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ScienceDirect
Biosensors and Bioelectronics 23 (2008) 1624–1630
BIOSENSORS & BIOELECTRONICS
www.elsevier.com/locate/bios

Electrochemical impedance spectroscopy for study of aptamer–thrombin interfacial interactions

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Received 1 August 2007; received in revised form 28 January 2008; accepted 29 January 2008
Available online 8 February 2008

Abstract

A simple and highly sensitive electrochemical impedance spectroscopy (EIS) biosensor based on a thrombin-binding aptamer as molecular recognition element was developed for the determination of thrombin. The signal enhancement was achieved by using gold nanoparticles (GNPs), which was electrodeposited onto a glassy carbon electrode (GCE), as a platform for the immobilization of the thiolated aptamer. In the measurement of thrombin, the change in interfacial electron transfer resistance of the biosensor using a redox couple of $[Fe(CN)_6]^{3-/4-}$ as the probe was monitored. The increase of the electron transfer resistance of the biosensor is linear with the concentration of thrombin in the range from 0.12 nM to 30 nM. The association and dissociation rate constants of the immobilized aptamer–thrombin complex were $6.7 \times 10^3\ M^{-1}\ s^{-1}$ and $1.0 \times 10^{-4}\ s^{-1}$, respectively. The association and dissociation constants of three different immobilized aptamers binding with thrombin were measured and the difference of the dissociation constants obtained was discussed. This work demonstrates that GNPs electrodeposited on GCE used as a platform for the immobilization of the thiolated aptamer can improve the sensitivity of an EIS biosensor for the determination of protein. This work also demonstrates that EIS method is an efficient method for the determination of association and dissociation constants on GNPs modified GCE.

Keywords: Biosensors; Electrochemical impedance spectroscopy; Aptamer; Thrombin; Gold nanoparticles; Glassy carbon electrode

1. Introduction

The detection and quantification of proteins play essential roles in fundamental research and clinical applications. Biosensors, which fuse the exquisite sensitivity and specificity of biological active substances with the suitable transducers, are simple, inexpensive analytical devices that may be able to provide escalating quantities of molecular information (Turner, 2000). In past three decades, antibody was widely utilized as molecular recognition substance for proteins with high affinity and specificity. However, antibodies have some limitations such as their production in vivo; limited target analytes; limited shelf lives and temperature-sensitive to undergo denaturation (O'Sullivan, 2002). Aptamers are artificially synthesized oligonucleotides and have high specificity and affinity for various target compounds such as drugs, enzymes, peptides and proteins (Osborne et al., 1997). Thus, aptamers have been applied in biosensors including optical sensors (Ho and Leclerc, 2004; Pavlov et al., 2004) and electrochemical biosensors (Ikebukuro et al., 2005; Radi et al., 2006). The field of electrochemical biosensors has grown rapidly in the past few years because they may provide fast, simple and inexpensive detection capabilities for biological binding events (Bakker and Qin, 2006).

Many work has been done in aptamer-based electrochemical biosensors for the determination of proteins, which are mainly based on the aptamers labeled using redox compounds such as methylene blue (Xiao et al., 2005) and catalysts such as horseradish peroxidase (Monica et al., 2006) and platinum nanoparticles (Polsky et al., 2006) as signal-produced labels. The labeling process of aptamer, however, is time-consuming and leads to the bioaffinity-reducing. Thus, increasingly attractive attention has been received to develop label-free biosensors for the determination of proteins. The label-free aptamer biosensors

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0956-5663/$ – see front matter © 2008 Published by Elsevier B.V.
doi:10.1016/j.bios.2008.01.029

for the determination of thrombin were developed using square wave technique (Floch et al., 2006), electrochemical impedance spectroscopy (EIS) technique (Radi et al., 2005; Cai et al., 2006; Xu et al., 2006) and ion-selective field-effect transistor technique (So et al., 2005). Among them, EIS is a very powerful tool for the analysis of interfacial properties related to biorecognition events occurring at the modified surfaces. EIS biosensors, which are based on the change of electron transfer resistance using a redox probe couple such as $[Fe(CN)_6]^{3-/4-}$, have received much attention due to high sensitivity. It was reported that an EIS biosensor for the determination of thrombin was based on self-assembled thiol-modified thrombin-binding aptamer on a gold electrode with a detection limit of 2.0 nM (Radi et al., 2005), and on micro-fabricated thin film gold coated glass with a detection limit of 0.10 nM (Cai et al., 2006). The sensitivity of EIS biosensor, however, should be improved for wider applications.

