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Development of an aptamer-based impedimetric bioassay using microfluidic system and magnetic separation for protein detection

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ABSTRACT

An aptamer-based impedimetric bioassay using the microfluidic system and magnetic separation was developed for the sensitive and rapid detection of protein. The microfluidic impedance device was fabricated through integrating the gold interdigitated array microelectrode into a flow cell made of polydimethylsiloxane (PDMS). Aptamer modified magnetic beads were used to capture and separate the target protein, and concentrated into a suitable volume. Then the complexes were injected into the microfluidic flow cell for impedance measurement. To demonstrate the high performance of this novel detection system, thrombin was employed as the target protein. The results showed that the impedance signals at the frequency of 90 kHz have a good linearity with the concentrations of thrombin in a range from 0.1 nM to 10 nM and the detection limit is 0.01 nM. Compared with the reported impedimetric aptasensors for thrombin detection, this method possesses several advantages, such as the increasing sensitivity, improving reproducibility, reducing sample volume and assay time. All these demonstrate the proposed detection system is an alternative way to enable sensitive, rapid and specific detection of protein.

1. Introduction

Aptamers are artificial oligonucleic acids which are in vitro selected through SELEX (systematic evolution of ligands by exponential enrichment) to bind specific target molecules (Ellington and Szostak, 1990; Tuerk and Gold, 1990). Compared with traditional receptor of antibodies, they have many advantages, including their remarkable target diversity, high binding affinity, convenient automated-synthesis, ease-of labeling, and high stability (Famulok and Mayer, 2011; Tombelli et al., 2005; Wang et al., 2012). Thus nowadays they have been applied to fabricate various kinds of biosensors such as electrochemical biosensors (Noonan et al., 2013; Xu et al., 2013), colorimetry biosensors (Liu et al., 2012; Wang et al., 2013), fluorescence biosensors (Sheng et al., 2011; Zhang et al., 2013), and piezoelectric biosensors (Wang and Li, 2013; Yao et al., 2009). Among them, impedance biosensor as one of the most powerful label-free biosensors has excellent advantages including high sensitivity, rapidity, ease to miniaturization and low cost (Daniels and Pourmand, 2007; Lisdat and Schafer, 2008; Pejcic and De Marco, 2006; Prodromidis, 2010). Several impedimetric aptasensors have been reported in recent years (Bogomolova et al., 2009; Cai et al., 2006; Chen et al., 2013; Deng et al., 2009; Li et al., 2010, 2008; Loo et al., 2012). Radi et al. (2005) developed an electrochemical impedance spectroscopy (EIS) biosensor based self-assembled thiol-modified thrombin-binding aptamer on a gold electrode with a detection limit of 2.0 nM. Cai et al. (2006) developed an EIS biosensor for the detection of thrombin using microfabricated thin film gold electrode with a detection limit of 0.10 nM.

The application of the microfluidic technique in impedimetric biosensing is one of the important trends in the development of impedimetric biosensors due to the exceptional merits over conventional devices in terms of sensitivity, stability, microscale bioanalysis and high throughput (Arlett et al., 2011; Varshney et al., 2007). Microfluidic system can improve the sensitivity of the impedance biosensor by integrating the working electrode into a microfluidic channel with a low height, by which can confine the analyte close to the electrode (Chen and White, 2011). In addition, the microfluidic system can improve the repeatability through effectively decreasing the chances of electrode fouling, which is the major problem in microelectrode based impedance detection. Finally, the microfluidic system facilitates control and manipulation of small volumes of liquid sample for impedance detection. Impedimetric aptasensors integrated microfluidic technique for the detection of analytes has been reported (Dapra et al., 2013; Kiilerich-Pedersen et al., 2013). However, the impedance measurement can be performed by binding

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the targets with aptamer modified on the surface of electrode, which caused the major problem that is the low capture efficiency of the immobilized surface for target. The problem of low capture efficiency can be resolved by developing impedance techniques that do not require immobilization of aptamer on the surface of electrodes. Instead, magnetic particles coated with specific aptamer can be used to capture the target.

Magnetic beads are known to be a powerful tool in separation and concentration of targets from complex specimen, which has been applied to fabrication of a variety of biosensors (Gijs, 2004; Lee et al., 2008). Their use improves the performance of the affinity interaction between the aptamer and the target including improving the sensitivity and reducing the assay time, because the affinity interaction occurred in solution instead of on the surface of electrode, which increased collision probability and contact time (Varshney and Li, 2007).

