

# Label-free protein recognition using an aptamer-based impedance measurement assay

Hong Cai, Thomas Ming-Hung Lee, I-Ming Hsing\*

*Department of Chemical Engineering, The Hong Kong University of Science and Technology,  
Clear Water Bay, Kowloon, Hong Kong*

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## Abstract

A novel aptamer-based biosensing assay for label-free protein detection and quantification by measuring the change in electrochemical impedance upon protein–aptamer complex formation was demonstrated. Our process starts with a self-assembly of the DNA aptamer on a microfabricated thin film gold electrode, followed by the recognition of the protein binding event via monitoring the interfacial electron transfer resistance with electrochemical impedance spectroscopy. The thrombin binding aptamer (TBA) has high binding affinity and specificity to its target protein and the impedance detection assay shows a reliable and sensitive quantification of thrombin with a detection range of four orders of magnitude and a detection limit of 0.1 nM.

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## 1. Introduction

The detection and quantification of proteins play essential roles in fundamental research as well as in clinical practice. To date, the antibody-based immunological assays are the most commonly used diagnostic formats, and methods used for protein detection are not as sensitive and specific as methods for detection of specific nucleic acid sequences. Aptamers, which are DNA or RNA molecules isolated from random-sequence nucleic acid libraries by “in vitro selection” [1,2], have been shown to be useful for the detection of protein analytes and can rival antibodies in diagnostic application. In this regard, numerous aptamers have been created for a broad range of proteins [3]. As an alternative to antibodies, aptamers bind targets with affinity and specificity comparable to those of monoclonal antibodies. Moreover, they have several inherent advantages over antibodies such as longer shelf-life, resistant to denaturation and degradation and can be more readily engineered for use

as biosensors. Also, since aptamers are nucleic acids, the work on developing DNA sensing assays will be applicable to the development of the aptamer-based protein recognition assays. Currently, several nucleic acid aptamer-based biosensors for protein detection have been developed [4–18]. The signal transduction approaches were mainly based on the quartz crystal microbalance (QCM) [6,7], surface plasmon resonance (SPR) [8], fluorescence approach [9–14] or other optical methods [15,16]. Until now, very few studies have reported the electrochemistry methods for the transduction of the protein recognition events on electrode surfaces [17,18].

In this work, we report a novel aptamer linked biosensor that can detect and differentiate label-free proteins by measuring the electrochemical impedance of the protein–aptamer complex. This approach for the protein detection inherits the merit of electrochemistry-based biosensor for the DNA detection [19–22] and addresses the issues of defolding/denaturation of protein detection on a solid surface. Our process starts with the self-assembly of the DNA aptamer, which has high binding affinity to the target protein, on the microfabricated thin film gold electrode, followed by the

\* Corresponding author. Tel.: +852 23587131; fax: +852 23580054.

E-mail address: [kehhsing@ust.hk](mailto:kehhsing@ust.hk) (I.-M. Hsing).

recognition of the formation of aptamer–protein complex via monitoring the changes in interfacial electron transfer resistance ( $R_{et}$ ) of electrochemical impedance spectroscopy.

## 2. Experimental

Two HPLC purified oligonucleotides were purchased from Invitrogen (Carlsbad, CA): thrombin binding aptamer (TBA) of 5'-thiol-TTT TTT GGT TGG TGT GGT TGG-3'; a scrambled sequence of 5'-thiol-TTT TTT GGT GGT TGT TGT GGT-3'. Sodium phosphate monobasic, sodium phosphate dibasic, sodium chloride, potassium chloride, magnesium chloride, mercapto-hexanol, and hexanol were purchased from Aldrich (Milwaukee, WI). Thrombin from human plasma (EC3.4.21.5), bovine serum albumin (BSA), and hemoglobin were obtained from Sigma (St. Louis, MO). The electrochemical system comprised a working electrode of gold-coated glass (Corning 7059, Corning Incorporated, Danville, VA, USA), a Ag/AgCl reference electrode (3M NaCl filling solution saturated with AgCl, EG&G, Princeton Applied Research, Oak Ridge, TN), and a platinum wire counter electrode. The 250 nm Au layer was patterned with standard photolithographic and wet chemical etching processes to define the active sensing area (1.275 mm<sup>2</sup>).

