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Quartz crystal microbalance detection of protein amplified by nicked circling, rolling circle amplification and biocatalytic precipitation

Xiaoru Zhang [a], Jiao Chen [a], Hongxia Liu [a], Shusheng Zhang [b,*]

[a] Key Laboratory of Sensor Analysis of Tumor Marker, Ministry of Education, College of Chemistry and Molecular Engineering, Qingdao University of Science and Technology, Qingdao 266042, PR China

[b] Key Laboratory of Detection Technology of Shandong Province for Tumor Marker, College of Chemistry and Chemical Engineering, Linyi University, Linyi 276005, PR China

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ABSTRACT

A novel quartz crystal microbalance (QCM) assay was described for sensitive detection of protein. Lysozyme was used as a model of protein. To enhance the sensitivity of this QCM biosensor, biocatalytic precipitation (BCP) reaction combined with strand-scission cycle and rolling circle amplification (RCA) were applied together for the first time. As a result of the multi-signal amplification in this aptasensor, the detection limit for lysozyme was down to 0.3 fM. What is more, this amplified QCM biosensor also showed good selectivity and practical usage in human serum.

1. Introduction

QCM sensor is a piezoelectric device that uses the frequency shifts on quartz crystals to measure the changes of mass on the surface (Janshoff et al., 2000). Measurements can be performed in real time without any supplementary labeling and has been widely applied in biochemical analysis (Pei et al., 2007). However QCM measurement is often insufficient to produce a large signal change for trace detection due to the relatively small number of targets specifically bound to the QCM surface (Abad et al., 1998). To further increase the mass load on the surface of quartz crystals and amplify the frequency shift aimed at the trace analysis, several signal amplification strategies have been developed (Tang et al., 2012), for example the usage of gold nanoparticle (Song et al., 2014), enzyme (Fei et al., 2011), liposome (Patolsky et al., 2000) or self-assembled DNA nanostructure (Tang et al., 2012) as an efficient mass amplifier. Among them, enzymatic biocatalytic precipitation (BCP), involving the formation of insoluble products on electronic transducers (Yang et al., 2014), has been utilized as an important amplification route for sensing and biorecognition events. Now, BCP reaction has been used in the field of faradaic impedance spectroscopy (Hou et al., 2014), photoelectrochemical measurement (Zhao et al., 2012; Zhang et al., 2014) as well as QCM analysis (Fei et al., 2011; Feng et al., 2007).

Rolling circle amplification (RCA) is a simple and efficient isothermal enzymatic process that utilizes unique DNA and RNA polymerases to generate a long single DNA/RNA strand within a short period of time (Ali et al., 2014, Ji et al., 2012). This amplification method is achievable at a constant temperature, in solution, on a solid support or in a complex biological environment without specialized instrumentation (Zhao et al., 2008). Herein, we develop a sensitive QCM biosensing platform by combining three effective amplification strategies—nicked circling, RCA and BCP reactions, to develop a new method for straightforward and cost-effective detection of protein *via* aptamer-based bioassay. To the best of our knowledge, such amplified QCM aptasensor has not yet been reported.

2. Experiment

2.1. Materials and apparatus

2.1.1. Materials

Deoxyribonucleoside 5′-triphosphates (dNTPs) mixture and all oligonucleotides as depicted in Table S1 of Supporting information were purchased from SBS Genetech. Co. Ltd. (China). The Phi29 DNA polymerase (10,000 U/mL) and T4 DNA ligase (5000 U/mL) were obtained from Thermo Scientific. The restriction endonuclease Nb.BbvCI (10,000 U/mL) was purchased from NEW ENGLAND Biolabs (NEB). 4-Chloro-1-Naphthol (4-CN), lysozyme,

* Corresponding author. Fax: +86 539 8766107.
E-mail address: shushzhang@126.com (S. Zhang).

