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Ultrasensitive detection of nucleic acids and proteins using quartz crystal microbalance and surface plasmon resonance sensors based on target-triggering multiple signal amplification strategy

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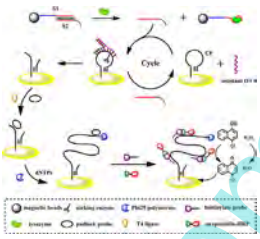
HIGHLIGHTS

- The detection of DNA and lysozyme was studied by quartz crystal microbalance and surface plasmon resonance sensors.
- The amplification process consists of TEHP, RCA and BCP.
- The proposed strategy exhibits an excellent specificity and is successfully applied in real sample assay.

GRAPHICAL ABSTRACT



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ABSTRACT

In this study, quartz crystal microbalance (QCM) and surface plasmon resonance (SPR) sensors were combined with template enhanced hybridization processes (TEHP), rolling circle amplification (RCA) and biocatalytic precipitation (BCP) for ultrasensitive detection of DNA and protein. The DNA complementary to the aptamer was released by the specific binding of the aptamer to the target protein and then hybridized with the capture probe and the assistant DNA to form a ternary "Y" junction structure. The initiation chain was generated by the template-enhanced hybridization process which leaded to the rolling circle amplification reaction, and a large number of repeating unit sequences were formed. Hybridized with the enzyme-labeled probes, the biocatalytic precipitation reaction was further carried out, resulting in a large amount of insoluble precipitates and amplifying the detection signal. Under the optimum conditions, detection limits as low as 43 aM for target DNA and 53 aM for lysozyme were achieved. In addition, this method also showed good selectivity and sensitivity in human serum.

1. Introduction

With the continuous development of analytical science, biosensing technology has been widely used in the life sciences in the field of various analytical testing processes. Biosensors have been developed for the analysis and detection of various biological small molecules, proteins, nucleic acids (DNA, RNA), ions, cells and so on [1–5], because of their fast detection, high sensitivity and specificity.

In order to improve the sensitivity of DNA biosensor, signal amplification technology has been widespread concern by the researchers [3,6,7]. Rolling circle amplification (RCA) is a trace

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molecular detection analysis method. After the RCA reaction, a single-stranded DNA having a large number of repeating sequence units complementary to the template strand is produced. The produced signal-stranded DNA facilitates binding to the signal probe, thereby enhancing the detection signal [8,9]. Recently, "Y" junction structure manipulated by the concept of a template-enhanced hybridization process (TEHP) has been used as an attractive strategy for quantitative DNA research. With the help of DNAzyme, the auxiliary probe and target can be regenerated and involved in another cycle to achieve signal amplification. The formation of a special structure can be transformed into a variety of signals for quantitative DNA detection [9–12].

Quartz crystal microbalance (QCM) was a sensing equipment whose principle was based on a phenomenon known as the reverse piezoelectric effect. When an alternating current (AC) voltage is applied to a pair of electrodes sandwiched between quartz crystals, the bulk acoustic waves of the quartz crystal produce the converse piezoelectric effect [13]. There are two main ways to transfer the analyte to the QCM electrode and measure the change in the resonant frequency of the quartz. One method known as the "dip and dry" method is to calculate the crystal resonant frequency by measuring the resonant frequency of the crystal twice. The second approach to delivering an analyte and measuring frequency changes includes flow injection analysis (FIA) systems.QCM biosensors were widely used in the detection of genes [14], proteins [15], cells [16], enzyme activities [17] and biomolecules [18] with the characteristics of real-time analysis and tag-free [19]. In the optical detection and analysis system, the surface plasmon resonance technique (SPR) is mainly used to quantitatively analyze biomolecules by changing the thickness of the medium or changing the refractive index near the metal surface, which has the advantages of rapid, real-time and in-situ detection of biomolecules [20–22].