One of the promising approaches to improve the sensitivity of DNA biosensors is to utilize nanomaterials such as gold nanoparticles (GNPs) for immobilization of the DNA probes (Wang et al., 2006). GNPs are superior to other types of nanoparticles for many reasons such as electrical conductivity, biocompatibility and ease of self-assembly through a thiol group. GNPs can also increase the surface area of electrode and the amount of immobilized DNA probes (Castañeda et al., 2007). In most of papers, GNPs were mainly prepared by chemical method and then modified on the surface of the electrodes through physical adsorption or chemical linking to construct a nanomaterial platform for biosensors. However, this approach needs a preparation of GNPs before the immobilization. The directly electrochemical deposition of GNPs onto the electrode surface is a very useful way to create a nanomaterial platform *in situ* for the fabrication of DNA biosensors (Li et al., 2005; Liu et al., 2005a).

Thrombin is the main effector protease of the coagulation cascade, a series of zymogen conversions that is triggered when circulating coagulation factors contact tissue factor (Coughlin, 2000). Thrombin plays a central role in a number of cardiovascular diseases and regulates many processes in inflammation and tissue repair at the vessel wall. Since the detection of thrombin in plasma or blood is clinically relevant, an aptamer-based sensor as an alternative diagnostic tool for thrombin analysis or blood coagulation investigation could gain great interest in the medical area (Centi et al., 2007).

The aim of this work is two-folds. One is to improve the sensitivity of EIS aptamer-based biosensor designed by signal enhancement using GNPs electrodeposited onto a glassy carbon electrode. Another is to investigate aptamer–thrombin interfacial interactions. The association and dissociation constants of the immobilized aptamer–thrombin complex were measured. To our best knowledge, EIS aptamer-based biosensors for thrombin at GNPs modified GCE have not been reported.

2. Materials and methods

2.1. Materials

Human α-thrombin (thrombin, $M_w = 36{,}700\ g\ mol^{-1}$, p*I* = 7.0–7.6) was purchased from Haematologic Technologies Inc. $HAuCl_4 \cdot 3H_2O$, bovine serum albumin (BSA), hemoglobin (Hb), hexanethiol (97%, HT) and hexammineruthenium(III) chloride were obtained from Sigma–Aldrich (USA). All reagents were of analytical grade, and Millipore Milli-Q water (18 ΩM cm) was used throughout. 0.10 M sodium phosphate buffer containing 10 mM KCl and 2 mM $MgCl_2$ (pH 7.00) was used as a binding buffer.

Three thrombin-binding aptamers (TBA) including TBA I (designed by Bock et al., 1992), TBA II (designed by Tasset et al., 1997) and TBA III (referred by Nutiu and Li, 2003) were synthesized by Shanghai Sangon Biological Science & Technology Company (Shanghai, China).

TBA I 5′-SH-$(CH_2)_6$–GGT **TGG** **TGT** GGT TGG-3′
TBA II 5′-SH-$(CH_2)_6$–AGT CCG T GGT **AGG** **GCA** GGT TGG GGT GAC T-3′
TBA III 5′-SH-$(CH_2)_6$–CCT GCC ACG CTC CGC TCA CTGT-GGT **TGG** **TGT** GGT TGG-3′

Underline means the binding sequence of aptamer and bold indicates the differences of the bases in the binding sequence.

2.2. Apparatus

Electrochemical measurements were performed on a CHI 660 electrochemical workstation (Chenhua Instruments Co., Shanghai, China). All experiments were carried out using a conventional three-electrode system with an EIS aptamer-based biosensor or a glassy carbon electrode (GCE, $\phi = 3$ mm) as working electrode, a platinum wire as the counter electrode and an Ag/AgCl (sat. KCl) as the reference electrode. All potentials were referred to this reference electrode. All experiments were carried out at room temperature (20 °C).

