the rapid growth of Ab-AuNPs. After 90 seconds, the absorbance value remained stable, indicated that the growth of Ab-AuNPs was stop and size was stable. In order to ensure the Ab-AuNPs growth completely, we would detect after Ab-AuNPs had growth 120s. As we can see, during the whole detection process, it only takes 10 min for antigen-antibody binding and 2 min for color rendering, so the detection can be completed within 15 min.

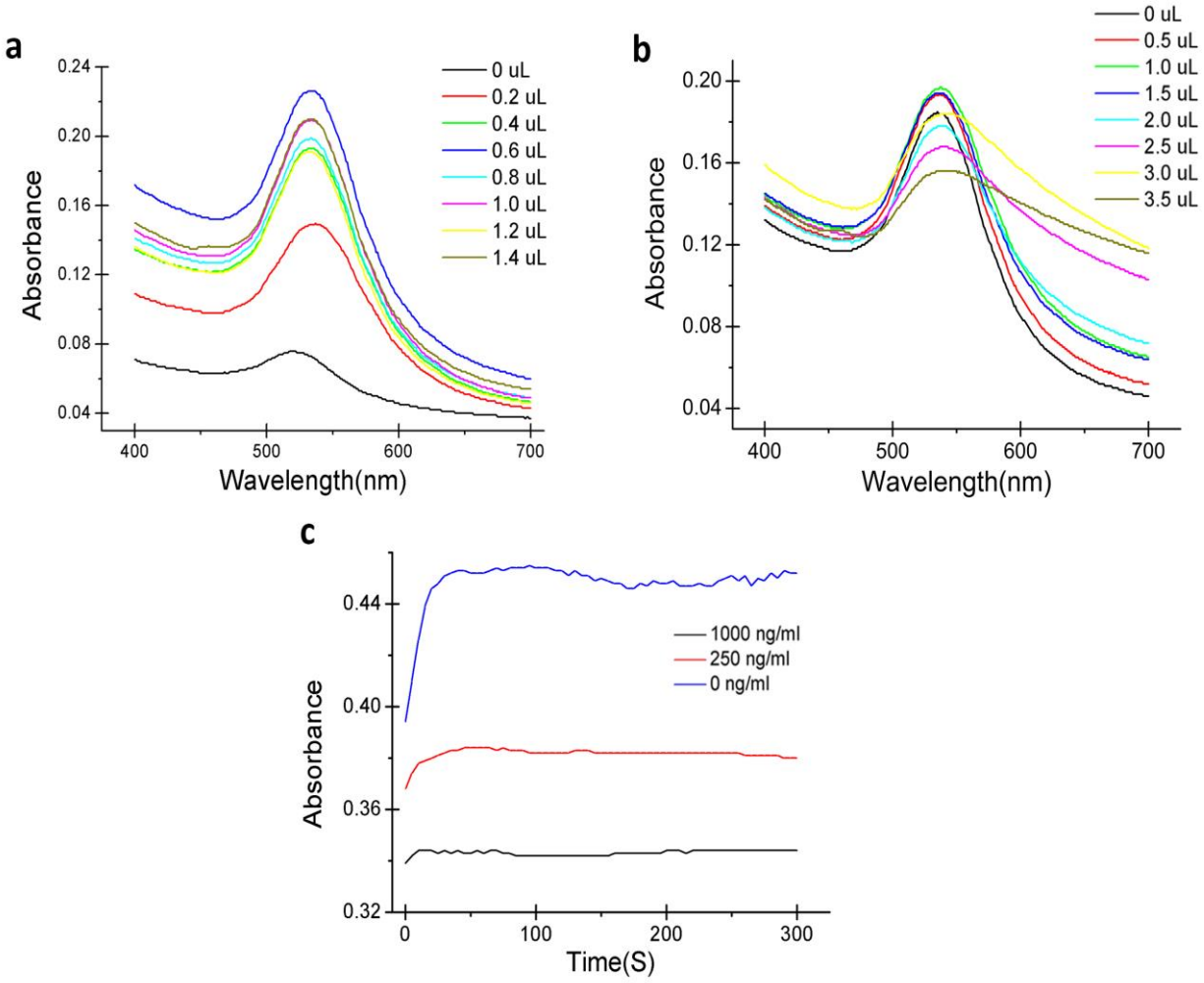

Fig. 3: Optimization of Ab-AuNPs growth condition. (a) Added different amounts of HAuCl4 to determine the optimal amount of HAuCl4. (b) Added different amounts of NH2OH to determine the optimal amount. (c) Added growth agent to different samples and tested the best growth time.