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thrombin, bovine serum albumin (BSA), and the IgG were purchased from Sigma-Aldrich. HRP conjugated Streptavidin was purchased from Sangon Biotech Co., Ltd. (China). The carboxyl-modified magnetic beads (MBs, 1.0–2.0 μm) were purchased from Tianjin Baseline ChromTech Research Centre (China). Milli-Q-deionized water was used throughout the experiments.

The stock solution of 4-CN was prepared by dissolving 4-CN in ethanol to give ethanolic solution of 0.05 M. Then, 1 mL ethanolic solution prepared above was diluted with 0.1 M phosphate buffered saline (PBS, pH 7.4) to 50 mL.

2.1.2. Apparatus

The resonance frequency was monitored by Q-Sense E1 QCM-D instrument (Q-Sense AB, Västra Frölunda, Sweden). QCM experiments were performed at a 5 MHz AT-cut quartz crystal (Q-Sense E1). The tapping mode AFM images were obtained using a Being Nano-Instruments CSPM-4000 (Benyuan, China) operating under ambient conditions. The imaging measurements were performed using AN-NSC10 Probe (NTI Co. Ltd., force constant 40 N/m, driving frequencies in the range of 220.4–394.6 kHz). The ratio of the setpoint and free cantilever vibration amplitudes Asp/A0 was maintained at 0.7–0.8. AFM was operated at a scan speed of 2 Hz.

Impedance measurements were carried out on CIMPS-1 electrochemical workstation (Germany). The supporting electrolyte is 10 mM PBS containing 0.1 M KCl and 2.5 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ as a redox couple.

2.2. Assembly on magnetic beads

Preparation of MB–aptamer–S1 conjugates was described in Supplementary material. During test, different concentrations of lysozyme (100 μL) were added to the conjugated MBs system. After shaking at 37 °C for 60 min and magnetic separation, the supernatant containing the released DNA S1 (100 μL) was obtained and kept at 4 °C before use.

2.3. Assembly on quartz crystal

Before use, the crystal chips were cleaned thoroughly by immersion in piranha solution (30% H_2O_2/70% concentrated H_2SO_4), followed by rinsing with deionized water and drying under a nitrogen stream. The pre-treated gold electrode was then incubated with 30 μL of capture probe DNA (S2, 100 nM) in pH 7.4 PBS at 37 °C for 16 h in a humidity chamber. After DNA S2 immobilization, 1% BSA was added and incubated for 2 h to passivate the electrode. After each step of fabrication process, the gold electrode was washed three times with pH 7.4 PBS and deionized water respectively, then dried under N_2 atmosphere.

A series of 25 μL volumes of reaction mixture containing the 21.5 μL DNA S1 supernatant obtained above, 2.5 μL 10 × NEB buffer and 1 μL Nb.BbvCI (10 U/μL) was dropped onto the electrode surface and incubated at 37 °C for 2 h.

2.4. RCA and BCP reaction

21.5 μL of padlock DNA (S3, 10 nM), 2.5 μL T4 DNA ligase buffer and 1 μL T4 DNA ligase (5 U/μL) were mixed in a centrifugal tube, then this mixture was transferred to the electrode surface and incubated at 22 °C for 1 h. Subsequently, the electrode was washed with PBS buffer and doubly distilled water, followed by drying under nitrogen gas. The RCA reaction was carried out by adding a mixture of 19 μL H_2O, 2.5 μL 10 mM dNTPs, 2.5 μL Phi29 DNA polymerase buffer and 1 μL Phi29 DNA polymerase (10 U/μL) onto the electrode. After incubation at 37 °C for 1.5 h, the electrode with the long single DNA strands produced by RCA was washed as before.

An aliquot of 25 μL biotin modified detection probe (S4, 10 nM) in PBS was dropped onto the electrode to form lots of DNA duplexes. After incubation at 37 °C for 30 min and washing, 25 μL HRP conjugated streptavidin (50 nM) was transferred to the electrode surface and incubated again at 37 °C for 30 min. Finally, the electrode was thoroughly rinsed as described above.