In this paper, we reported an aptamer based impedimetric biosensing methods using microfluidic system and magnetic separation for protein detection. Thrombin and its specific aptamer were chosen as the model for testing the detection approach. Aptamer coated magnetic beads were used to capture and separate thrombin, and concentrated to a desired volume with deionized water (DI water) for impedance measurement in a microfluidic flow cell with embedded interdigitated array microelectrode. The impedance change due to the conductivity change induced by the charges of thrombin surface was proportional to the concentrations of thrombin. The approach combined the advantages of the microfluidic system for control and manipulation of small volumes of liquid sample for impedance detection and magnetic beads for separation and concentration of targets from complex specimen, which provided an alternative way for protein detection with a lower detection limit and shorter assay time compared with the other impedimetric biosensors.

2. Experimental

2.1. Microfluidic flow cell with embedded interdigitated array microelectrode

Flow cell was made of PDMS. A sample port and a waste port were punched symmetrically on the top of the flow cell (as shown in Fig. 1A). The gold interdigitated array microelectrode (Electrode chip 2050.5) integrated in the flow cell was produced in the ABTECH Scientific, Inc (Virginia, USA).

2.2. Reagents and materials

The biotin labeled anti-thrombin aptamer was synthesized by Sagon Inc. (Shanghai, China) and had the following sequence: 5′-biotin-TTT TTT TTG GTT GGT GTG GTT GG-3′. The aptamer stock solution (100 μM) was prepared with deionized water and was kept frozen. Prior to use, the aptamer solution was thermally treated at 95 °C for 10 min, followed by cooling for 10 min. Previous studies have shown that this thermal treatment disrupted any pre-existing higher order structure.

Human α-thrombin, lysozyme, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Streptavidin coated magnetic beads with 2.8 μm in diameter were obtained from Invitrogen (Carlsbad, CA). All other reagents were of analytical reagent grade. All experiments were conducted at room temperature (unless specified otherwise). Binding buffer solution contains 20 mM Tris–HCl, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ with the pH value of 7.4. The biotin labeled aptamer was diluted with binding buffer solution to 0.5 μM. The thrombin was prepared to various concentrations (0.01, 0.1, 1, 2.5, 5, 7.5 and 10 nM) in the binding buffer solution. Millipore Milli-Q water (18 ΩM · cm) was used throughout.

