Biosensors and Bioelectronics 126 (2019) 767–772

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Simultaneous detection of three biomarkers related to acute myocardial infarction based on immunosensing biochip

Kaige Zhao[a,1], Min Tang[a,1], Huaisheng Wang[b], Zhenxian Zhou[c], Yafeng Wu[a,*], Songqin Liu[a,*]

[a] Jiangsu Engineering Laboratory of Smart Carbon-Rich Materials and Device, School of Chemistry and Chemical Engineering, Southeast University, Nanjing 211189, China
[b] Department of Chemistry, Liaocheng University, Liaocheng, Shandong 252059, China
[c] Nanjing Second Hospital, China

ARTICLE INFO

Keywords:
Nanoprobes
Immunosensing biochip
Simultaneous detection
Chemiluminescence imaging
Acute myocardial infarction

ABSTRACT

An immunosensing biochip for simultaneous detection of three biomarkers related to acute myocardial infarction (AMI) was developed based on anionic soybean peroxidase (SBP) functionalized nanoprobe and chemiluminescent imaging. The nanoprobes (Ab2-SiO_2-SBP) were fabricated by co-immobilization of SBP and one of the detection polyclonal antibodies, anti-cardiac troponin I antigen (anti-cTnI), anti-creatine kinase-MB (anti-CK-MB) and anti-myoglobin (anti-Myo), on the silica nanoparticle surface. The detection sensitivity was enhanced since the large surface area of silica carriers increased the loading of SBP for per sandwiched immunoreaction. The immunosensing biochip designed as 3 × 8 wells array was constructed by simultaneously immobilizing three capture monoclonal antibodies on the same one microtiter well with 2 × 3 active spots. In the presence of target protein, the nanoprobes will be attached onto the spots with high specificity through the sandwiched immunoreactions, which triggered the chemiluminescence (CL) signals on each sensing site of the microtiter plates and allowed to CL imaging of three biomarkers in one well at the same time. Therefore, the proposed biochip was a promising convenient strategy for simultaneous detection of cTnI, CK-MB and Myo, which showed potential application for multianalyte determination in clinical diagnostics.

1. Introduction

Acute myocardial infarction (AMI) is a kind of clinical Cardiovascular disease which could cause a severe public health problem in a global context (White and Chew, 2008; Wu et al., 2007). Therefore, the diagnosis and prevention of cardiovascular disease are important and necessary for health care systems (Apple et al., 2007; Wu et al., 2007). The clinical investigations revealed some cardiac biomarkers such as myoglobin (Myo), cardiac troponin I (cTnI), creatine kinase-MB (CK-MB), b-type natriuretic peptide (BNP) and so on, were related to myocardial infarction and could be detected in serum (Lee et al., 2012; Zhang et al., 2018a). Among these, Myo is the fundamental protein to check at the onset of infarction (Montague and Kircher, 1995; Wu et al., 2007). Due to the unique cardiac specificity and selectivity of Cardiac troponin I (cTnI), it was regarded as the new “gold standard” for CVDs diagnosis (Sheng et al., 2017; Wu et al., 2007). Creatine kinase-MB (CK-MB) is related to recurrence of myocardial infarction, which has also been demonstrated an important clinically sensitive and selective cardiac markers in the screening of AMI (Adams et al., 1993; Wu et al., 2007).

Currently, several immunoassay methods, including fluorescence (Caulum et al., 2007; Hayes et al., 2009), electrochemistry (Chua et al., 2009; Purvis et al., 2003), surface plasmon resonance (Kurita et al., 2006; Masson et al., 2004) and electrochemiluminescence (Giannitsis et al., 2010; Shen et al., 2011), have been employed for detection the cardiac biomarkers in patient serum. Some of these methods received high sensitivity, good selectivity, label-free, high-throughput for the detection of AMI-related biomarkers, and even some approaches have been practically used in clinical diagnostic. Nevertheless, these methods can not realize simultaneous quantification of multiple cardiac biomarkers at the same time (Chon et al., 2014; Lee et al., 2012). Therefore, designing a novel immunosensing biochip was of great significance for simultaneous detection of cTnI, CK-MB and Myo in blood, which provided many advantages over separate determination of each marker, and observably increased the probability of an early diagnosis of AMI.

Horseradish peroxidase (HRP) was demonstrated as an excellent catalyst for the luminol-H_2O_2 chemiluminescence system. When adding

* Corresponding authors.
E-mail addresses: wuyafeng_1985@163.com (Y. Wu), liusq@seu.edu.cn (S. Liu).
[1] These authors contributed the equal work.

https://doi.org/10.1016/j.bios.2018.11.044
Received 31 August 2018; Received in revised form 3 November 2018; Accepted 27 November 2018
Available online 06 December 2018
0956-5663/

luminescence enhancers, a strong luminescence signal will generate and reach to the maximum value in the first few minutes, but then rapidly decays faster with time (Kapeluich et al., 1997). However, when the anionic SBP catalyzes the luminol-H_2O_2 luminescence system, the luminescence signal decays at a relatively slow rate and can generate a long-time luminescence signal (Alpeeva and Sakharov, 2005; Marchis et al., 2012; Sakharov et al., 2006, 2010). With the addition of co-enhancers (3-(10′-phenothiazinyl) propane-1-sulfonate (SPTZ), 4-morpholinopyridine (MORP)) in the SBP-luminol-H_2O_2 chemiluminescence system, the CL signal was efficiently increased (Marzocchi et al., 2008; Vdovenko et al., 2009; Sakharov and Vdovenko, 2013; Zhao et al., 2015a, 2015b; Liu et al., 2016). In addition, SBP has the advantages of good thermal stability, structural stability, catalytic activity, and ability to maintain activity over a wide range of pH making it widely available (Tang et al., 2018; Zhao et al., 2015a, 2015b).