The working Au electrode was treated with a solution containing  $5 \times 10^{-6}$  M of TBA or the scrambled sequence in 0.1 M PBS<sup>+</sup> buffer (phosphate buffer containing 10 mM NaCl, 5 mM KCl, 1 mM MgCl<sub>2</sub>, pH 7.4) for 12 h. Proper folding of the aptamer has been provided by heating the solution to 90 °C for 3 min and then cooling by immersion into the ice bath. After washing with the PBS<sup>+</sup> buffer, the DNA-functionalized Au-surface was treated with mercapto-hexanol ( $1 \times 10^{-3}$  M in hexanol) for 1 h to yield the DNA assembly on the electrode surface. The resulting monolayer-functionalized electrode was incubated 20 min with the protein analyte (thrombin, various concentrations in 0.1 M PBS<sup>+</sup> buffer) for protein–aptamer interaction. As in control experiments, the TBA-functionalized electrode was treated with non-specific target BSA (0.5 mg mL<sup>-1</sup>), or hemoglobin (0.1 mg mL<sup>-1</sup>) for the same period of time. The electrode was rinsed with 0.1 M PBS<sup>+</sup> buffer and applied for the electrochemical measurements.

The electrochemical measurements were performed by using an Autolab PGSTAT electrochemical impedance analyzer and potentiostat/galvanostat (Eco Chemie, The Netherlands) connected to a computer (Eco Chemie software: Frequency Response Analyser (FRA 4.9) and General Purpose Electrochemical System (GPES 4.9) for impedance and cyclic voltammetry, respectively). All the impedance measurements were performed in 10 mM NaCl/0.1 M phosphate buffer solution, pH 7.0, in the presence of 10 mM K<sub>3</sub>[Fe(CN)<sub>6</sub>]/K<sub>4</sub>[Fe(CN)<sub>6</sub>] (1:1 mixture) as a redox probe, at room temperature. The measurements were performed in the frequency range of 1.0 Hz to 100 kHz and upon biasing the working electrode at  $E = 0.19$  V. The impedance spectra

were plotted in the form of complex plane diagram (Nyquist plots). The experimental impedance data were simulated using electronic equivalent circuits. A typical shape of a faradaic impedance spectrum includes a semicircle region lying on the  $Z_{re}$ -axis followed by a straight line. The semicircle portion, observed at higher frequencies, corresponds to the electron-transfer-limited process, whereas the linear part is characteristics of the lower frequency range and represents the diffusion-limited electron-transfer process. The semicircle diameter equals the electron transfer resistance,  $R_{et}$ . This resistance controls the electron-transfer kinetics of the redox-probe at the electrode interface; its value is extracted from the respective computer simulated spectra and taken as measurement signal.

## 3. Results and discussion

In this paper, thrombin, a critical enzyme in the blood coagulation system with a well-established aptamer structure from SELEX [23,24] was chosen as a target protein to demonstrate our strategy. The core sequence of this aptamer is 5'-GGT TGG TGT GGT TGG. In phosphate buffer solution containing potassium ions, this aptamer folds into a chair-like structure with two G-tetrad stacks by two TT loops and a single TGT loop. The formation of the quadruplex structure plays an important role in binding to thrombin [25]. In order to immobilize the aptamer probe on the gold electrode surface with a reduced hindrance effect, an active end thiol group and the six additional nucleotides (T<sub>6</sub>) spacer was modified to the 5'-end of the 15-mer DNA aptamer. The electrochemistry-based detection strategy is outlined in Fig. 1, where the thiolated TBA was self-assembled as a monolayer on a micro-fabricated Au electrode surface. Unreacted surface sites on the electrode were further blocked by mercapto-hexanol. The surface coverage of TBA is determined by the electrochemical method described by Tarlov and co-workers [26] and it corresponds to a value of  $6.5 \times 10^{-12}$  mol/cm<sup>2</sup>. The resulting monolayer-functionalized electrode was allowed to interact with the thrombin analyte. Fig. 2 shows the

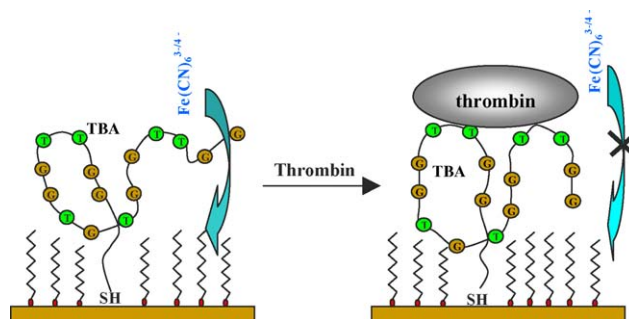


Fig. 1. Label-free electrochemical sensing of thrombin using thrombin binding aptamer (TBA) functionalized gold electrode and electrochemical impedance spectroscopy as a means of transduction.