In the present study, a novel quartz crystal microbalance combined with surface plasmon resonance measurement is demonstrated to sensitive and selective detection of DNA and lysozyme. The detection process consists of aptamer recognition strategy, template enhanced hybridization processes, rolling circle amplification and biocatalytic precipitation. The amplification process was triggered aptamer recognition of target molecule, allowing the released complementary strand to be used as the catalyst of TEHP. With the help of DNAzyme, the RCA reaction was occurred. The large number of HRP are captured on the electrode surface which further stimulate the biocatalytic precipitation (BCP) [23] onto the electrode surface for signal amplification, producing insoluble products on the electrode surface that changes the sensor response greatly.

2. Experimental

2.1. Materials and reagents

The oligonucleotides required for the experiment and HRP conjugated Streptavidin were purchased from Sangon Biotech Co. Ltd. (Shanghai, China), and the DNA sequences required are shown in Table S1(see supplementary material), Phi29 DNA polymerase (5 U μL^{-1}) and T4 ligase (5 U μL^{-1}) were purchased from Thermo Scientific. Mixture of four dNTPs (2.5 mM) and 4-Chloro-1-Naphthol (4-CN) were obtained from the SBS Genetech. Co. Ltd. (Shanghai, China), and the Nb.BbvCI (10000 U mL^{-1}) were purchased from the NEW ENGLAND Biolabs (NEB). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were purchased from Shanghai Aladdin Biochemical Technology Co. Ltd. Lysozyme, thrombin and Bovine serum albumin (BSA) were purchased from Sigma-Aldrich. (Shanghai, China). The Carboxyl-modified magnetic beads (size: 0.4–0.5 μm) were purchased from Tianjin Baseline Chromtech Research Centre (China). Hybridization buffer (pH 7.4) contained Tris (10 mM), ethylenediaminetetraacetic acid (EDTA) (1 mM), and NaCl (100 mM). Rinsing buffer (pH 7.4) contained Tris (10 mM), EDTA (1 mM), NaCl (100 mM), and Tween-20 (0.1%). All other reagents used in the experiments were of analytical grade.

2.2. Apparatus and methods

Measurements were made using a Q-Sense E1 QCM-D instrument (Q-Sense AB, Västra Frölunda, Sweden), which could provide real-time response at multiple video frequencies. Gold coated quartz crystals having a fundamental resonance frequency of 5 MHz were purchased from Q-Sense. Atomic force microscopy (AFM) images were taken with the Being Nano-Instruments CSPM-4000 (Benyuan, China). SPR detection was performed on a SPRNavi200 (BioNavis, Finland). All measurements were carried out at room temperature unless otherwise indicated. The gold electrode surface was cleaned with hydrogen peroxide (30%), ammonia–water (25%), and ultrapure water at a volume ratio of 1: 1: 5. The electrode was placed on a washing rack and immersed in a cleaning beaker of electrode cleaning solution for 15 min at 75 °C. Electrode was rinsed with the ultrapure water and then blown dry with the nitrogen gas. 30 μL of capture probe DNA (CP, 100 nM) was incubated with a pretreated gold electrode at 37 °C for about 16 h. The gold electrode was washed three times with rinsing buffer and ultrapure water respectively, and then dried with nitrogen gas. After immobilization of CP DNA, 1% BSA was incubated for 2 h to passivate the gold electrode. Again, the gold electrode was washed with rinsing buffer and ultrapure water and then dried with nitrogen gas.