2.5. QCM measurement

During QCM measurement, 300 μL of the BCP solution containing 1 mM 4-CN and 1 mM H_2O_2 was pumped into reaction pool. The resonance frequency of the QCM was recorded until equilibrium was reached. The temperature was controlled at 25 °C throughout the measurement.

3. Results and discussion

3.1. Strategy for amplified QCM detection and its feasibility

This strategy can be used for the detection of nucleic acid as well as protein when aptamer was used. By combination of DNA nicked circling, RCA and BCP, a sensitive QCM detection of lysozyme was developed and described in Fig. 1A. Aptamer immobilized on MBs and its complementary oligonucleotides S1 were first hybridized to form a rigid duplex. When target lysozyme was added, due to the recgnition between aptamer and lysozyme, the single-stranded DNAs S1 were released and acted as triggers in following reaction. The released DNA S1 was then introduced to the detection system and hybridized with hairpin DNA S2 immobilized on gold electrode. The formed duplex DNA (S1/S2) contained a restriction endonuclease Nb.BbvCI recognition site, which led to the scission of DNA S2. Then the thermally unstable structure DNA S1 spontaneously dissociated and amplification was accomplished by the hybridization of free DNA S1 with another hairpin DNA S2, which led to the strand-scission cycle. In this way, one trigger DNA S1 results in the cleavage of many DNA S2 and large quantities of hairpin fragments are formed.

After incubating hairpin fragment immobilized on the gold electrode with padlock probe S3 and T4 ligase, the circular template was formed. The following RCA reaction could be initiated by adding Phi29 DNA polymerase and dNTPs to form a long single-stranded DNA, which stimulated the binding of abundant biotin-labeled DNA S4. Finally, the streptavidin-HRP could be captured onto the assembled 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 and the formed precipitation deposited on the gold electrode surface was determined by QCM. Through the nicked circling, rolling cycle amplification reaction and biocatalytic precipitation, the QCM signals were significantly enhanced. Thus, a sensitive QCM biosensor for the detection of lysozyme was obtained.

As a proof-of-concept experiment, a series of control experiments was monitored (shown in Fig. 1B). In the absence of lysozyme, no DNA S1 was produced. While, in the presence of target lysozyme but no nicking enzyme Nb.BbvCI, T4 DNA ligase and Phi29 DNA polymerase, the formed duplex DNA (S1/S2) could not be cut. In both of these cases, RCA primer probe cannot be liberated and the RCA reaction cannot be preceded, let alone the incorporation of HRP probes. Therefore, the frequency shifts were negligible (curves *a* and *b*). In contrast, by the combination of DNA nicked circling, RCA and BCP amplification system, even when the concentration of lysozyme was as small as 1.0×10^{-13} M, frequency shift increased greatly (curve *c*), which indicating the proposed QCM strategy could be amplified by cycle system and enzymatic biocatalytic reaction.