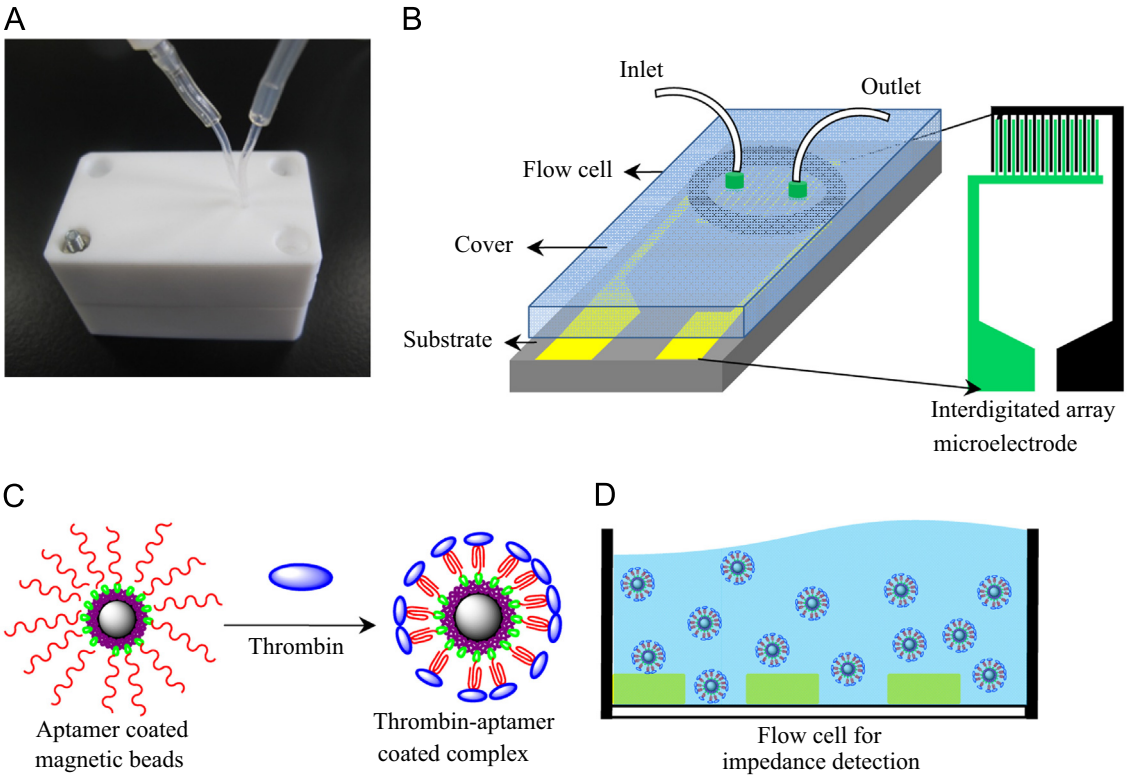


Fig. 1. (A) Picture of the microfluidic instrument; (B) schematic diagram of the microfluidic instrument including flow cell and interdigitated array microelectrode; (C) schematic diagram of the stepwise preparation of the thrombin-aptamer coated magnetic bead complex; and (D) schematic diagram of impedance detection in the flow cell.

2.3. Fabrication of microfluidic impedimetric sensing device

The magnetic stand (Invitrogen, Carlsbad, CA) was used for magnetic separation of magnetic beads in 1.5 mL microcentrifuge tubes. The streptavidin coated magnetic beads (20 μL) were washed using water for three times. And 80 μL of biotin-labeled aptamer solution was mixed with streptavidin coated magnetic beads for 15 min at room temperature. After aptamer immobilization, 200 μL of biotin (0.1 mM) was used to block unbind sites on the surface of magnetic beads. Excess biotin was washed out by the binding buffer solution. 300 μL of different concentrations of thrombin was mixed with aptamer modified magnetic beads, respectively. Following the reaction, the thrombin-aptamer coated magnetic beads were washed three times with DI water with magnetic separation, and were concentrated in 100 μL of DI water. Finally the thrombin-aptamer coated magnetic beads suspension was injected into the flow cell with the help of a syringe pump at a flow rate of 10 μL min^{-1} for impedance detection. For the specificity testing, 10 nM lysozyme and BSA were treated with the same procedure.

2.4. Impedance measurements

Impedance measurements were performed using a Solartron Analytical model 1260 Impedance-Gain-Phase Analyzer in combination with a model 1287 Electrochemical Interface (Solartron Analytical, Farnborough, UK). For all impedance measurements, a sine-modulated AC potential of 50 mV was applied and the impedance spectroscopy was obtained at the frequency range from 1 Hz to 900 kHz.

3. Results and discussion

3.1. Design of the microfluidic impedimetric sensing device

The microfludic system is connected to impedance detector using a USB data line (Fig. 1A). The circular flow cell with a diameter of 5.5 mm and a volume of 23.7 μL is made of PDMS via a soft lithography method (Fig. 1B). An inlet and an outlet are punched at the two sides of the flow cell. A gold interdigitated array microelectrode that is composed of two sets of interconnected microband electrodes is embedded in the flow cell. The interdigitated array microelectrode has 50 digits pairs interconnected microband with the microband width of 20 μm, microband length of 4.985 mm and microband gap of 20 μm.

The fabrication steps of thrombin-aptamer coated magnetic beads are shown in Fig. 1C. Biotin labeled aptamers were combined with streptavidin modified magnetic beads via the biotin–streptavidin interaction. After that step, the unbound sites were blocked with the biotins in order to avoid the nonspecific signal. Then the target thrombin was combined with the aptamer coated magnetic beads to form the thrombin-aptamer coated magnetic beads complexes, which were transferred into the DI water for impedance detection (Fig. 1D).

3.2. Detection of thrombin using the microfludic impedimetric bioassay

Bode plot is significantly suitable to study the relationship of impedance with frequency. Fig. 2A shows the Bode plots of impedance spectra for DI water (curve a), aptamer coated magnetic beads suspension in DI water (curve b), thrombin-aptamer coated magnetic beads complexes with the concentration of 10 nM thrombin suspension in DI water (curve c), respectively. Compared to the impedance value of DI water, the impedance of aptamer coated magnetic beads in DI water decreased significantly, because the aptamer is a single strand nucleic acid with abundant negative charges on its phosphate backbones. In our experiment, the conductivity of the DI water is in a range of 1–2 μS cm^{-1}. When aptamer coated magnetic beads were suspended in such low conductive DI water, they could increase the conductivity of the suspension due to their surface charges, which contribute to the decrease of the impedance value measured. Likewise, the thrombin-aptamer coated magnetic bead complexes showed a further decrease in impedance value due to the electrical nature of thrombin surface. It has been reported that 79 out of 89 charged amino acids in thrombin are exposed on the surface of the thrombin biomolecule, which contributes to a high number of polar and charged molecules on the surface of thrombin (Bode et al., 1989).