In this paper, a novel immunosensing biochip was constructed to achieve simultaneous detection of three cardiac markers (cTnI, CK-MB, Myo) related to AMI disease. Firstly, silica spheres with large specific surface area were used for co-immobilization of SBP and detection antibodies. The large specific surface area facilitated the immobilization of a large amount of antibodies and enzymes, benefitting for improving the sensitivity and high-throughput capacity. Three kinds of monoclonal capture antibodies (anti-cTnI, anti-CK-MB and anti-Myo) were simultaneously immobilized on the surface of a nitrocellulose filter membrane (NC membrane) to form the biochip with 3 × 8 wells. After sandwiched immunoreactions, the nanoprobes were brought to active sensing sites corresponding to the target protein, which catalyzed the oxidation of luminol and generated the chemiluminescence (CL) signal. This biochip realized fast and high-throughput detection of the three cardiac markers, which provided a promising protocol for simultaneous determination of panel of markers in clinical applications.

2. Material and methods

2.1. Materials and reagents

Soybean peroxidase (SBP, EC 1.11.1.7, RZ≤ 3.29, 2229 IU per mg dry weight) was purchased from Bio-Research Products, Inc. (USA). Cardiac troponin I antigen (cTnI), creatine kinase-MB (CK-MB), myoglobin (Myo) and their primary antibodies (mouse monoclonal antibody, capture antibody): anti-cTnI 3.51 mg/mL, anti-CK-MB 2.38 mg/mL and anti-Myo 6.4 mg/mL; secondary antibodies (mouse polyclonal antibody, detection antibody): anti-cTnI 1.85 mg/mL, anti-CK-MB 5.2 mg/mL and anti-Myo 1.5 mg/mL, were received from R&D Systems Industries Int. (USA). Ethanol, glutaraldehyde, ammonium hydroxide, sodium hydrate, sodium dihydrogen phosphate, disodium hydrogen phosphate, tris (hydroxymethyl) amino-methane (Tris) and hydrogen peroxide (H_2O_2) were obtained from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Bovine serum albumin (BSA, ≥96%), tetraethoxysilane (TEOS), 3-aminopropyltriethyloxy silane (APTES), 3-(10′-phenothiazinyl) propane-1-sulfonate (SPTZ), 4-morpholinopyridine (MORP) and luminol were received from Sigma-Aladdin (Shanghai, China). Tumor necrosis factor-alpha (TNF- < alpha >), carbohydrate antigen 19–9 (CA19-9) and alpha-fetoprotein (AFP) were purchased from Sigma-Aladdin (Shanghai, China). Double distilled water was used in all experiments. Phosphate buffered saline (PBS, pH 7.4) was prepared by mixing 0.01 M NaH_2PO_4, 0.01 M Na_2HPO_4 and 0.15 M NaCl. 50 mM pH 8.5 Tris-HCl buffer was prepared with Tris and HCl. Washing solution (TBST) was prepared by adding of 0.3% Tween-20 into 50 mM pH 7.5 Tris-HCl.

Cool CCD imaging analyzer (AE-1000 YQ001, Beijing BGI-GBI Biotech Co. Ltd., China) was used to collect CL signals in all measurements. An exposure time of one min was selected for all measurements. The spots automatically obtained by CCD were identified by Acquire Control. The CL intensity per spot was calculated using the data analysis software of MetaMorph, which related to the pixel intensity.

2.2. Preparation of nanoprobe by coupling of the secondary antibody and SBP on SiO_2 nanoparticles

SiO_2 nanoparticles were prepared by the previous method (Chen et al., 2009a, 2009b; Qian et al., 2010). Briefly, 20 mL EtOH, 6 mL deionized water, 4 mL ammonia was vigorously stirred for 5 min. Then 30 mL anhydrous alcohol containing 1.7 mL TEOS was slowly added into the above ethanol solution and allowed to react for 75 min at 25 °C. The reaction mixture was centrifuged for another 10 min, washing with deionized water and ethanol for three times. Therefore, the SiO_2 nanoparticles with diameter of ~350 nm were obtained for the following uses.

Then, 1 g as-prepared SiO_2 nanoparticles and 15 mL toluene were added to a 50-mL three-necked flask and ultrasonically dispersed for 15 min 100 μL of APTES was added into the flask, and the mixture was refluxed at 110 °C with continuous stirring for 20 h. The resulting silica nanoparticles functionalized with amino groups (SiO_2-NH_2) were collected by centrifugation at 8000 rpm for 10 min, followed by respectively washing with ethanol and deionized water for three times and dispersing in deionized water to a final volume of 10 mL for future use.

The nanoprobe (Ab2-SiO_2-SBP) was prepared according to the amide reactions with glutaraldehyde as a linkage (Fig. 1A). For the preparation of anti-cTnI-SiO_2-SBP, anti-Myo-SiO_2-SBP or anti-CK-MB-SiO_2-SBP, 2 mL of 2.5% glutaraldehyde was added into 2 mL above SiO_2-NH_2 solution and reacted at 4 °C for 1 h. The precipitate was obtained by centrifugation of the reaction mixture at 10,000 rpm for 10 min and re-dispersed in 500 μL PBS. After that, 240 μL 100 μg/mL anti-cTnI (or 240 μL 100 μg/mL anti-Myo or 240 μL 100 μg/mL anti-CK-MB) and 400 μL 1 mg/mL SBP were simultaneously added and allowed to react at 4 °C for 18 h. The suspension was centrifuged at 9000 rpm for 10 min to remove the unbound enzymes and antibodies. Finally, 4 mL 3% BSA was added and reacted for 1 h to block the non-specific binding sites of silica surface. The resulting nanoprobe Ab2-SiO_2-SBP was washed with PBS and re-dispersed in 2 mL PBS for later use.