2.3. Fabrication of aptamer-based biosensor

Schematic representation of aptamer-based biosensor with fabrication steps and performance is showed in Fig. 1. This designed biosensor was fabricated by two steps including an electrochemical deposition step and an immobilization step. The procedure for the deposition of GNPs at GCE was adapted from the reference (El-Deab et al., 2003). The GCE surface was polished with 0.3 μm alumina slurry and then ultra-sonicated in water for 5 min. The polished GCE was immersed in 0.5 M H_2SO_4 containing 0.5 mM $HAuCl_4$, which was degassed with N_2 stream for at least 20 min before the electrochemical deposition. A constant potential of +1.1 V (vs. Ag/AgCl, sat. KCl) was applied for 60 s and then a constant potential of 0 V was applied for 30 s.

The solution of 20 μg mL^{-1} TBA I (or TBA II, TBA III) prepared by the binding buffer (pH 7.00) was heated at 90 °C for 3 min and then cooled in ice bath to obtain a proper folding configuration of TBA I according to the reference (Liss et al., 2002). 20 μL of this treated solution was dropped onto the surface of freshly prepared GNPs on GCE and incubated for 16 h at room temperature. After the resulting electrode was washed using the binding buffer (pH 7.00) to remove absorbed TBA I,

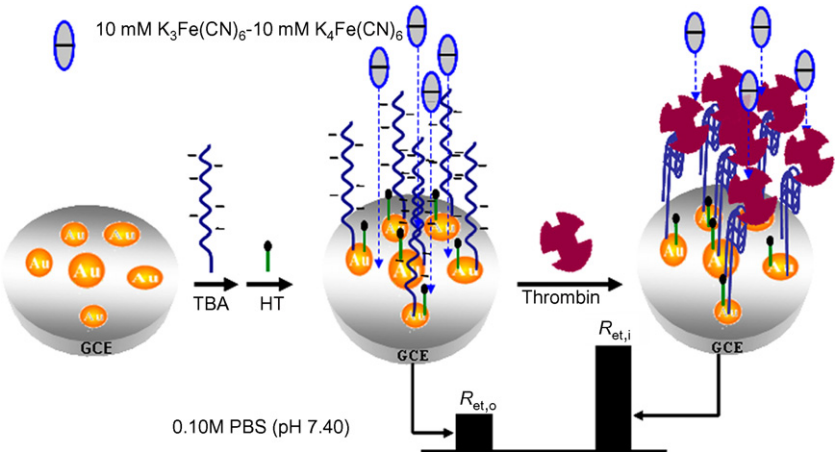

Fig. 1. Schematic representation of aptamer–based biosensor with fabrication steps and performance.

20 μL of 1 mM ethanolic hexanethiol solution was placed on the surface of the electrode for 40 min at room temperature. The obtained electrode was washed with water and used as the EIS aptamer-based biosensor in this work.

2.4. Electrochemical measurement

Twenty microlitres of a fixed concentration of thrombin was dropped on an EIS aptamer-based biosensor fabricated, and allowed to interaction the aptamer with thrombin for 40 min. The biosensor was rinsed with 0.10 M sodium phosphate buffer (PBS, pH 7.40) and placed into an electrochemical cell. EIS measurement were performed in 5.0 mL of 0.10 M PBS (pH 7.40) containing 10 mM $K_4[Fe(CN)_6]$–10 mM $K_3[Fe(CN)_6]$–10 mM NaCl. The amplitude of the applied sine potential in each case was 5 mV, whereas +0.20 V of the direct current potential was limited to the formal potential of the redox couple $[Fe(CN)_6]^{3-/4-}$. The electrochemical impedance spectra were recorded within the frequency from 100 kHz to 1.0 Hz with a sampling rate of 12 points per decade. The concentration of thrombin was quantified by the change of electron transfer resistance of the biosensors.

3. Results and discussion

3.1. Characterization of aptamer-based biosensor

GNPs deposited electrochemically on the surface of GCE was described in Section 2.3. The scanning electronic microscopic image of the resulting electrode showed that the GNPs were discrete and the size distribution of GNPs was observed in the range 10–60 nm.