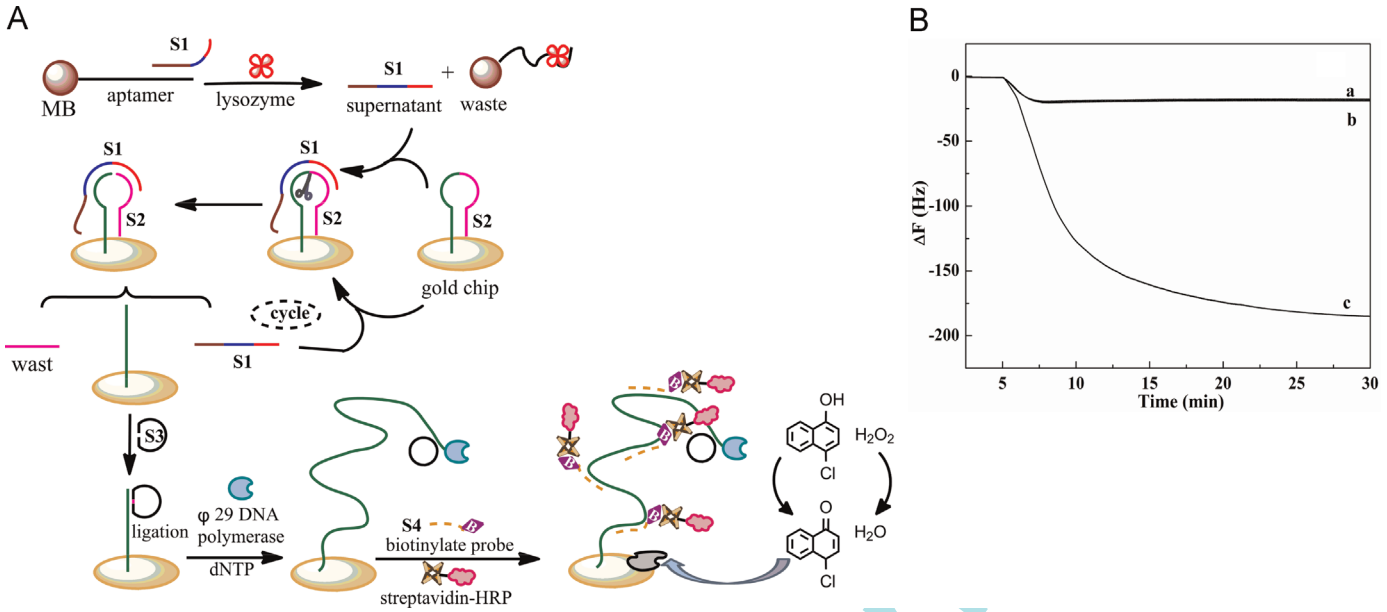

Fig. 1. (A) Schematic diagram of the amplified detection of lysozyme. (B) Real-time frequency shifts of amplified QCM biosensor to control samples: (a) In the absence of target lysozyme; (b) In the presence of 1.0×10^{-13} lysozyme, but in the absence of nicking enzyme, ligase and polymerase; and (c) In the presence of target lysozyme, nicking enzyme, ligase and polymerase, the concentration of lysozyme is 1.0×10^{-13} M.