2.3. Immobilization of DNA onto magnetic beads

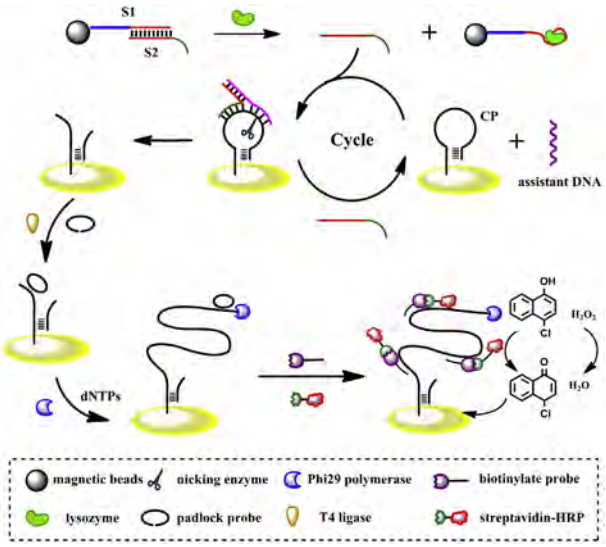
The process of the immobilization of DNA onto magnetic beads (MBs) was enforced as follows: First, 30 μL Carboxyl-modified magnetic beads were washed three times with 100 μL imidazole-hydrochloric acid buffer (0.1 M, pH 6.8), and then activated in 100 μL imidazole buffer containing EDC (0.2 M) by gentle shaking for 30 min. 15 μL amino-modified DNA S1 (100 nM) was mixed with the activated magnetic beads by gentle mixing for 16 h at 37 °C. The DNA MB-S1 were rinsed three times with 200 μL rinsing buffer, and then magnetically separated. Finally, Then DNA S2 was added to MB-S1 and the mixture was gently stirred at 37 °C for 1 h, hybridized to form MB-S1-S2 complex. The excess DNA reagent was removed by washing three times and magnetic separation.

2.4. Template enhanced hybridization process

The TEHP reaction was initiated by addition of 15 μL assistant DNA (100 nM), 1 μL of Nb.BbvCI (10 U μL^{-1}), 3 μL 10 × NEB buffer and varying concentrations of target DNA, and incubated for 90 min at 37 °C. The target DNA was obtained as follows: varying concentrations of lysozyme was added to MB-S1-S2 and the reaction was incubated at 37 °C for 1 h under shaking. After magnetic separation, the supernatant which contained target DNA was kept at 4 °C before use. The well was washed three times with 100 μL of rinsing buffer and three times with 100 μL of ultrapure water, respectively.

2.5. Rolling circle amplification reaction

21.5 μL of pad-lock probe (100 nM), 1 μL T4 ligase (5 U μL^{-1}) and T4 DNA ligase buffer were applied to the gold electrode and incubated at 37 °C for 1 h. Excess reagents containing non-



Scheme 1. Schematic representation of DNA and proteins detection based on target-triggered multiple signal amplification strategy.

complementary DNA were removed by washing three times with rinsing buffer and twice with ultrapure water. The RCA was performed in reaction solution which contained 2 μL of dNTPs (10 mM), 2.5 μL phi29 DNA polymerase, 3 μL phi29 buffer and ultrapure water. The reaction solution was added to the gold electrode and incubated at 37 °C for 2 h, and then gold electrode was cleaned as above. The RCA products were hybridized with 30 μL biotin modified signal probe (100 nM) on the substrate at 37 °C for 1 h. After rinsing, 30 μL of HRP conjugated streptavidin (50 nM) was transferred to the electrode surface and incubated at 37 °C for 30 min.

2.6. BCP reaction and QCM detection

In the QCM detection, 500 μL of the BCP solution containing 4-CN (1 mM) and H_2O_2 (1 mM) were added to the system reaction solution. The resonance frequency of the QCM was recorded until equilibrium was reached. The experimental temperature was controlled at 37 °C.

3. Results and discussion

3.1. Design of strategy

In this experiment, a new method for ultrasensitive detection of DNA and protein based on TEHP, RCA and BCP was designed, and detection principle of lysozyme was shown in Scheme 1. The aptamer S1 is immobilized on the magnetic beads and hybridizes with its complementary strand S2 to form a duplex. In the presence of lysozyme, the conformation of S1 was changed to bind lysozyme molecules and DNA S2 (i.e., the target DNA) spontaneously released from duplex which further triggered the TEHP. The target DNA, assistant DNA and capture probe DNA hybridized to form a stable "Y" junction structure. One portion of the target DNA was complementary to the assistant probe and the other portion was complementary to the capture probe. This structure had a specific recognition site for the nicking endonuclease Nb.BbvCI and was cleaved to form a CP fragment. Cyclic amplification was achieved by hybridization of free assistant DNA and target DNA with another CP, which formed a large number of CP fragments under the nicking endonuclease Nb.BbvCI. On the contrary, in the absence of target DNA, although assistant DNA had some matched bases with the CP, they could not form a stable double-stranded structure due to the short matched nucleotide bases.