3.4 The results of WFPI method detection cTnI protein standard.

According to the above method, we tested different concentrations of a cTnI standard protein (0, 15.63, 31.25, 62.50, 125, 250, 500, and 1000 ng/mL) and used a microplate reader to detect the UV-vis spectra. As we can see from Fig. 4a, when the sample contained cTnI protein, after the reaction the solution was blue, when the sample lacked cTnI protein, after the reaction the solution was red. With the increased of cTnI content, both the color of the solution and its corresponding UV-vis absorption had evident changes (Fig. 4a-b). Since the Ab-AuNPs absorption peak after growth was

at 532 nm, the absorption at 532 nm was increased with the cTnI concentration increased (Fig. 4c). Fig. 4d was the calibration curve for cTnI detection, exhibits a nearly linear dependence at 15.63-250 ng/mL with a LOD of 13.70 ng/mL.

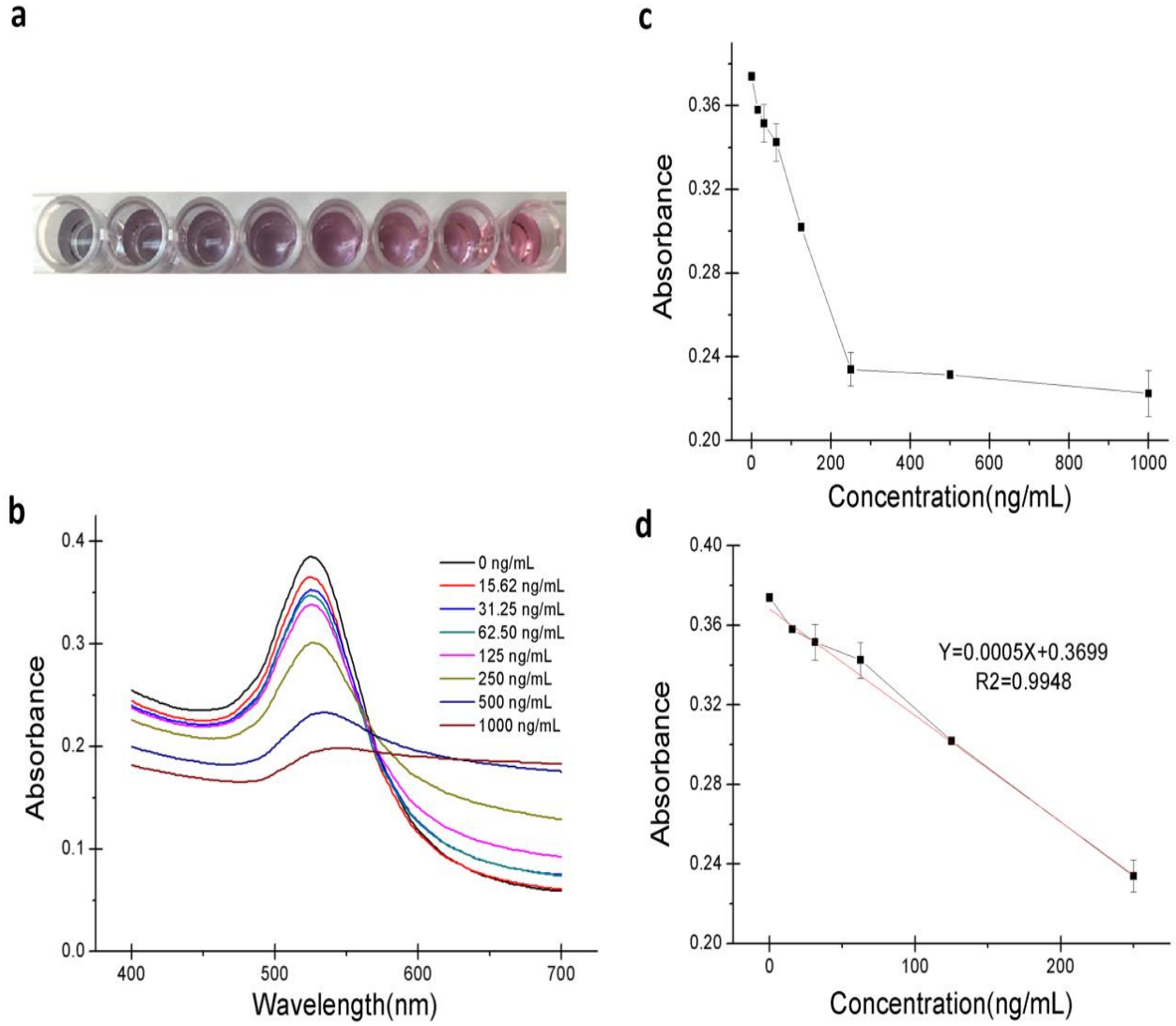

Fig. 4: Detection of cTnI protein standard. (a) Corresponding photography of solutions. (b) The change of the UV-vis spectra. (c) Plots of 532nm versus various concentrations of cTnI. (d) Calibration curve for cTnI detection.

3.5 The result of clinical samples detection.

In order to identify whether the WFPI was suitable for the actual patient to detect, we used this method to detect 32 serum samples containing 16 normal serums and 16 patient serums. We can see a clear color difference between the patient serums (blue) and normal serums (red) from S3, the positive detection rate of this method was 93.7% (Supplementary Table 1). So the WFPI method was suitable for the actual patient to detect.