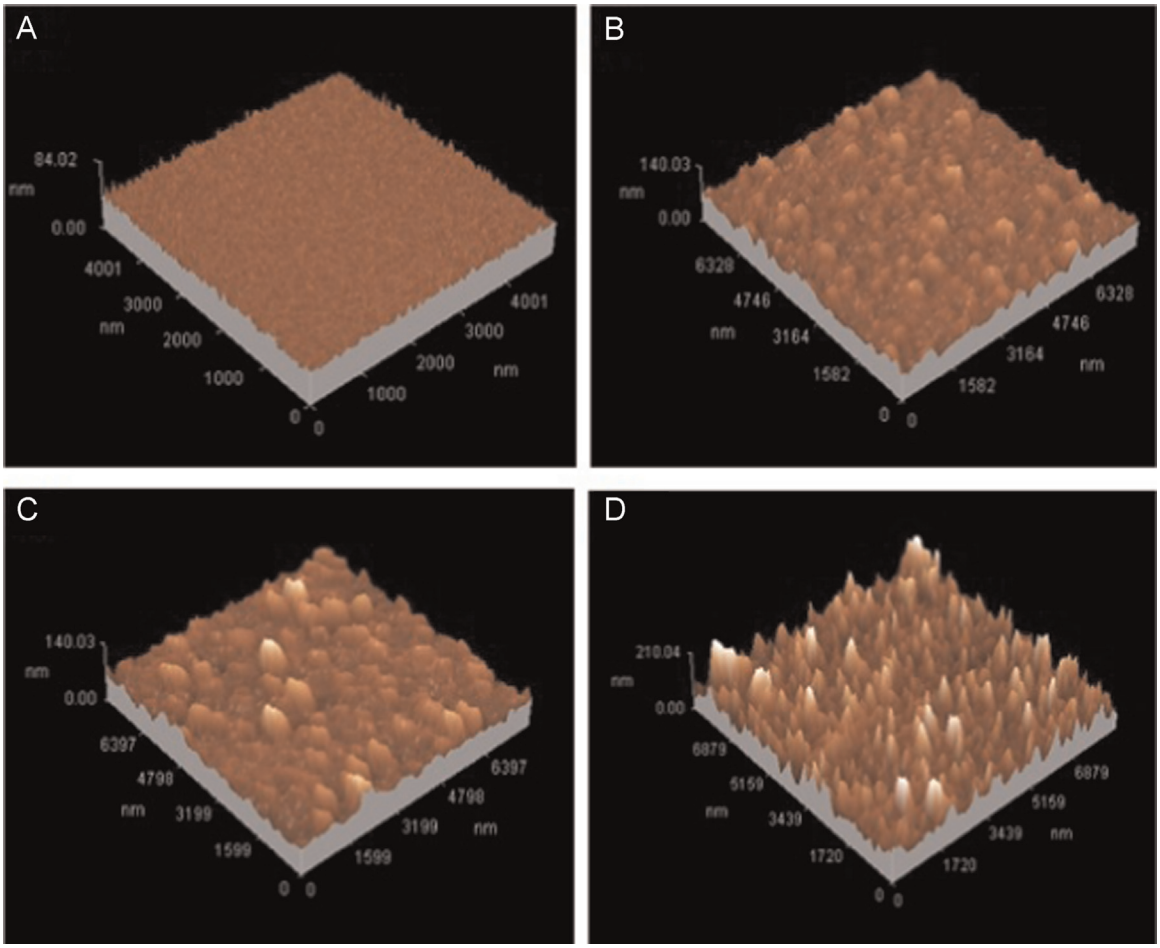

Fig. 2. AFM topography images of the crystal surface. (A) The capture probes immobilized on the surface. (B) After BSA blocking and further the RCA reaction. (C) After labeling of HRP conjugated streptavidin. (D) After the final 20 min HRP induced BCP. The concentration of lysozyme was 1.0×10^{-13} M.

3.2. EIS measurement

The electrochemical impedance spectroscopy (EIS) could provide further information on the impedance changes of the electrode surface during the modification process. As seen from Fig. S1 of Supplementary material, only a small semicircle could be observed at the bare electrode. With the increase of electrode material, which could block the electron transference of the redox probe at the electrode interface, the diameter of the semicircle was growing bigger. Therefore, after the immobilization of the capture probe S2, blocking the electrode with BSA, RCA reaction and hybridization with HRP probe, the Ret increased gradually as the experiment proceeded. However, the resistance was heavily increased after BCP reaction compared with all other lines. This might attribute to the distinct blocking ability of these insoluble products produced by biocatalyzed precipitation reaction. On the basis of EIS measurements, it is reasonably conclusive that the immobilization process is successful.

3.3. AFM measurement

Surface morphology on chip can be comfirmed by AFM. As shown from Fig. 2A, when immobilized capture probes on the gold surface, the surface was relatively smooth. After blocked with BSA and further the RCA reaction, the surface morphology showed much more unevenly distributed "islands" than before (Fig. 2B), indicating that BSA effectively blocked the residue area of gold electrode. When labeling of HRP conjugated streptavidin, some larger aggregates of protein could be observed (Fig. 2C). Finally, after BCP reaction, the surface morphology showed significantly enhanced roughness, which indicated the successful BCP on the gold surface (Fig. 2D).