After TEHP, the circular template was formed by specific ligation of the padlock probe under T4 ligase. The RCA reaction was initiated by the addition of Phi29 DNA polymerase and dNTPs, and a single

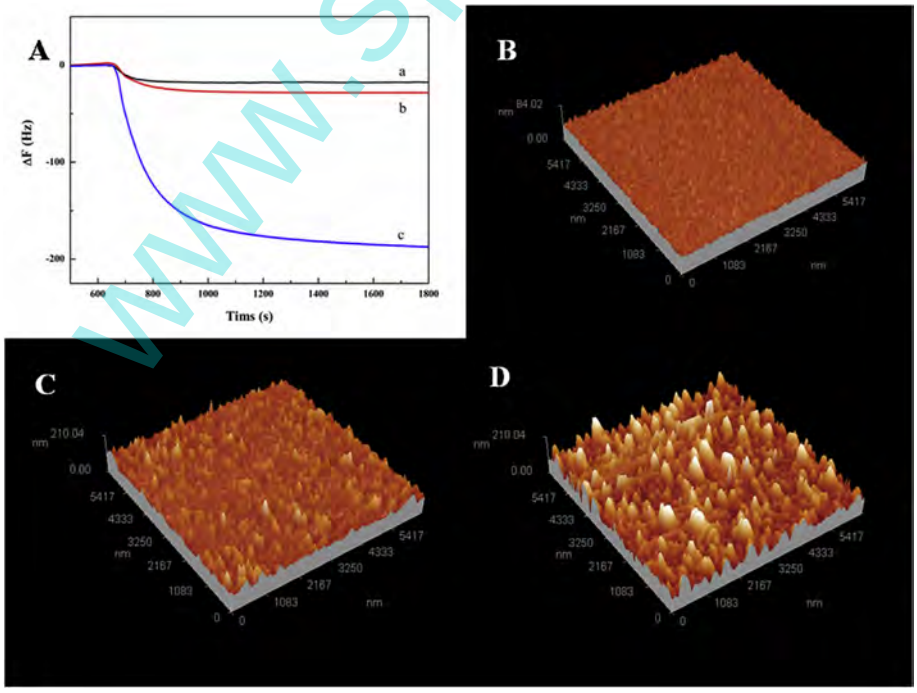


Fig. 1. (A) Real-time frequency shifts of amplified QCM biosensor to control samples: (a) in the absence of target DNA; (b) in the absence of T4 DNA ligase, polymerase and the nicking enzyme Nb.BbvCI; (c) in the presence of target DNA, polymerase, nicking enzyme, and the concentration of target DNA is 1.0×10^{-12} M. AFM topography images of the crystal surface: (B) The capture probes immobilized on the surface. (C) After the RCA reaction. (D) After the final biocatalytic precipitation reaction.