In order to monitor each immobilization step and binding step, corresponding cyclic voltammograms and impedance spectra were recorded. Fig. 2 shows the Nyquist plots of impedance spectra at different electrodes. The bare GCE exhibited very small semicircle at high frequencies (curve *a*). The GNPs deposited GCE resulted in an almost straight line at high frequencies (curve *b*). When TBA I was self-assembled onto the GNPs, the R_{et} increased to 920 Ω (curve *c*). This is attributed to fact that self-assembled TBA I having a single strand nucleic acid with negative charges on its phosphate backbone makes an electrostatic repulsive force to $[Fe(CN)_6]^{3-/4-}$. When the aptamer-based biosensor was constructed, in which a short-chain alkyl molecule HT was employed to occupy the left bare sites on GNPs for eliminating the non-specific adsorption, led to the R_{et} further increased from 920 Ω to 1450 Ω (curve *d*). After thrombin was captured onto the surface of TBA I-based biosensor, the R_{et} greatly increased to 1800 Ω (curve *e*). This is probably attributed to the fact that an insulating layer on the surface of TBA I-based biosensor is generated and the transfer of redox couple $[Fe(CN)_6]^{3-/4-}$ to the surface of GCE is prohibited. This

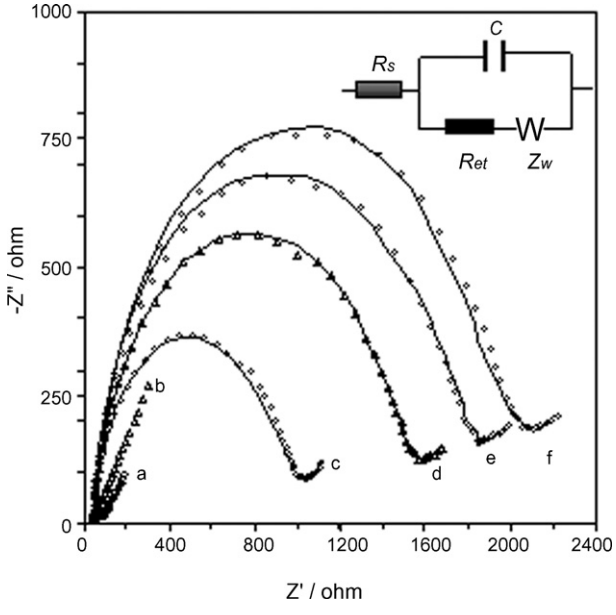

Fig. 2. Nyquist plots of impedance spectra obtained in 0.10 M PBS (pH 7.40) containing 10 mM $K_3Fe(CN)_6$–10 mM $K_4Fe(CN)_6$ at (a) bare GCE; (b) GNPs deposited on GCE; (c) TBA I/GNPs/GCE; (d) TBA I/HT/GNPs/GCE; (e) 0.60 nM thrombin/TBA I/HT/GNPs/GCE; (f) 6.0 nM thrombin/TBA I/HT/GNPs/GCE after interaction with for 40 min. Fitted data (solid line).The biased potential was 0.20 V. The frequency was from 100 kHz to 1.0 Hz and the amplitude was 5.0 mV. The inset shows the equivalent circuit applied to fit the impedance spectroscopy.

suggests that the GNPs play two roles including electron transport controlling and biomolecules self-assembling. These results obtained from EIS are in a good agreement with the results obtained from the cyclic voltammograms (data not shown). It was found that the change of the R_{et} in EIS was much more sensitive than the change of peak currents in cyclic voltammograms. Therefore, EIS detection model is utilized in this work to obtain a high sensitivity.

In order to obtain useful parameters including electron transfer resistance (R_{et}), electrode/electrolyte interface capacitance (C), the ohmic resistance of the electrolyte solution (R_s) and the Warburg impedance (Z_w), the experimental impedance data were fitted with commercial software (CHI 660). A modified Randle's equivalent circuit (Patolsky et al., 1999) was found to fit adequately the data obtained over the measured frequency range. The fitting of measured spectra (line) to the equivalent circuit is also shown in Fig. 2, which indicates a good agreement between the circuit model and the measurement system, especially in the high-frequency range. Therefore, the equivalent circuit model is applied to the measurement system in this study.