3.4. Sensitivity and selectivity of the assay

The frequency shift was correlated to the amount of BCP product, which was related to the RCA product as well as target concentration. As shown in Fig. 3A, on the basis of the optimized conditions, the frequency change increased gradually with the increase of the lysozyme concentration, which implied that more and more BCP products were obtained. Since the frequency change tended to stabilize after 25 min (the real BCP reaction time is up to c.a. 20 min), the frequency shifts of 25 min was used for data analysis. As shown in Fig. 3B, the normalized frequency change intensity subtracted blank was linearly related to the logarithm of the lysozyme concentrations ranging from 1.0×10^{-15} to 5.0×10^{-12} M, with a linear correlation coefficient of 0.9964. The RSD for 11 repetitive measurements of 1.0×10^{-14} M lysozyme

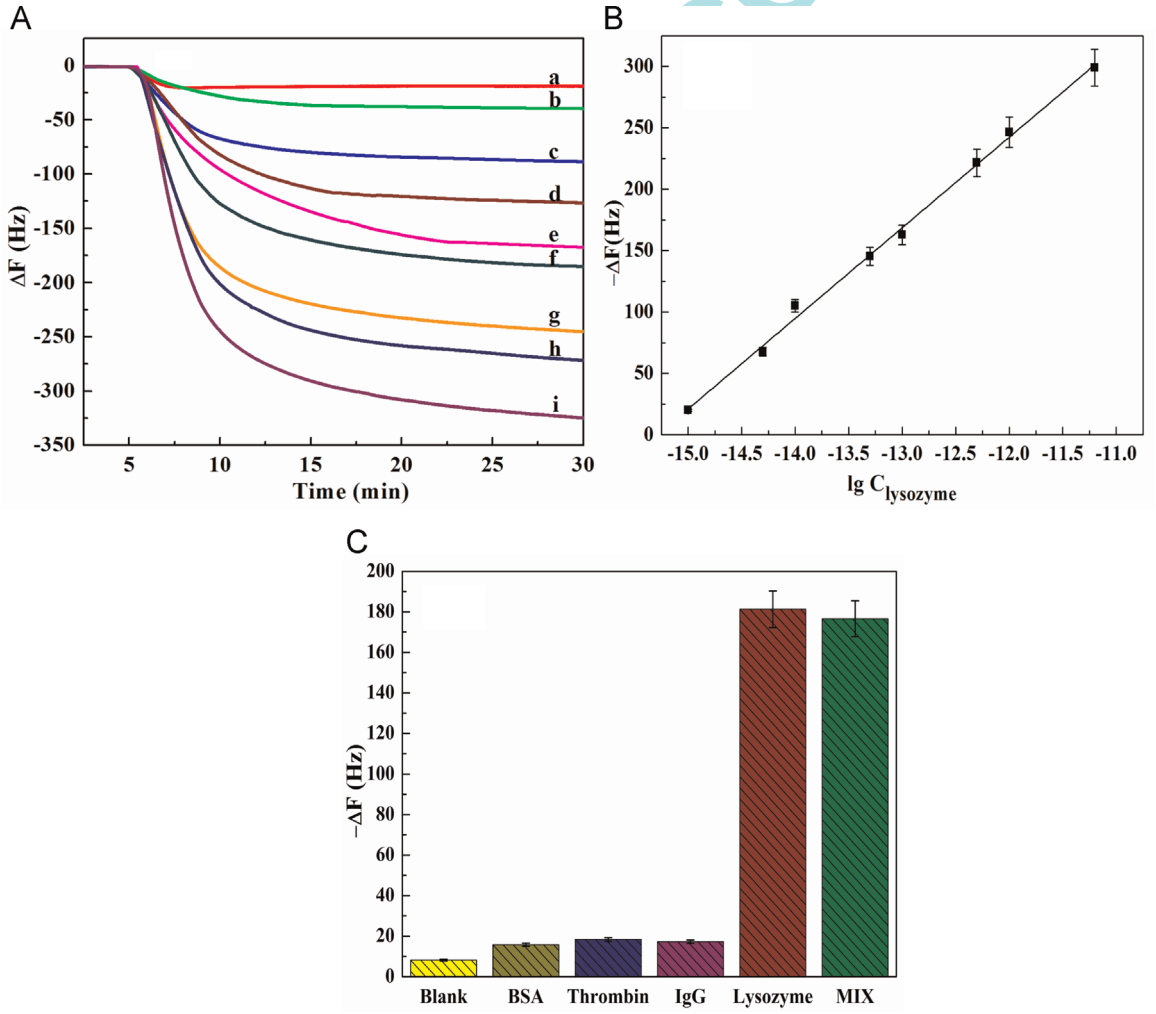

Fig. 3. (A) Frequency responses of amplified QCM biosensor for detection of lysozyme. The concentrations of lysozyme were as follows: (a) 0, (b) 1.0×10^{-15}, (c) 5.0×10^{-15}, (d) 1.0×10^{-14}, (e) 5.0×10^{-14}, (f) 1.0×10^{-13}, (g) 5.0×10^{-13}, (h) 1.0×10^{-12}, and (i) 5.0×10^{-12}. (B) Linear relationship between the frequency shifts and the lysozyme concentrations. Error bars are standard deviation of three repetitive measurements. (C) Frequency responses after the addition of lysozyme or other proteins. The concentration of lysozyme: 1.0×10^{-13} M. Concentration of BSA, thrombin and IgG: 1.0×10^{-10} M.

was 6.2%, indicating a good reproducibility of the assay. The detection limit of 0.3 fM could be estimated using 3σ. The sensitivity for lysozyme of the proposed method is better than most methods developed previously. For example, it is about 10 times lower than strand-displacement amplified SERS detection developed in our previous work (Li et al., 2012; Ye et al., 2013), much lower than some electrochemical assay (Deng et al., 2009; Xia et al., 2013; Cheng et al., 2007) and DNAzyme based optical detection (Teller et al., 2009; Li et al., 2007), but a bit higher than target-triggered peroxidase DNAzyme-associated aptasensor (0.1 fM, Fu et al., 2011) and much higher than Rayleigh light scattering spectroscopy of individual nanoplasmonic aptasensors (7 aM, Truong et al., 2013). However, the main drawback of the present method is that it is time consuming (about 7 h). For saving time, one solution is to use circled DNA instead of padlock DNA. Thus, the process of nicked circling and RCA can be operated in one pot without considering the different operation temperature for T4 ligase.