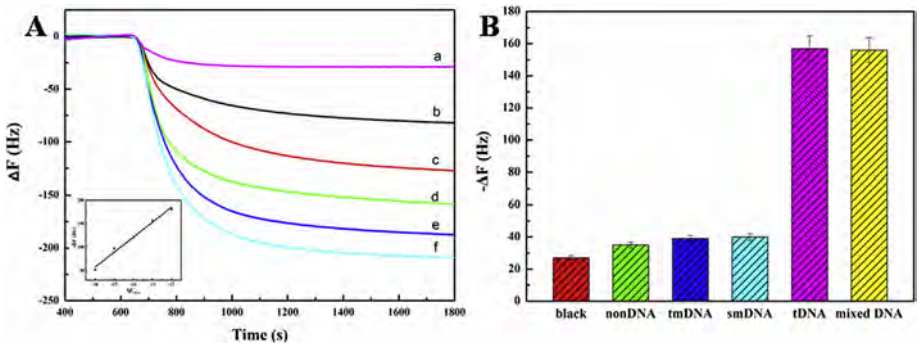


Fig. 2. (A) Real-time frequency responses of amplified QCM biosensor for detection of DNA. The concentrations of target DNA were as follows: (a) 0, (b) 1.0×10^{-16} M, (c) 1.0×10^{-15} M, (d) 1.0×10^{-14} M, (e) 1.0×10^{-13} M, (f) 1.0×10^{-12} M. Inset: Linear relationship between the frequency shifts and the DNA concentrations. Error bars are standard deviation of three repetitive measurements. (B) The frequency response after addition of target DNA or mismatched DNA. The concentration of target DNA: 1.0×10^{-14} M. The concentration of mismatched DNA: 1.0×10^{-12} M. The mixed DNA contained 1.0×10^{-14} M target DNA and other mismatched DNA at the concentrations of 1.0×10^{-12} M.

strand of DNA containing a large number of repeat sequences was generated. The biotin-modified signal probe hybridized to these repeats and bound the streptavidin-HRP to the surface of the gold electrode through the recognization between biotin and streptavidin. In the presence of H_2O_2, the HRP-biocatalyzed oxidation of 4-chloro-1-naphthol occurred. Therefore, a large amount of precipitate deposited on the surface of the gold electrode, sensor response signal has been significantly strengthened.

3.2. Feasibility of the assay

In order to prove the feasibility of DNA detection program, a control experiment was conducted. In the absence of target DNA S2, the system was unable to generate template-enhanced hybridization and RCA reactions. Therefore, the frequency shift was very small (Fig. 1A curve a). In the presence of the target DNA S2 but without the nicking endonuclease Nb.BbvCI, T4 ligase and Phi29 DNA polymerase, the RCA primer probe was not exposed and RCA couldn't occur. The result was negligible (Fig. 1A curve b). In the presence of very low concentrations of target DNA, the frequency shift was significantly increased, indicating that the amplification reactions of TEHP, RCA and BCP were occurred (Fig. 1A curve c).

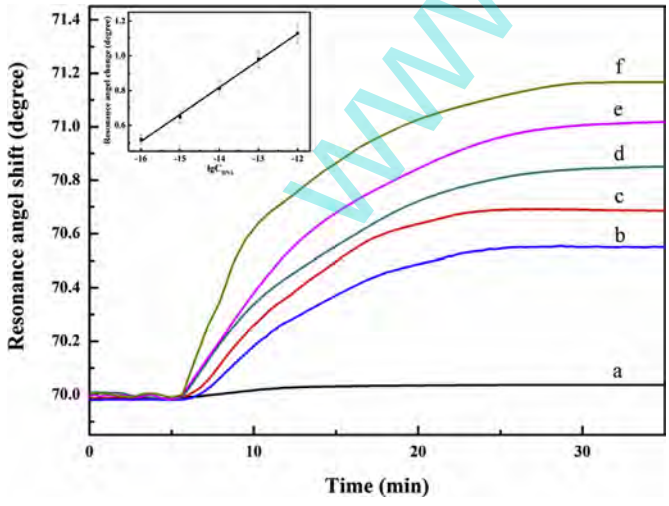


Fig. 3. Real-time sensor responses of amplified SPR biosensor for detection of DNA. The concentrations of target DNA were as follows: (a) 0, (b) 1.0×10^{-16} M, (c) 1.0×10^{-15} M, (d) 1.0×10^{-14} M, (e) 1.0×10^{-13} M, (f) 1.0×10^{-12} M. Inset: Linear relationship between the sensor response signal and the DNA concentrations. Error bars are standard deviation of three repetitive measurements.

3.3. AFM measurement

The gold surface morphology was determined by AFM. The capture probe was immobilized on the gold surface as shown in Fig. 1B. After the RCA reaction, the surface of the gold showed many of uneven distribution of the "island" (Fig. 1C). Finally, after the end of the biocatalytic precipitation reaction, the gold surface showed a high "peak" indicating that the biocatalytic precipitation was successfully captured onto the gold surface (Fig. 1D).