2.3. Construction of the immunosensing biochip and their chemiluminescent imaging

As shown in Fig. 1B and Fig. S1, the fabricated biochip contains 24 parts (3 rows × 8 columns) and each part contains 6 active spots (2 rows × 3 columns). To avoid the interference between the spots, the distance between two spots was 2 mm (center to center). For each part, the first column was spotted with cTnI primary antibodies (anti-cTnI), the second column was spotted with anti-CK-MB and the third column was spotted with anti-Myo. The concentration of primary antibodies was 0.1 mg/mL, prepared with spotting buffer (0.1 mM PBS containing 0.6 mg/mL sucrose and 10 μg/mL methyl violet). 20 nL primary antibody solution was automatically spotted on the corresponding site of NC membrane by Biodot AD6000 at 24 °C and a humidity of 50%. After immobilized primary antibodies on the NC membrane, it was moved to a humidity chamber at 4 °C overnight. Then the waterproof transparency (made of Silicone and PVC) was coated on the edge section of each part to form 24 wells. Finally, 150 μL of 5% BSA in pH 7.4 PBS was added in each well and incubated for 2 h to block the excessive binding sites on NC membrane and to reduce background signal. The microtiter plate modified with primary antibodies contains 24 wells (at each well, the first column was anti-cTnI, the second column was anti-CK-MB and the third column was anti-Myo), which was stored in the dark at 4 °C for future use.

The strategy of chemiluminescent (CL) imaging immunoassay for simultaneous detection of cTnI, CK-MB and Myo was illustrated in Fig. 1B. 90 μL samples was added into each well of the microtiter plate, incubated under gentle shaking at room temperature for 30 min, washed with TBST for three times. Then, 90 μL the mixture of Ab2-SiO_2-SBP with same volume were respectively added to each well and incubated for another 30 min, followed by washing with TBST for three

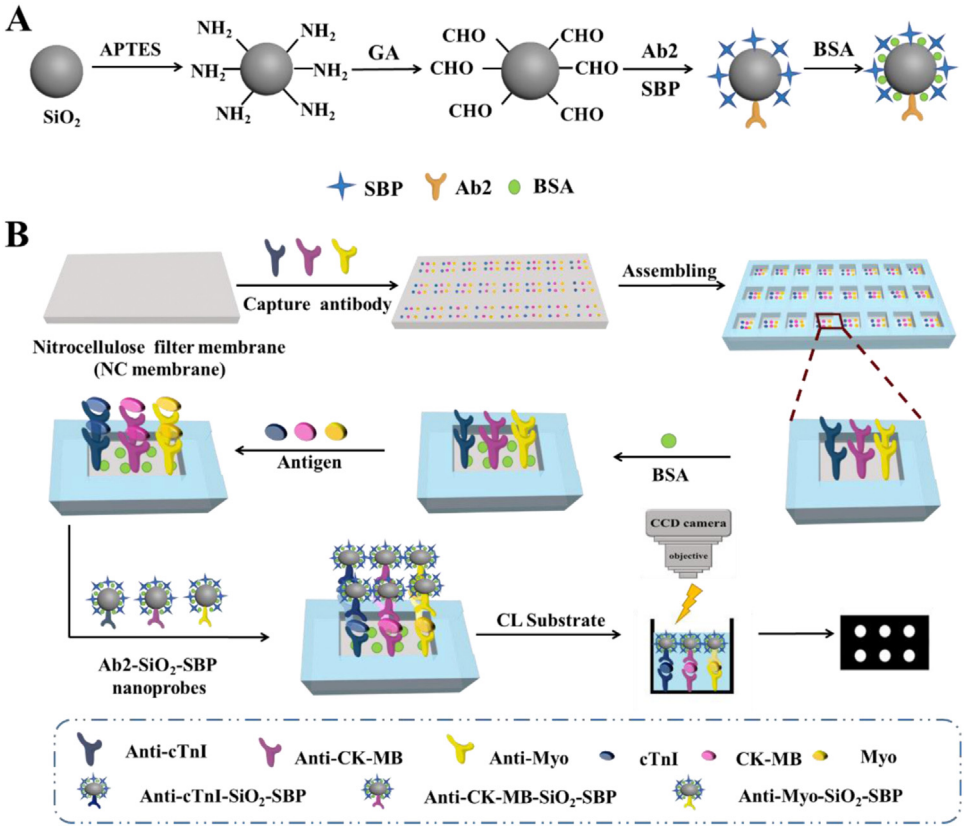

Fig. 1. The scheme of (A) the preparation of Ab2-SiO_2-SBP and (B) the general preparation procedures of immunosensing biochip and CL imaging immunoassay procedure.

times. Finally, 30 μL chemiluminescent substrate mixture solution containing 2 mM luminol, 10 mM H_2O_2, 0.8 mM SPTZ, 0.2 mM MORP and 50 mM Tris-HCL were delivered into the sensing wells to trigger the CL reaction. The CL spots were simultaneously collected by CCD (exposure time was set as 3 min) and automatically analyzed by Acquire Control. Our method realized simultaneous detection of three biochemical markers related to myocardial diseases on a single chip.