Control experiments were performed to determine the selectivity of the present QCM sensor towards lysozyme. As shown in Fig. 3C, significantly higher shift of frequency was observed with the lysozyme than with other non-target proteins (bovine serum albumin (BSA), thrombin, and IgG), even though the concentration of lysozyme was 1000 times lower than non-target proteins, indicating a good specificity of the QCM aptasensor.

In order to assess the feasibility of the proposed QCM aptasensor, the amount of lysozyme in diluted human serum was detected. The serum sample was centrifuged to remove solid suspension and diluted 10^7-fold with buffer. The calculated content of lysozyme for human serum was 3.34×10^{-7} M. The recovery for the spiked lysozyme was performed by adding different concentrations of lysozyme into the 10^7-fold diluted serum. As shown in Table S2 of Supplementary material, the recovery was between 91.0 and 96.8% and the RSD was lower than 6.3% ($n=3$). The ability to detect the analysts in complex matrixes demonstrates the potentialities for practical sensing investigations.

4. Conclusion

In conclusion, we presented a novel system for the sensitive determination of proteins based on amplified QCM method. By means of DNA nicked circling, rolling circle amplification and the biocatalyzed precipitation of an insoluble product, a detection limit of 0.3 fM for lysozyme was obtained with satisfied specificity. In addition to QCM detection, this rolling circle amplified biocatalyzed precipitation strategy can also be used in other transducer, such as faradaic impedance spectroscopy, photoelectrochemistry and SPR measurements. Furthermore, the developed strategy can be extended easily for the detection of various analytes, such as nucleic acid, protein and biological small molecules, thus finds potential application in biomedical research and clinical diagnostics.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2014.10.055.

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