To choose the best representative frequency, we compared the impedance difference of the thrombin-aptamer magnetic bead complexes with the concentration of 10 nM thrombin (represented by the percent of impedance change $|Z_{sample} - Z_{control}|/Z_{control} \times 100\%$) in the frequency region of 1 Hz–900 kHz. As shown in Fig. 2B, the impedance difference showed increase in the frequency region from 1 Hz to 90 kHz followed by the decrease in the frequency region from 90 kHz to 900 kHz (Fig. 2B). The maximum difference in the percent of impedance change was at 90 kHz. Thus we selected 90 kHz as the test frequency to investigate impedance change (represented by $|Z_{sample} - Z_{control}|$) for further experiments in this work.

3.3. The equivalent circuit

The data of impedance spectroscopy can be simulated with an equivalent circuit including ohmic resistance (R_s) of the solution between two electrodes and constant phase elements (CPE) (Fig. 3 inset). R_s accounts for change in conductivity of the bulk medium and charge transport across the bulk solution. CPE represents the

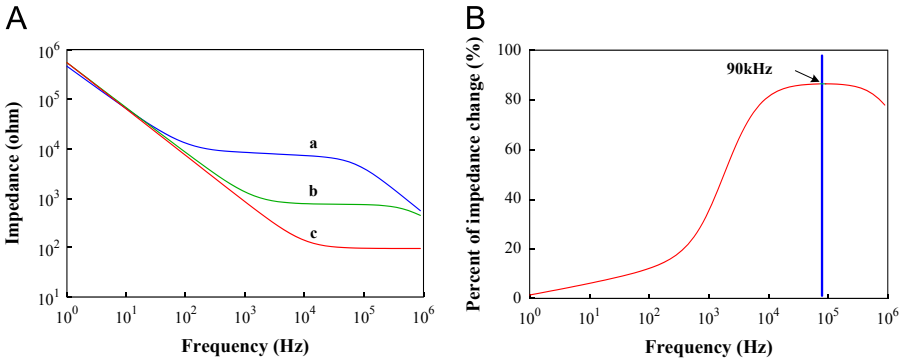
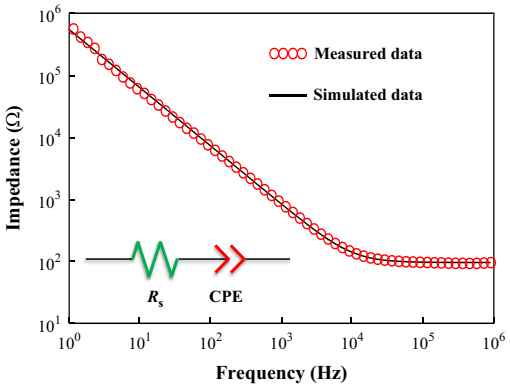


Fig. 2. (A) Bode diagrams of impedance spectra of DI water (a), aptamer coated magnetic beads in DI water (b), thrombin-aptamer coated magnetic beads complexes (the thrombin concentration is 10 nM) in DI water (c); and (B) the difference in impedance of thrombin binding with the concentration of 10 nM in the range of frequency from 1 Hz to 900 kHz.

effect of ionic species on the capacitance near the surface of an electrode. Fig. 3 shows the Bode impedance spectroscopy of the thrombin-aptamer coated magnetic bead complexes with the concentration of 10 nM thrombin in DI water (red circle line). To validate the equivalent circuit, 50 data points on the impedance measured spectroscopy were automatically selected by the software and used as input to the equivalent circuit, generating a fitting impedance spectrum (Fig. 3 solid line). The mean and maximum modulus of impedance errors were 0.98% and 1.63%, respectively, which indicated that the equivalent circuits provided a feasible model to describe the impedance characteristics of the system in DI water. By fitting the data, the value of each electrical element in the equivalent circuit was obtained (Fig. 3). The most significant change was found in R_s due to the increase in conductivity of the water medium caused by the presence of thrombin-aptamer coated magnetic beads in DI water.