3. Results and discussion

3.1. Detection principle with silica nanoprobes

The SiO_2 nanoparticles with diameter of ~350 nm and good monodispersity was prepared using Stöber method (Stober et al., 1968) (Fig. S2). After modifying with APTES, the zeta potential was changed from −39.34 for silica to 11.54 mV for SiO_2-NH_2 (Fig. S3). With glutaraldehyde as a linkage, SBP and the detection antibodies (Ab2) were co-immobilized on the surface of silica spheres to form three kinds of Ab2-SiO_2-SBP nanoprobes (anti-cTnI-SiO_2-SBP, anti-CK-MB-SiO_2-SBP and anti-Myo-SiO_2-SBP) (Chen et al., 2009a; Wu et al., 2009). The loading capacity of Ab2 or SBP on SiO_2 was monitored by a UV–visible spectrometer at 280 nm or 420 nm and approximately up to 112 mg/g or 183 mg/g, respectively. The detection sensitivity was dramatically enhanced because the large surface area of silica carriers (124 m^2/g, measured from BET) increased the amount of signal report molecules bound per sandwiched immunoreaction. Here, the signal report molecules were SBP, which displayed much better chemiluminescent performances in luminol-H_2O_2 system than horseradish peroxidase (Alpeeva and Sakharov, 2005; Marchis et al., 2012; Sakharov et al., 2006, 2010; Vdovenko et al., 2010; Tang et al., 2018; Zhao et al., 2015a, 2015b).

The anionic SBP catalyzes the oxidation of luminol according to the "ping-pong" mechanism:

$$SBP + H_2O_2 \rightarrow SBP(I) + H_2O \quad (1)$$

$$SBP(I) + A \rightarrow SBP(II) + A^{\bullet} \quad (2)$$

$$SBP(II) + A \rightarrow SBP + A^{\bullet} + H_2O \quad (3)$$

$$A^{\bullet} \rightarrow A + h\nu \quad (4)$$

Here, SBP(I) and SBP(II) are the intermediate compounds, while A and $A^{\bullet}$ are luminol and its radical product by one electron catalytical oxidation reaction, respectively. The immobilized SBP is first chemically oxidized to SBP(I). The intermediate compound of SBP(I) is then reacted with luminol to form SBP(II) and the excited species of luminol. The intermediate compound SBP(II) will react with luminol to produce the excited species of luminol, while SBP return to its initial state. Eventually the excited luminol species converts into 3-aminophthalate by emitting light and the CL intensity is monitored.

As a proof of concept, the mixture of three target antigens and their nanoprobes Ab2-SiO_2-SBP were subsequently added into the wells which contained capture antibodies and incubated for 30 min, respectively. The CL substrate containing luminol and H_2O_2 was added and a powerful CL signal was observed due to the efficient catalysis of H_2O_2-luminol system by SBP (Fig. 2A, band c, and Fig. 2B, band c, Fig. 2B just showed the data with cTnI as a model protein). Control measurements showed that no CL signal was observed after addition of H_2O_2 without luminol (Fig. 2A and B, band a), or only a weak CL signal was observed when only luminol added without H_2O_2 (Fig. 2A and B, band b). In the presence of only MORP or SPTZ, the CL intensity of luminol-H_2O_2 system was increased ~5.4% or 47%, respectively (Fig. 2B, band d and e). When SPTZ and MORP were simultaneously contained in luminol-H_2O_2 system, the CL intensity enhanced by 470% compared with no enhancer in the CL substrate (Fig. 2B, band f). This indicated that SPTZ