3.2. Performance of aptamer-based biosensors

3.2.1. Linear range and detection limit

The quantitative behavior of the biosensors fabricated with three TBA probes under the optimized self-assembly time was assessed. The impedance spectra from the biosensors fabricated with TBA I probe are shown in Fig. 3. From the inset of Fig. 3, it

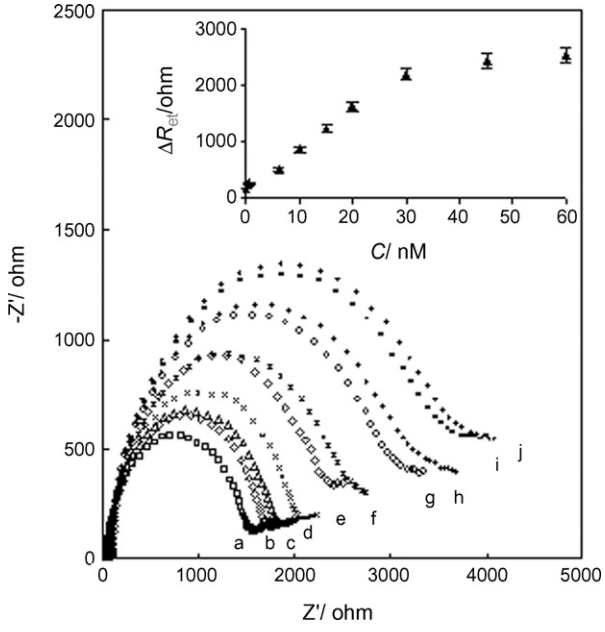

Fig. 3. Nyquist plots of impedance spectra of aptamer-based biosensors fabricated with TBA I after interaction with different concentrations of thrombin for 40 min. (a) 0; (b) 0.12 nM; (c) 0.6 nM; (d) 6.0 nM; (e) 10 nM; (f) 15 nM; (g) 20 nM; (h) 30 nM; (i) 45 nM; (j) 60 nM. The measurement conditions and the electric circuit are same as in Fig. 3. The inset shows the dependence of ΔR_{et} on the concentration of thrombin. $\Delta R_{et} = R_{et,i} - R_{et,0}$. $R_{et,i}$ is the electron transfer resistance in the presence of thrombin and $R_{et,0}$ is that in the absence of thrombin.

can be seen that the R_{et} has a fine linear relationship with the concentration of thrombin in the range from 0.12 nM to 30 nM. The regression equation was $\Delta R_{et} = 234.3 + 70.7 \times 10^{-9} c$ (unit of c, M), and the regression coefficient (r^2) was 0.9924. The detection limit for thrombin was 0.06 nM. This detection limit is lower than that (2.0 nM) reported by Radi et al. (2005). The precision was estimated to be 4.5% of relative standard deviation for five successive measurements of 6.0 nM thrombin. Therefore, a high sensitivity and a good reproducibility of the developed biosensors were obtained.

We compared the sensitivity of biosensor fabricated using GNPs electrodeposited on GCE with that using gold disk electrode and gold thin film coated GCE, which was prepared by ion-sputtering technique (Bal-tec SCD-005 sputter coater, Ar, 0.05 mbar, 40 mA during 60 s, sample distance: 5 cm). It was found that the sensitivity (a slope of linear relationship between R_{et} and the concentration of thrombin, 70.7×10^{-9}) of the biosensor fabricated using GNPs electrodeposited on GCE was higher than that using gold disk electrode (54.1×10^{-9} and gold thin film coated GCE (59.3×10^{-9}). This is attributed to the fact that the surface density of TBA I probe immobilized on the surface of GNPs electrodeposited on GCE (1.2×10^{13} molecule cm^{-2}) is higher that that on gold disk electrode (4.7×10^{12} molecule cm^{-2}) and gold thin film coated GCE (5.2×10^{12} molecule cm^{-2}). The surface density of TBA I were quantified by chronocoulometry in 10 mM Tris–HCl containing 50 mM $[Ru(NH_3)_6]^{3+}$ according to the method employed in reference (Steel et al., 1998). Additionally, it was found that the background of R_{et} for biosensors using gold thin film and gold electrode was much higher than that of the biosensors using GNPs on GCE. This is attributed to the fact that the surface of the gold electrodes including the gold thin film coated GCE and gold electrode is occupied by short-chain alkyl molecule HT, while TBA and HT cannot be self-assembled onto the surface of GCE. A high sensitivity of designed biosensor, therefore, were obtained.