3.4. Sensitivity of the DNA assay

Under the optimal experimental conditions described in the Supplementary material, different concentrations of target DNA were determined by QCM. As the concentration of target DNA increased, the final frequency signal increased significantly, and the influence of target DNA concentration on frequency shift was shown in Fig. 2A. As shown in Fig. 2A (inset), the linear relationship between the frequency shift and the logarithm of the removal of blank DNA concentration ranged from 1.0×10^{-16} M to 1.0×10^{-12} M, with a linear correlation coefficient of 0.9942. The detection limit was estimated to be 43 aM (3σ, n = 11), which was lower than that reported previously in the literature (Table S2 of Supplementary material). A relative standard deviation (RSD) was calculated to be 4.6% by serially measuring 1.0×10^{-15} M target DNA for 11 repetitive determinations, indicating that a high accuracy and reproducibility of the proposed method were acceptable. The limit of quantification (LOQ) was 0.14 fM (10σ) under the optimum conditions.

3.5. Selectivity of the DNA analysis

The selectivity of the target DNA was examined by a control experiment. As shown in Fig. 2B, single base mismatched DNA, triple mismatched base sequence DNA and complete mismatched sequence DNA were selected for detection. Although the concentration of all the mismatched base sequence DNA were two orders of magnitude higher than the concentration of the target DNA, the frequency shift signal was much lower than the target DNA. When the three mismatched bases were mixed, the frequency shift signal did not change with respect to the target DNA, indicating that the method had good selectivity to the target DNA.

3.6. Surface plasmon resonance detection

The target-triggered multiple signal assay in combination with

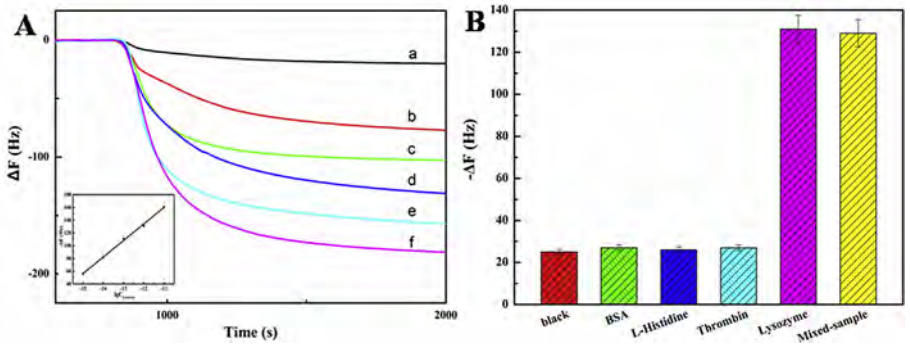


Fig. 4. (A) Real-time frequency responses of amplified QCM biosensor for detection of lysozyme. The concentrations of lysozyme were as follows: (a) 0, (b) 1.0×10^{-15} M, (c) 1.0×10^{-14} M, (d) 1.0×10^{-13} M, (e) 1.0×10^{-12} M, (f) 1.0×10^{-11} M. Inset: Linear relationship between the frequency shifts and the lysozyme concentrations. Error bars are standard deviation of three repetitive measurements. (B) Selectivity analysis of the proposed aptasensor. The concentration of lysozyme: 1.0×10^{-13} M. The concentration of BSA, L-histidine and thrombin: 1.0×10^{-11} M. The mixed sample contained 1.0×10^{-13} M lysozyme and BSA, L-histidine and thrombin at the concentrations of 1.0×10^{-11} M.

Table 1
Comparison of different methods for lysozyme detection.