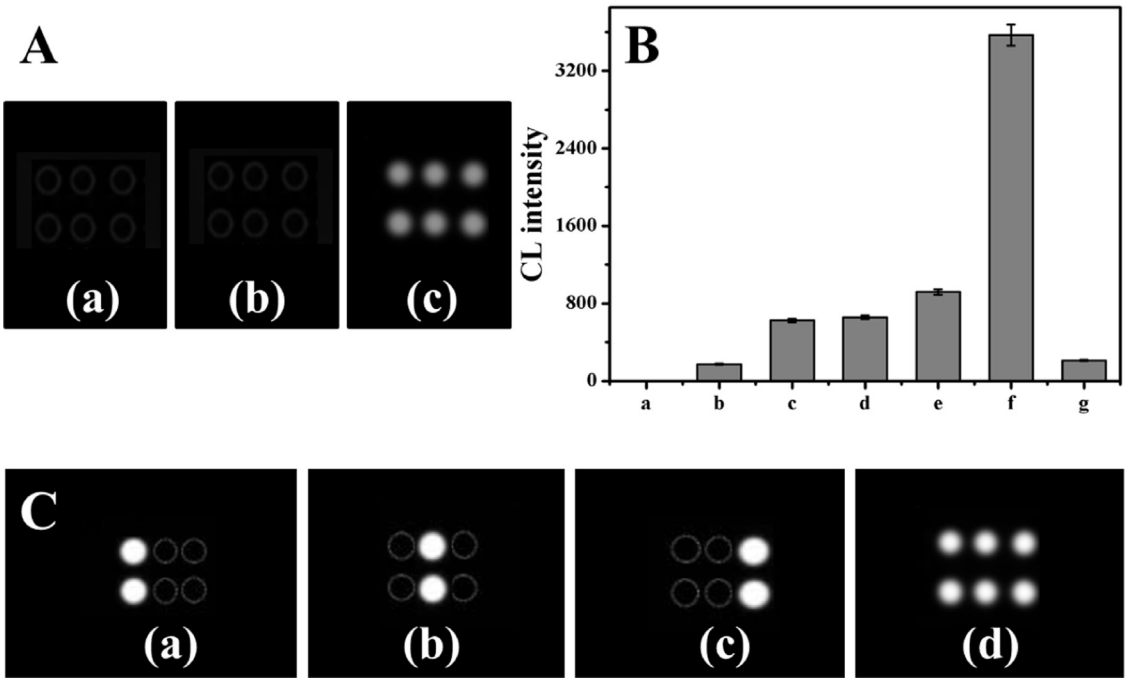

Fig. 2. (A) CL images of the nanoprobes functionalized immunosensing biochip with different chemiluminiscent substrates: Tris-HCL containing (a) only H_2O_2, (b) only luminol, (c) luminol and H_2O_2. (B) With cTnI as model, the CL intensity under different conditions. (a-f) The CL intensity from different chemiluminiscent substrates Tris-HCL containing (a) only H_2O_2, (b) only luminol, (c) luminol and H_2O_2, (d) luminol, H_2O_2 and MORP, (e) luminol, H_2O_2 and SPTZ, (f) luminol, H_2O_2, MORP and SPTZ, (g) with the chemiluminiscent substrate of (f), while the anti-cTnI-SBP was used instead of anti-cTnI-SiO_2-SBP. (C) CL images of primary antibodies modified immunosensing biochips incubated (a) only cTnI, (b) only CK-MB, (c) only Myo, (d) cTnI + CK-MB + Myo, then followed by the incubation with the mixture solution of Ab2-SiO_2-SBP to form sandwiched immunocomplex. All the CL imagings were collected in 50 mM Tris-HCL containing 2 mM luminol, 10 mM H_2O_2, 0.8 mM SPTZ and 0.2 mM MORP.

and MORP have a significant enhancement to the chemiluminescence signal of the SBP-catalyzed luminol-H_2O_2 system. To confirm the signal amplification effect of SiO_2, anti-cTnI-SBP was used instead of anti-cTnI-SiO_2-SBP, the other conditions were the same, the CL signal lowered ~15 folds (Fig. 2B, band g).

The concept for simultaneous detection of three cardiac biomarkers was proved by the as-prepared immunosensing biochip. As shown in Fig. 2C, when the sample contained all three kinds of antigens (cTnI, CK-MB and Myo), after the mixture of Ab2-SiO_2-SBP and chemiluminescent substrate were subsequently added, six spots were lighted (Fig. 2C, d). While if the sample contained only cTnI or CK-MB or Myo, only the spots in the corresponding column were lighted. For cTnI, only the first column was lighted (a), for CK-MB, only the second column was lighted (b) and for Myo, only the third column was lighted (c). So there was no cross-reactivity between antigens.

Control experiments were conducted by using primary antibodies modified biochips with only antigen (cTnI, CK-MB and Myo) or only Ab2-SiO_2-SBP (anti-cTnI-SiO_2-SBP, anti-CK-MB-SiO_2-SBP and anti-Myo-SiO_2-SBP), SiO_2 modified with only SBP or only Ab2, according to the same procedure as biochip test. As shown in Fig. S4, only weak CL signal was observed at the corresponding spots. All these results demonstrated that the CL signals came from the sandwiched immunoreactions among the capture antibody, antigen, and Ab2-SiO_2-SBP and there was no obvious nonspecific adsorption.

3.2. High-throughput and simultaneous detection of three cardiac biomarkers

The sensitivity of the biochip depends on the composition and concentration of the chemiluminiscent substrates, which has great influence on the CL signal intensities and should be optimized. At a constant luminol concentration, the CL signal individually increased with the increasing of concentrations of H_2O_2 or SPTZ or MORP in the mixture solution containing other two substrates at constant concentration while one substrate concentration changed (Fig. S5). The trend was reached a maximum value at 10 mM H_2O_2 or 0.8 mM SPTZ or 0.2 mM MORP. Therefore, the mixture solution containing 2 mM luminol, 10 mM H_2O_2, 0.8 mM SPTZ and 0.2 mM MORP was chosen as the CL substrate.

The detection sensitivity also depended on the ratio of SBP and Ab2 loaded on the surface of SiO_2 nanoparticles, which is tunable during the competitive epoxy-amino coupling reaction. Anti-cTnI was used as example, as shown in Fig. S6, when the ratio of SBP to anti-cTnI was 5:3, the CL signal was the largest, further increase the ratio resulted in the reduced CL response.

Under the optimal detection conditions, the CL intensity were linearly proportional to the logarithm of the concentrations of analytes over the ranges of 0.02–80 ng/mL for cTnI, 0.2–120 ng/mL for CK-MB and 0.3–480 ng/mL for Myo, respectively (Fig. 3). The linear curve fits a regression equation of $I_{CL} = 2243.3 + 611.7 \log c$ (ng mL^{-1}) with R^2 of 0.9944 for cTnI, $I_{CL} = 1412.7 + 638.9 \log c$ (ng mL^{-1}) with R^2 of 0.992 for CK-MB and $I_{CL} = 2137.4 + 530.7 \log c$ (ng mL^{-1}) with R^2 of 0.9911 for Myo, respectively. Here I_{CL} is the CL intensity, c is the target concentration in the incubation solution. The detection limits were calculated to be 0.006 ng/mL for cTnI, 0.067 ng/mL for CK-MB and 0.1 ng/mL for Myo, respectively (based on signal-to-noise ratio S/N = 3). Compared with the previously reported method, the as-prepared immunosensing biochip obviously showed a wide linear range and low detection limit, which provided an alternative strategy for simultaneously sensitive detection of three biomarkers related to AMI (Table 1).