At last, we used WFPI and ELISA methods to detect the same 20 patients' serum samples. Fig. 5a-b was the result of the used ELISA method to detected cTnI protein standard when the cTnI content was higher, the 450 nm absorbance value became larger, and when the cTnI concentration was between 15.63 ng/mL to 250 ng/mL the OD450 was linearly correlated with concentration. Fig. 5c shown the cTnI concentrations in the same patient's serum detected by these two methods were close, and these two methods result had a good correlation.

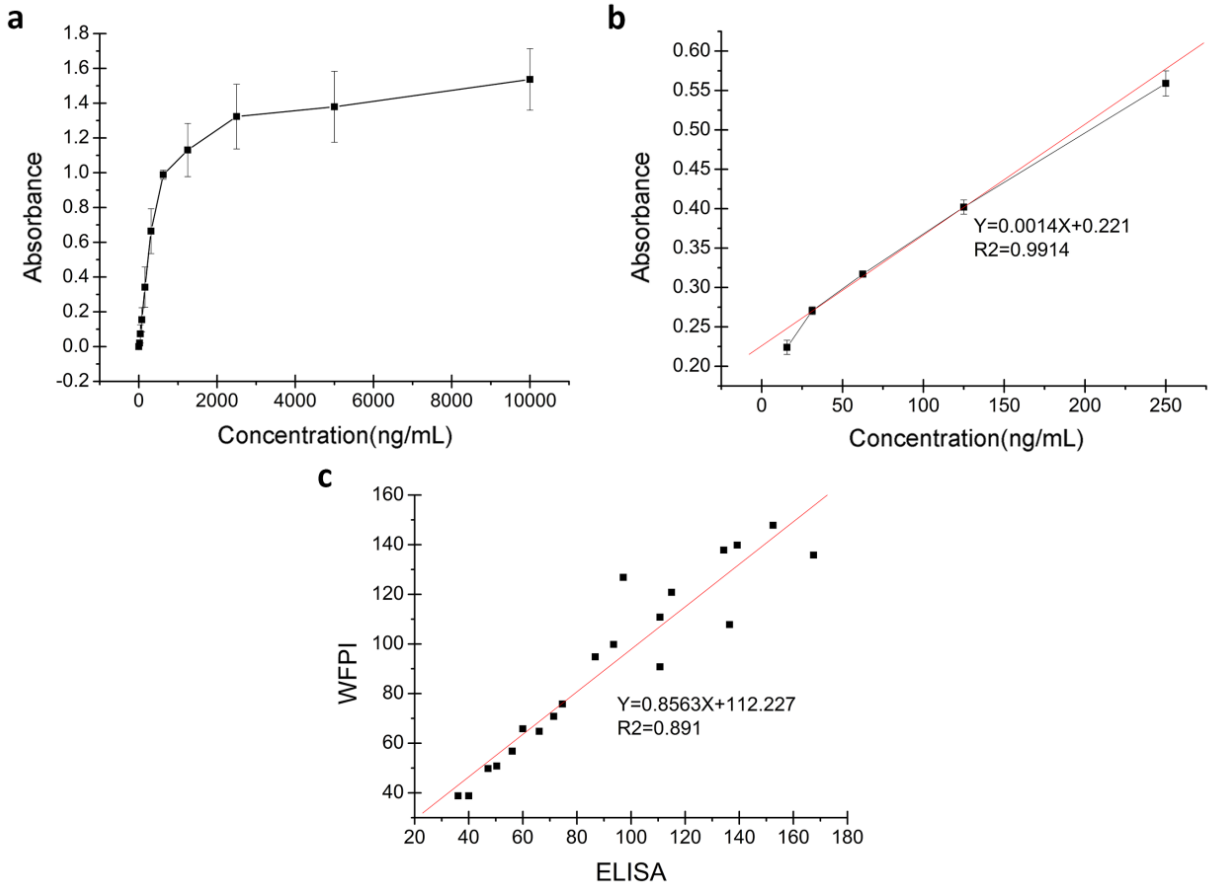

Fig. 5: Clinical samples detection. (a) Plots of 480 nm versus various concentrations of cTnI. (b) ELISA calibration curve for cTnI detection. (c) The fitting graph used two methods to detect the same patient samples.

4. Conclusion

Here we have established a WFPI method which can be finished in 15 min, and exhibits a nearly linear dependence at 15.63-250 ng/mL with a LOD of 13.70 ng/mL, could be used for the early detection of acute myocardial infarction. This method was stable, reproducible, and didn't react with other indicators of acute myocardial infarction, giving it a high specificity. Our method was simple and fast, the results were easily observed with the naked eye didn't require expensive instruments. It was suitable for POCT as well as for high-risk patients to use at home, which could be a valuable tool for AMI detection.

Acknowledgements

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