Sample	R_s (Ω)	CPE-T	CPE-P
Aptamer modification	700.9	3.5029E-7	0.91052
Thrombin binding	97.12	3.2442E-7	0.93817
Change	-86.14%	-7.39%	3.03%

Fig. 3. Bode diagram of electrochemical impedance spectroscopy of the biosensing method together with the fitting spectroscopy. *Inset*: the equivalent circuit including constant phase elements (CPE), and solution resistance (R_s); simulated values of all elements in the equivalent circuit after aptamer modification, and after thrombin binding, as well as the percentage of their changes. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

3.4. Optimization of parameters

To avoid the fluctuation of the signal caused by flow while minimizing the consumed sample volume, we adopted the stationary status for more consistent measurements to pursue a better detection limit and dynamic range. Sample was injected into the flow cell at the flow rate of 10 μL min^{-1} and stopped for a period of time to measure impedance. As shown in Fig. 4A, the impedance change drops rapidly after the pumping is stopped and it does not change after stopping for 25 s. Thus we chose the idling time of 25 s for further measurements.

Solution medium is of significant importance for impedance detection, thus we studied the impedance signal of the thrombin-aptamer coated magnetic bead complexes in different solution mediums including water, PBS, and mannitol. Compared to the mannitol and PBS solution, the DI water got the largest impedance change signal (Fig. 4B). This is due to the factor that the conductivity of the DI water is lowest (1–2 μS cm^{-1}). When thrombin-aptamer coated magnetic bead complexes were suspended in such low conductive solution medium and reached a sufficient concentration, they could significantly increase the conductivity of the suspension due to the charges of the protein surface. Compared with DI water, the mannitol with higher conductivity (~20 μS cm^{-1}) contributed to higher background, which leaded to less impedance signal. In PBS solution, the charges of the protein surface cannot lead to any change in the conductivity of the bulk solution due to the quite high background conductivity of PBS (~58 mS cm^{-1}).

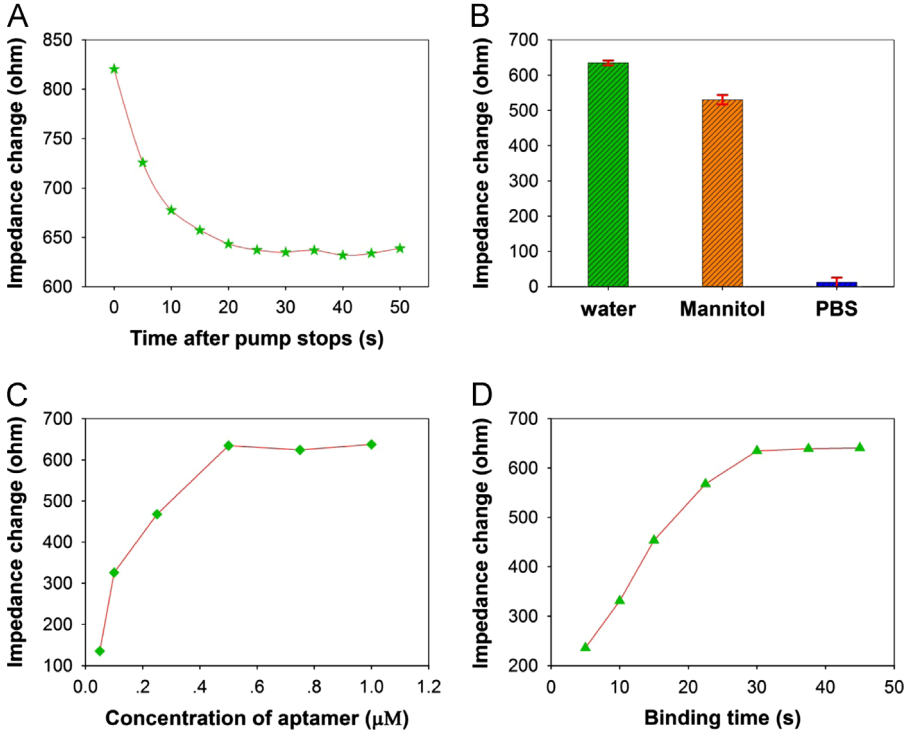


Fig. 4. (A) Impact of idle time on impedance measured at 90 kHz after pump is stopped. Sample is pumped at a flow rate of 10 μL min^{-1}; (B) effect of different mediums for impedance detection including water, PBS and mannitol; (**C**) effect of the aptamer concentration on impedance signal; and (D) effect of binding time between aptamer coated magnetic beads with thrombin at the concentration of 10 nM.