3.3. Reproducibility, stability and specificity of the biochip

The reproducibility of the biochip was tested by separately detection three antigens in five wells on the same biochip at concentration of

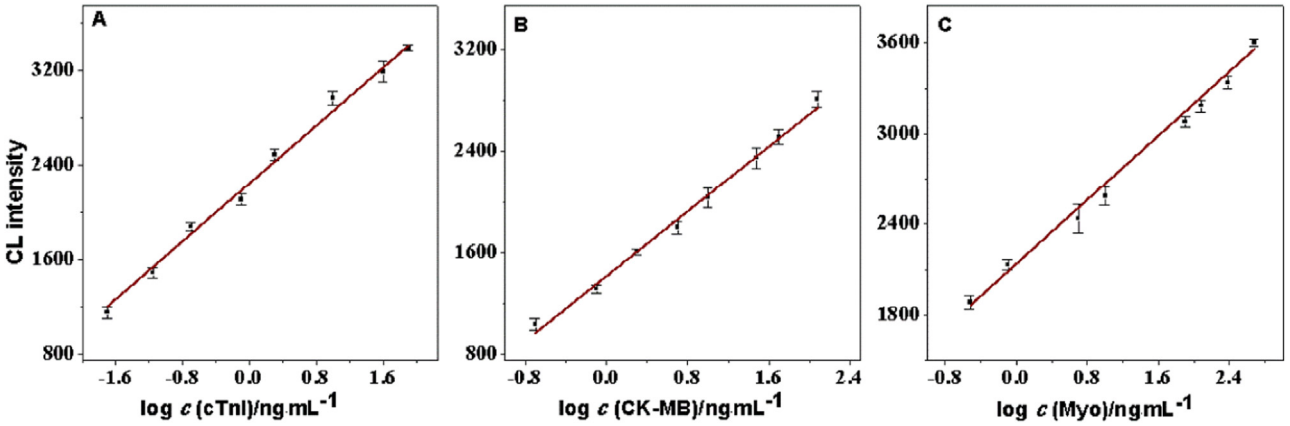

Fig. 3. Plots of CL intensity versus the concentrations of (A) cTnI with varied concentration of 0.02, 0.07, 0.2, 0.8, 2, 10, 40 and 80 ng/mL, (B) CK-MB at concentrations of 0.2, 0.8, 2, 5, 10, 30, 50 and 120 ng/mL, (C) Myo at concentrations of 0.3, 0.8, 5, 10, 80, 120, 240 and 480 ng/mL.

50 ng/mL cTnI, CK-MB and Myo, respectively. The maximum values of relative standard deviation (RSD) was calculated to be 2.5% for cTnI, 2.2% for CK-MB and 3.3% for Myo, respectively (Fig. S7A). The reproducibility of the biochip was also tested by using five different chips at concentration of 50 ng/mL cTnI, CK-MB and Myo, respectively. The maximum values of RSD were 3.5% for cTnI, 5.8% for CK-MB and 4.1% for Myo, respectively (Fig. S7B). After storing the biochip at 4 °C for three months, the CL intensity remained 97% of its initial response for cTnI, 95% for both CK-MB and Myo, respectively, showed an acceptable storage stability (Fig. S7C).

In order to verify the good specificity of the biochip, four other antigens (100 ng/mL TNF-α, 100 ng/mL CA19-9, 100 ng/mL AFP, 100 ng/mL BSA) and artificial plasma were respectively added to the microtiter plates to test whether other antigens co-existed in normal serum could interfere the detection of cTnI, CK-MB and Myo. As shown in Fig. 4A, after adding other antigens or artificial plasma, the influence of the detected CL intensity almost can negligible. Thus, the proposed biochip possessed good selectivity without apparent interference from nonspecific adsorption.

In addition, the total process of detecting the cardiac marker takes at most 70 min, including 60 min incubation time for two-step sandwich immunoreaction,10 min for washing and exposure for the CCD signal collection. Thus, the biochip has multiple advantages including a quick assay time, an easy assay procedure, small sample consumption and simultaneous detection of three different cardiac markers.

3.4. Simultaneous detection of cardiac biomarkers in actual serum

The proposed immunosensing biochip was applied to simultaneously detect cTnI, CK-MB and Myo in human serum samples. Five different samples were collected from patients in the Nanjing Drum Tower Hospital. After appropriate dilution of the patient's plasma, a series of cTnI, CK-MB and Myo with known concentration were separately added into the diluted plasma samples. As shown in Table S1, the recovery rates for the detection of cTnI in serum samples from 90% to 105%, 92% to 106.4% for CK-MB and 94.6% to 107% for Myo. Indicating that this method has good reliability for detection of cardiac markers (cTnI, CK-MB, Myo) in real serum samples.

In order to further evaluate the analytical reliability and clinical application potential of the microtiter plates, the concentrations of cTnI, CK-MB, Myo in serum samples were tested by comparing the results obtained from the prepared biochip with commercial ELISA kit test. Eight different human serum samples were collected from patients with acute myocardial infarction in the Nanjing Drum Tower Hospital. As shown in Fig. 4B, the values determined using the biochip for cTnI, CK-MB and Myo were in good agreement with the clinical experimental data (ELISA). Our approach could be a promising convenient strategy for cTnI, CK-MB and Myo detection and acute myocardial infarction diagnosis.