In order to obtain a high sensitivity of designed biosensors, the linear range and detection limit of other two biosensors fabricated with TBA II and TBA III were also checked. The results showed that two biosensors fabricated with TBA II and TBA III exhibited a linear response of the R_{et} to the increase of thrombin concentration in the range from 0.12 nM to 30 nM, respectively. The regression equation was $\Delta R_{et} = 502.3 + 108.2 \times 10^{-9}$ c (unit of c, M) with a regression coefficient of 0.9953 for TBA II and $\Delta R_{et} = 714.3 + 91.5 \times 10^{-9}$ c (unit of c, M) with a regression coefficient of 0.9929 for TBA III, respectively. The lowest detection limit of 0.03 nM was obtained by the biosensor with TBA II. From the slopes of three regression equations, the sensitivity sequence, TBA II > TBA III > TBA I was obtained. The different sensitivity is probably related to different binding ability of different TBA with thrombin, which will be discussed later.

3.2.2. Selectivity of aptamer-based biosensor

To assess the specificity of the biosensor with TBA I, fourfold excess of BSA and Hb was examined as the same protocol, respectively. The result showed that the increase of the R_{et} for

thrombin ($\Delta R_{et} = 630\,\Omega$) is most obvious, while the changes of resistance are nearly negligible for BSA ($\Delta R_{et} = 30\,\Omega$) and Hb ($\Delta R_{et} = 20\,\Omega$) (Figure not showed). This indicates that the specificity of the biosensor, therefore, was obtained.

The stability of the GNPs electrodeposited on GCE stored in water at 4 °C was estimated by discontinuously determining the EIS response in 0.10 M sodium phosphate buffer (pH 7.40) containing 10 mM $K_3Fe(CN)_6$−10 mM $K_4Fe(CN)_6$ every day. The result showed that the change in ΔR_{et} was less than 5% within 10 days. This indicates that the detachment of the GNPs is negligible within 10 days. This is probably attributed to the fact that interaction between GNPs and the surface of glassy carbon electrode is combination of physical adsorption with Au–O–C bond. The carbon on the surface of GCE was oxidized to C–OH and C=O groups when GCE was pretreated by applying potential of +1.1 V for 60 s. The storage stability of the biosensors was tested. It was found that the response of the biosensors with TBA I to 6.0 nM thrombin was not significantly changed when the biosensors were stored at 4 °C for 2 weeks. A stability of the biosensors, therefore, was evident.

3.3. Kinetic studies of immobilized aptamers interaction with thrombin

Fig. 4 shows the impedance spectra of the biosensor with TBA I at different binding time from 0 to 130 min. The concentration of 60 nM thrombin was used in this kinetic study in order to maintain sufficient target analyte. The inset of Fig. 4 shows the dependence of R_{et} on binding time and "washing time". From the inset of Fig. 4, it can be clearly seen that R_{et} increases gradually with increasing binding time from 0 to 95 min. This is consistent with the electrostatic repulsion of the redox indicator from the electrode interface by the formation of the TBA I-thrombin complexes, thereby introducing a barrier for interfacial electron transfer. After 95 min, R_{et} reached a plateau, indicating that the association reaction reached the saturation. $R_{et,eq}$ was obtained from the average of fitted values from Fig. 4, curve 11–13. For the dissociation process, upon washing the surface of the biosensor with 0.10 M PBS (pH 7.40), the obtained R_{et} significantly decreased with an increase of washing time up to 70 min (from 130 min to 200 min). Actually, the association rate constant k_a was calculated from the data from 0 to 130 min in the inset of Fig. 4, while the disassociation rate constant k_d was calculated from the data from 130 min to 200 min in the inset of Fig. 4, when thrombin was removed from the surface of the biosensor by washing with a suitable buffer. By plotting of $\ln R_{et}/R_{et,eq}$ as a function of the washing time, a straight line was obtained. The regression equation was $\ln R_{et}/R_{et,eq} = -1.0 \times 10^{-4}\ t$ with a regression coefficient of 0.9624. From the slope of the regression equation, k_d can be calculated to be $1.0 \times 10^{-4}\ s^{-1}$, which is close to the value reported ($6.3 \times 10^{-4}\ s^{-1}$) (Baldrich et al., 2005). By plotting of $\ln \Delta R_{et}/R_{et,eq}$ as a function of the binding time, a straight line was obtained. The regression equation $\ln \Delta R_{et}/R_{et,eq} = -5.0 \times 10^{-4} t - 0.61$ with a regression coefficient of 0.9692. From the slope of the regression equation, k_a can be deduced to be $6.7 \times 10^3\ M^{-1}\ s^{-1}$. Clearly, the association constant K_a can be easily calculated from the Eq. $K_a = k_a/k_d$. Thus, the association constant K_a was $6.7 \times 10^7\ M^{-1}$ and the dissociation constant K_d was 15 nM. K_d obtained in this work is nearly close to that (26 nM) reported (Tasset et al., 1997). This indicates that the proposed method for the determination of kinetic parameters is credible. As kinetic study of TBA I, the rate constants of TBA II with thrombin was also measured (Fig. S 1). The result showed that k_d was $5.0 \times 10^{-5}\ s^{-1}$ and k_a was $1.2 \times 10^4\ M^{-1}\ s^{-1}$. Thus, K_d obtained was 4.2 nM, which was consistent with that obtained (5.88 nM) by quartz crystal sensor method (Hianik et al., 2005).