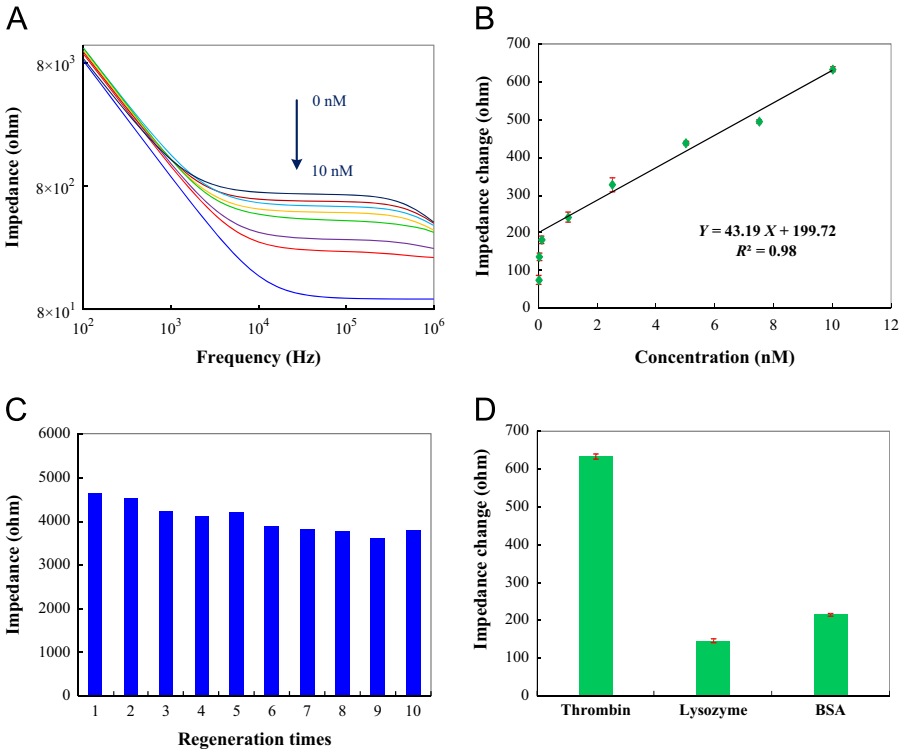


Fig. 5. (A) Bode diagrams of impedance spectra of thrombin-magnetic beads in DI water with the thrombin concentrations of 0, 0.01, 0.1, 1, 2.5, 5, 7.5 and 10 nM. Frequency range: 1 Hz–900 kHz. Amplitude: 50 mV; (B) the linear relationship between the concentrations of thrombin and the impedance measured at 90 kHz. Error bars are standard deviations of 3 measurements; (C) regeneration condition with 1 mL DI water after ten times; and (D) the specificity test of the assay for thrombin. The thrombin, BSA, and lysozyme were all tested at a concentration of 10 nM.

The modified amount of aptamer on the surface of the magnetic beads is the key element in the aptasensor fabrication, since the parameter has a profound effect on the detection sensitivity. Fig. 4C shows that the impedance change signal increased upon increasing the concentrations of aptamers from 0 to 0.5 μM and then reached a saturation state. Thus 0.5 μM of aptamer was chosen as the best aptamer concentration in the further measurement. In addition, the optimal binding time of aptamer coated magnetic beads with the target thrombin was investigated. As shown in Fig. 4D, the impedance change signal increased with the increasing binding time at the range from 5 to 30 min and did not change significantly at the range from 30 to 45 min, thus we chose the binding time of 30 min as the optimum.

3.5. Analytical performance of the microfluidic impedimetric bioassay

Under the optimal conditions, Bode plots for different concentrations of the target thrombin (0–10 nM) were recorded. As shown in Fig. 5A, the impedance values decreased with the increasing thrombin concentrations at the range from 0 to 10 nM. Moreover, the impedance change signal at 90 kHz displayed a good linearity with the concentrations of the target protein in the range from 0.1 to 10 nM (Fig. 5B). The linear equation was calculated as $Y(\Omega) = 43.19X + 199.72$ with the correlation coefficient of 0.98 (R^2).