4. Conclusions

In this work, the simultaneous detection of three cardiac biomarkers (cTnI, CK-MB and Myo) related to acute myocardial infarction (AMI) was successfully carried out based on nanoprobes using a homemade immunosensing biochip, showing high sensitivity and specificity, good stability and sensing reproducibility. Nanoprobes were prepared by co-immobilization of SBP and the detection polyclonal antibodies on the surface of the silica nanoparticles. The detection sensitivity was dramatically enhanced because the large surface area of silica carriers increased the loading of SBP for per sandwiched immunoreactions and the excellent chemiluminescent performances of SBP in luminol-H_2O_2

Table 1
Comparison of the developed immunoassay with other immunoassays reported in literatures for the detection of cTnI, CK-MB and Myo.

	Measurement methods	Linear range(ng/mL)	Detection limit(ng/mL)	References
cTnI	Electrochemical immunoassay	0.8–5.0	0.5	(Vdovenko et al., 2010)
	Optomagnetic immunoassay	0.03–6.5	0.03	(Dittmer et al., 2010)
	Enzyme-linked immunosorbent asssys	0.1–100	0.027	(Cho et al., 2009)
	Electrochemiluminescence	0.02–80	0.006	This work
CK-MB	fluorescent microsphere lateral flow assay	500–2000	0.63	(Zhang et al., 2018a, 2018b)
	capillary-based chemiluminescence immunoassay	2.4–300	0.8	(Li et al., 2018)
	solution immersed silicon (SIS) assay	0.1–100	0.1	(Diware et al., 2017)
	Electrochemi luminescence	0.2–120	0.067	This work
Myo	capillary-based chemiluminescence immunoassay	0.33–81	1.2	(Diware et al., 2017)
	conductometric biosensor	5–2500	1.4	(Torabi et al., 2007)
	solution immersed silicon (SIS) assay	0.1–100	1	(Lee et al., 2011)
	Electrochemiluminescence	0.3–480	0.1	This work

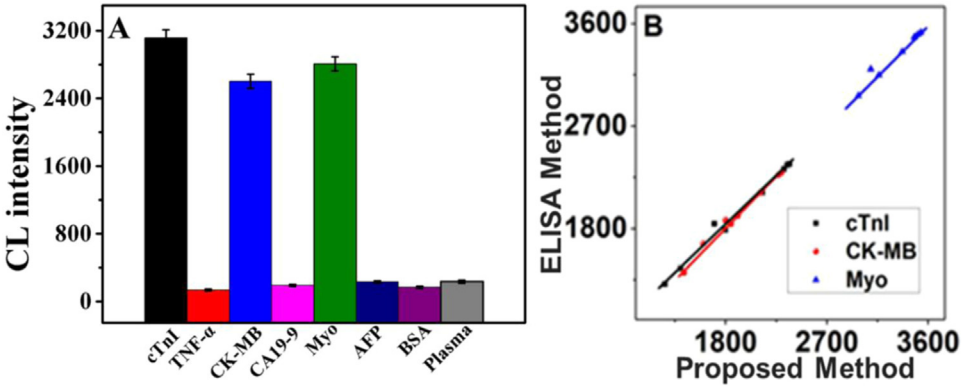

Fig. 4. (A) Specificity study of the proposed biochip. The CL intensity of the immunosensing biochip in the presence of 50 ng/mL cTnI, 100 ng/mL TNF-α, 50 ng/mL CK-MB, 100 ng/mL CA19-9, 50 ng/mL Myo, 100 ng/mL AFP, 100 ng/mL BSA and artificial plasma, respectively. (B) Comparison of the results of the proposed immunosensor chip with the clinical standard method (ELISA).

system. The detection of three biomarkers in real serum showed an acceptance precision with the standard clinical methods (ELISA). Therefore, the proposed immunosensing biochip was a promising convenient strategy for cTnI, CK-MB and Myo detection and provided a promising protocol for simultaneous determination of panel of markers in clinical applications.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (Nos. 21635004, 21627806 and 21705018), the Fundamental Research Funds for the Central Universities 2242017K3DN11).

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bios.2018.11.044.