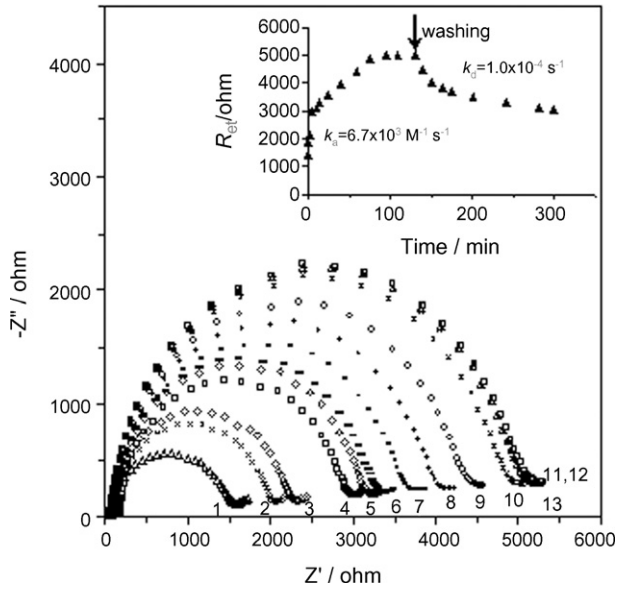

Fig. 4. Nyquist plots of aptamer-based biosensors fabricated with TBA I measured in 0.10 M PBS (pH 7.40) containing10 mM $K_3Fe(CN)_6$–10 mM $K_4Fe(CN)_6$ after the biosensor was immersed in 0.10 M PBS (pH 7.40) containing 60 nM thrombin for different association times (1) 0 min; (2) 1 min; (3) 3 min; (4) 5 min; (5) 10 min; (6) 15 min; (7) 25 min; (8) 40 min; (9) 60 min; (10) 75 min; (11)95 min; (12)110 min; (13) 130 min. R_{et} in the inset is obtained from the fitting of impedance spectrum.

3.4. Association constants of immobilized aptamer with thrombin

The equilibrium constants of the binding reaction of three aptamers self-assembled on the surface of GNPs modified GCE with interaction of thrombin, were determined (Fig. S 2) according to the EIS method reported by Szymánska et al. (2007). According to Eq. (1),

$$\frac{R_{et,i} - R_{et,0}}{R_{et,0}} = \frac{\Delta R_{et}}{R_{et,0}} = K_a c \quad (1)$$

by plotting of $\Delta R_{et}/R_{et,0}$ as a function of the concentration of thrombin, a straight line was obtained. From the slope of the regression equation, the association constant K_a can be calculated. The dissociation constant K_d obtained in this work and reported from references are listed in Table 1. From Table 1, it can be seen that K_d of TBA I (20 nM) is the same as K_d of TBA III (20 nM), but it is higher than that of TBA II (10 nM). This gives a good reason for a high sensitivity obtained by the biosensor fabricated with TBA II. This indicates that the asso-

Table 1
Dissociation constants of aptamer–thrombin complex obtained by different methods