IUPAC defines the detection limit as background signal plus 3 times the background standard deviation. The background signal of aptamer modified magnetic beads appears as low as 108.35 Ω (mean ± standard deviation). The impedance change signal of thrombin at the concentration of 0.01 nM is 139.88 Ω, which is significantly higher than the detection limit signal. The characterized detection limit of this approach for the detection of thrombin was 0.01 nM. The detection limit of this method is comparable with many other impedimetric biosensors for the detection of thrombin (Table 1). Compared with the other aptamer based biosensors for the detection of thrombin, it can be seen that the microfluidic impedimetric bioassay possesses improved or comparable performance for the detection of thrombin, nevertheless our sensor is suitable for microscale bioanalysis with high throughput.

3.6. Regeneration and specificity of the microfluidic impedimetric bioassay

Obtaining a clean electrode surface is critical for biosensor regeneration and preserving low measurement variability, and thus also contributes to reducing detection cost and improving detection sensitivity. To investigate the regeneration condition, after 10 nM thrombin-magnetic beads complexes were detected, the electrode was cleaned by filling the flow wells with 0.25, 0.5, 0.75 and 1 mL of DI water at a flow rate of 100 μL min^{-1}, respectively. The result found that the impedance increased with the increasing volume of the DI water, and 1 mL of DI water was sufficient for the generation of a clean electrode surface (data not shown). In addition, the impedance at 90 kHz of the generated electrode surface with 1 mL DI water during 10 repeated cycles was shown in Fig. 5C. The relative standard deviations (RSD) of the impedance of generation electrode for 10 regeneration cycles were found to be less than 8.4%. This result indicated that the biosensor could be regenerated easily with DI water in 10 min (avoiding complex washing steps) and reused for a large number of times without impact on the performance of the biosensor.

The specificity of the sensing device was investigated by testing non-target proteins including lysozyme and BSA. As shown in Fig. 5D, the impedance change signal for the target thrombin at the concentration of 10 nm is much higher than that of non-target proteins (lysozyme and BSA) with the same concentration, indicating the

Table 1
The sensing performance of the microfluidic impedimetric aptasensor along with those reported in the literatures.

Method	Technique	LOD (nM)	Linear range	References
Electrochemical	Impedance	0.01	0.1–10 nM	This work
	EIS	0.093	0.5–40 nM	Zhang et al. (2013)
	EIS	0.01	10–50 nM	Loo et al. (2012)
	EIS	0.013	0.1–30 nM	Li et al. (2011)
	EIS	0.03	0.12–30 nM	Li et al. (2008)
	EIS	2.0	5.0–35.0 nM	Radi et al. (2005)
	EIS	0.1	1 nM–1 μM	Cai et al. (2006)
	EIS	0.1	2.5–100 nM	Xu et al. (2005)
Optical	SERS	0.1	0.1 nM–1 μM	Cho et al. (2008)
	SPR	12	0.19–0.27 μM	Jalit et al. (2013)
	Fluorescence	1	1–20 nM	Chi et al. (2011)

EIS: Electrochemical impedance spectroscopy. SERS: Surface enhanced raman scattering.

excellent specificity of the bioassay for thrombin detection arising from the high specificity of the anti-thrombin aptamer.

3.7. *Real sample analysis*

The developed sensing device was applied to the analysis of thrombin in real complex samples. The serum 1:5 diluted with PBS buffer was spiked with thrombin at different concentrations. The analytical results showed the acceptable relative standard deviation and quantitative recoveries (Table S1). This implied that the present bioassay possesses a promising feature for the analytical application in complex biological samples.

4. Conclusion

In this study a novel microfluidic impedimetric bioassay coupling of magnetic separation was successfully developed and evaluated for rapid and specific detection of thrombin. The use of the microfluidic system can improve the sensitivity and repeatability by sample confinement in a microfluidic cell with low height and avoiding electrode fouling. In addition, aptamer modified magnetic beads were used to separate and concentrate the thrombin from the sample, which provided excellent specificity and further improved the sensitivity of the biosensor. Compared with the reported impedimetric aptasensors for thrombin detection, this approach possesses several advantages, such as the increasing sensitivity, reducing sample volume and the assay time. All these demonstrate that the proposed detection system is an alternative way to enable low-cost, rapid, and highly specific detection of protein and may be easily applied for the detection of other kinds of proteins by immobilizing different specific aptamers on the surface of magnetic beads.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2014.03.015.

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