References

Adams, J.E., Abendschein, D.R., Jaffe, A.S., 1993. Circulation 88, 750–763.
Alpeeva, I.S., Sakharov, I.Y., 2005. J. Agric. Food Chem. 53, 5784–5788.
Apple, F.S., Jesse, R.L., Newby, L.K., Wu, A.H.B., Christenson, R.H., 2007. Circulation 115, 352–355.
Caulum, M.M., Murphy, B.M., Ramsay, L.M., Henry, C.S., 2007. Anal. Chem. 79, 5249–5256.
Chen, C.L., Li, Y., Liu, S.Q., 2009a. J. Electroanal. Chem. 632, 14–19.
Chen, L.Y., Chen, C.L., Li, R.N., Li, Y., Liu, S.Q., 2009b. Chem. Commun. 2670–2672.
Cho, I.H., Paek, E.H., Kim, Y.K., Kim, J.H., Paek, S.H., 2009. Anal. Chim. Acta 632, 247–255.
Chon, H., Lee, S., Yoon, S.Y., Lee, E.K., Chang, S.I., Choo, J., 2014. Chem. Commun. 50, 1058–1060.
Chua, J.H., Chee, R.E., Agarwal, A., Wong, S.M., Zhang, G.J., 2009. Anal. Chem. 81, 6266–6271.
Dittmer, W.U., Evers, T.H., Hardeman, W.M., Huijnen, W., Kamps, R., de Kievit, P., Neijzen, J.H., Nieuwenhuis, J.H., Sijbers, M.J., Dekkers, D.W., Hefti, M.H., Martens, M.F., 2010. Clin. Chim. Acta 411, 868–873.
Diware, M.S., Cho, H.M., Chegal, W., Cho, Y.J., Kim, D.S., Won, O., S., Kim, K.S., Paek, S.H., 2017. Biosens. Bioelectron. 87, 242–248.
Giannitsis, E., Kurz, K., Hallermayer, K., Jarausch, J., Jaffe, A.S., Katus, H.A., 2010. Clin. Chem. 56, 254–261.
Hayes, M.A., Petkus, M.M., Garcia, A.A., Taylor, T., Mahanti, P., 2009. Analyst 134, 533–541.
Kapeluich, Y.L., Rubtsova, M.Y., Egorov, A.M., 1997. J. Biolumin. Chemilumin. 12, 299–308.
Kurita, R., Yokota, Y., Sato, Y., Mizutani, F., Niwa, O., 2006. Anal. Chem. 78, 5525–5531.
Lee, I., Luo, X.L., Cui, X.T., Yun, M., 2011. Biosens. Bioelectron. 26, 3297–3302.
Lee, I., Luo, X.L., Huang, J.Y., Cui, X.T., Yun, M., 2012. Biosensors 2, 205–220.
Li, Y.X., Guo, H., Hu, Y.M., Qi, X.Q., 2018. Int. J. Electrochem. Sci. 13, 287–295.
Liu, A.R., Zhao, F., Zhao, Y.W., Shangguan, L., Liu, S.Q., 2016. Biosens. Bioelectron. 81, 97–102.
Marchis, T., Cerrato, G., Magnacca, G., Crocella, V., Laurenti, E., 2012. Biochem. Eng. J. 67, 28–34.
Marzocchi, E., Grilli, S., Della Ciana, L., Prodi, L., Mirasoli, M., Roda, A., 2008. Anal. Biochem. 377, 189–194.
Masson, J.F., Obando, L., Beaudoin, S., Booksh, K., 2004. Talanta 62. pp. 865–870.
Montague, C., Kircher, T., 1995. Am. J. Clin. Pathol. 104, 472–476.
Purvis, D., Leonardova, O., Farmakovsky, D., Cherkasov, V., 2003. Biosens. Bioelectron. 18, 1385–1390.
Qian, J., Zhang, C.Y., Cao, X.D., Liu, S.Q., 2010. Anal. Chem. 82, 6422–6429.
Sakharov, I.Y., Vdovenko, M.M., 2013. J. Anal. Biochem. 434, 12–14.
Sakharov, I.Y., Alpeeva, I.S., Efremov, E.E., 2006. J. Agric. Food Chem. 54, 1584–1587.
Sakharov, I.Y., Berlina, A.N., Zherdev, A.V., Dzantiev, B.B., 2010. J. Agric. Food Chem. 58, 3284–3289.
Shen, W., Tian, D.Y., Cui, H., Yang, D., Bian, Z.P., 2011. Biosens. Bioelectron. 27, 18–24.
Sheng, Q.L., Qiao, X.J., Zhou, M., Zheng, J.B., 2017. Microchim. Acta 184, 1573–1585.
Stober, W., Fink, A., Bohn, E., 1968. J. Colloid Interf. Sci. 26, 62–69.
Tang, M., Zhou, Z.X., Li, S.G., Zhao, F., Liu, S.Q., 2018. Talanta 180, 47–53.
Torabi, F., Far, H.R.M., Danielsson, B., Khayyami, M., 2007. Biosens. Bioelectron. 22, 1218–1223.
Vdovenko, M.M., Della Ciana, L., Sakharov, I.Y., 2009. Anal. Biochem. 392, 54–58.
Vdovenko, M.M., Zubkov, A.V., Kuznetsova, G.I., Della Ciana, L., Kuzmina, N.S., Sakharov, I.Y., 2010. J. Immunol. Methods 362, 127–130.
White, H.D., Chew, D.P., 2008. Lancet 372, 570–584.
Wu, A.H.B., Jaffe, A.S., Apple, F.S., Jesse, R.L., Francis, G.L., Morrow, D.A., Newby, L.K., Ravkilde, J., Tang, W.H.W., Christenson, R.H., Cannon, C.P., Storrow, A.B., 2007. Clin. Chem. 53, 2086–2096.
Wu, Y.F., Chen, C.L., Liu, S.Q., 2009. Anal. Chem. 81, 1600–1607.
Zhang, D., Huang, L., Liu, B., Ni, H.B., Sun, L.D., Su, E.B., Chen, H.Y., Gu, Z.Z., Zhao, X.W., 2018a. Biosens. Bioelectron. 106, 204–211.
Zhang, J., Lv, X.F., Feng, W., Li, X.Q., Li, K.J., Deng, Y.L., 2018. Microchim. Acta 185, 364.
Zhao, F., Chai, D., Lu, J.S., Yu, J.C., Liu, S.Q., 2015a. Anal. Bioanal. Chem. 407, 6117–6126.
Zhao, F., Zhu, W., Su, J., Xue, Y.C., Wei, W., Liu, S.Q., 2015b. Curr. Anal. Chem. 11, 80–86.