Detection modes	Transducer	Dynamic range	Detection limit
autonomous DNA machine	Electrochemical	1pM–80pM	0.52 pM [24]
DNA base electrooxidation	Electrochemical	0.86–2.6 μM	18 nM [25]
[Fe(CN)6]III-probe impedance	Electrochemical	3.5–17.5 pM	0.14 pM [26]
perylene-probe aggregation	Fluorescent	0.5–10.5 nM	70 pM [27]
CPE-aptamer-SiNP	Fluorescent	1.5–22.5 μM	25 nM [28]
aptamer-based network of DNA-related reaction cycle	Fluorescent	10–200 fM	3.6 fM [29]
magnetic-nanoparticle aggregation	magnetic relaxation switch	0.5–80 nM	0.5 nM [30]
peroxidase DNAzyme-associated aptasensor	colorimetric	0.1 fM–1 pM	0.1 fM [31]
conjugate EuIII complex and aptamer-wrapped carbon nanotubes	luminescent	10 nM–2.0 μM	0.9 nM [32]
vertically ordered mesoporous silica film-assisted label-free	electro chemiluminescence	0.1 nM–1.0 μM	0.06 nM [33]
tris(bipyridine)ruthenium(II) complexcontaining multiple cyclodextrins	electro chemiluminescence	0.1 nM–0.5 μM	48 pM [34]
Present study	QCM	1 fM–10 pM	53 aM

biocatalytic precipitation amplification was further implemented in the SPR biosensor. Surface Plasmon Resonance (SPR) technology was mainly through the metal surface near the detection of changes in dielectric thickness or refractive index changes in the quantitative analysis of biomolecules. The SPR biosensor showed amplified responses to target DNA of various concentrations (Fig. 3). As shown in Fig. 3 (inset), the linear relationship between the logarithm of target DNA concentration and SPR response signal values ranged from 1.0×10^{-16} M to 1.0×10^{-12} M, with a linear correlation coefficient of 0.9992. The detection limit of 44 aM was estimated using 3σ. These results showed that the system was successfully applied to the SPR biosensor technology for the target DNA detection.

3.7. Analytical performance of lysozyme detection

A series of lysozymes with different concentrations were tested in order to demonstrate that the proposed strategy had a high sensitivity for protein detection. The QCM biosensor showed amplified responses to lysozyme of various concentrations (Fig. 4A). A linear relationship between the frequency shift and the logarithm of lysozyme concentrations ranged from 1.0×10^{-15} M to 1.0×10^{-11} M (Fig. 4A inset), with a linear correlation coefficient of 0.9929. The detection limit was estimated to be 53 aM by using 3σ, which was lower than that reported previously (Table 1). The LOQ was 0.18 fM under the optimum conditions.

In order to further study the selectivity of QCM biosensors, a series of controlled experiments were performed with three non-target proteins: bovine serum albumin (BSA), L-histidine and thrombin. Results as shown in Fig. 4B, by comparing QCM detection signals of target lysozyme with non-target protein, even if the concentration of non-target protein is 100 times of the lysozyme concentration, the detection signal obtained was much lower than that of low concentration lysozyme detection signal. When the three non-target proteins were mixed, the frequency shift signal did not change with respect to the lysozyme. So the QCM aptamer sensor exhibited good selectivity based on the specificity of the aptamer to target proteins and high affinity.

3.8. Practical determination of lysozyme in serum samples

In order to verify the feasibility of the method in practical clinical application, the detection of lysozyme in human serum was carried out. Serum samples were centrifuged and serially diluted prior to analysis, followed by addition of lysozyme at various concentrations to human serum samples. As show in Table S3 of Supplementary material, the spiked recovery was 95.5–97.4% with a relative standard deviation (RSD) of less than 6.1%. The experimental results show that the method has the potential of clinical application and can be used for the analysis and detection of complex biological samples.

4. Conclusion

In this study, a novel ultra-sensitive detection system was proposed, which combined multiple signal amplification methods of TEHP, RCA and BCP. The detection limits as low as 43 aM for target DNA and 53 aM for lysozyme were obtained. Due to the high affinity and specificity of the aptamer for the target molecule, the assay provided excellent selectivity and good analytical performance in the actual human serum samples, providing a useful tool for the analysis of clinical and complex biological samples. In

addition, given the unique and attractive characteristics, the QCM and SPR biosensor systems provided a novel method for analysis of DNA and proteins with appropriate aptamer.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.aca.2017.04.047.

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