Aptamer	Site of thrombin	K_d (nM)	Method/technique	Ref.
TBA I (15 b)	Exosite I	20	Equilibrium, EIS	This work
		15	Kinetic approach, EIS	
TBA I (31 b)	Exosite I	240	Non-equilibrium, capillary electrophoresis	Berezovski et al., 2003
TBA I (17 b)	Exosite I	5.20	Non-equilibrium, titration curves	Li et al., 2002
		4.87	Non-equilibrium, fluorescence	
TBA I (15 b)	Exosite I	450	Affinity probe capillary electrophoresis	German et al., 1998
TBA I (19 b, 20 b, 21 b)	Exosite I	10	Equilibrium, fluorescence	Hamaguchi et al., 2001
TBA II (29 b)	Exosite II	10	Equilibrium, EIS	This work
		4.2	Kinetic approach, EIS	
TBA II (32 b)	Exosite II	5.88[a]	Equilibrium, quartz crystal microbalance	Hianik et al., 2005
		25[a]	Equilibrium, differential pulse voltammetry	
		11.11[a]	Equilibrium, fluorescence	
TBA II (29 b)	Exosite II	0.5	Nitrocellulose filter binding	Tasset et al., 1997
TBA III (37 b)	Exosite I	20	Equilibrium, EIS	This work

[a] The values K_d were calculated from the corresponding reference.

ciation constant K_a of the aptamer with target analyte protein is mainly dominated by the binding sequence of the aptamer and binding sites of thrombin. This result also can be explained by the configurations of aptamers and thrombin. It was reported that human α-thrombin (thrombin) has two positively charged sites termed Exosite I and II on the opposite sides of the protein (Cao and Tan, 2005). The binding sequence of TBA I is the same as that of TBA III, which binds with the Exosite I of thrombin. The binding sequence of TBA II binds with the Exosite II of thrombin (Tasset et al., 1997).

Compared the dissociation constant K_d (15 nM for TBA I, 4.2 nM for TBA II) obtained by the kinetic approach with that K_d (20 nM for TBA I, 10 nM for TBA II) obtained by concentration equilibrium approach, the dissociation constant K_d obtained from the former is slightly lower than that derived from the latter. However, the difference is within the experimental accuracy (Liu et al., 2005b). K_d of TBA I obtained is coincident with that obtained by Bock (25 nM) (Bock et al., 1992) and is slightly higher than that reported by Hamaguchi using fluorescence method (10 nM) (Hamaguchi et al., 2001). This indicates that our results should be believed. However, K_d of TBA I obtained by Szymánska method (20 nM) was much lower than that obtained by Berezovski et al. (2003) (240 nM) and much lower than that obtained by German et al. (1998) (450 nM). This large discrepancy is probably ascribed to the fact that the binding reaction occurres in solution. This also implies that orient immobilized aptamer on GNPs modified GCE benefits efficiently binding with thrombin. K_d of TBA I obtained by our method (20 nM) is slightly higher than that obtained by Li et al. (2002) (5.20 nM and 4.87 nM). This is attributed to the different sequence of the used aptamers and different experimental methods and test conditions including pH, ion strength, temperature and sensor platform. K_d of TBA II obtained by our method (10 nM) is in the range obtained by Hianik using differential pulse voltammetry (25 nM) and quartz crystal sensor method (5.88 nM) on a modified gold electrode (Hianik et al., 2005). K_d of TBA III obtained by our method (20 nM) is first reported and reliable although it cannot be compared with the reported value. Thus, it might be concluded that EIS is very suitable technique for the determination of equilibrium constants of the binding reaction between aptamer self-assembled on a GNPs modification electrode and thrombin.

4. Conclusions

The study described herein has developed a simple and highly sensitive EIS biosensor for the determination of thrombin. EIS technique rather than linear potential sweep technique can achieve a high sensitivity of the biosensor developed. The sensitivity of EIS biosensor designed for target protein can be obtained by using a high binding constant of aptamer. This work demonstrates that GNPs electrodeposited on GCE used as a platform for the immobilization of the thiolated aptamer can improve the sensitivity of an EIS biosensor for the determination of protein. This work also demonstrates that EIS method is an efficient method for the study of aptamer–protein interfacial interactions on GNPs modified GCE.

Acknowledgements

Financial supports from the National Science Foundation of China (No. 90607016 and No.20375025) are gratefully acknowledged.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2008.01.029.

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