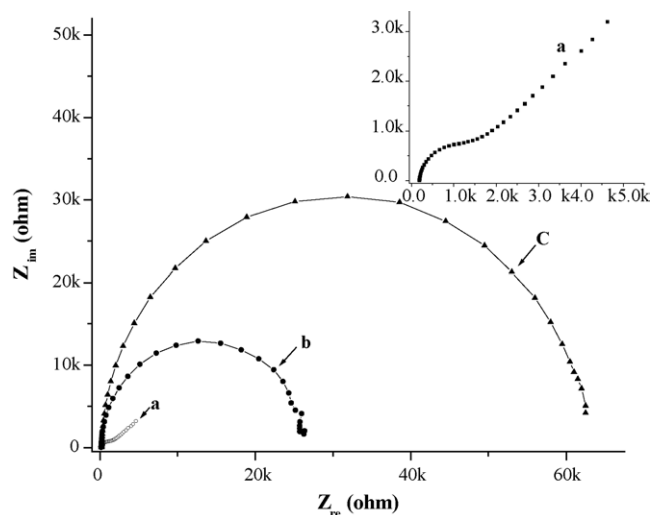


Fig. 2. Impedance spectra (Nyquist plots,  $Z_{im}$  imaginary impedance,  $Z_{re}$  real impedance) of (a) bare microfabricated Au electrode, (b) TBA-functionalized Au electrode after blocking with mercapto-hexanol (1.0 mM; 60 min) and (c) after interaction with the protein analyte, thrombin (concentration 1.0  $\mu$ M, 20 min). Inset: impedance spectra of (a) the bare Au electrode. All measurements were performed using 1.275 mm<sup>2</sup> Au electrodes in a 0.1 M phosphate buffer (pH 7.2 with 10 mM NaCl) containing 10 mM  $[\text{Fe}(\text{CN})_6]^{3-}/[\text{Fe}(\text{CN})_6]^{4-}$  (1:1 mixture); bias potential 0.19 V vs. Ag/AgCl, frequency range 1.0 Hz to 100 kHz, alternate voltage 5 mV.

impedance spectra (in the form of a Nyquist plot,  $Z_{im}$  versus  $Z_{re}$ ) using  $[\text{Fe}(\text{CN})_6]^{3-}/[\text{Fe}(\text{CN})_6]^{4-}$  as the redox-probe, observed upon the assembly of the TBA monolayer on the electrode (curve b), and reaction with the target thrombin (bulk concentration 1.0  $\mu$ M, curve c). The experimental electrochemical impedance spectra were fitted by the computer simulated spectra using an electronic circuit based on the Randles and Ershler theoretical model [27]. This equivalent circuit includes the ohmic resistance of the electrolyte solution,  $R_s$ , the Warburg impedance,  $Z_w$ , resulting from the diffusion of the redox-probe, the double-layer capacitance,  $C_{dl}$ , and the electron-transfer resistance,  $R_{et}$ . The latter two components,  $C_{dl}$  and  $R_{et}$ , represent interfacial properties of the electrode, which is highly sensitive to the surface modification. When a non-homogeneous surface layer exists, constant phase element (CPE,  $\text{CPE} = A^{-1}(j\omega)^{-n}$ ) can be introduced into the circuit instead of a capacitance. The CPE becomes equal to the  $C_{dl}$  when  $n = 1$ . The experimental electrochemical impedance spectra can be well fitted with equivalent electronic circuit ( $R_s(\text{CPE}[R_{et}Z_w])$ ) including  $R_s$ ,  $Z_w$ , CPE and  $R_{et}$ . The bare Au electrode exhibits a very small semicircle domain, suggesting a very low electron-transfer resistance to the redox probe dissolved in the electrolyte solution (inset figure). The assembly of the TBA monolayer onto the electrode support increases  $R_{et}$  to 25.0 k $\Omega$  (curve b). This is attributed to the electrostatic repulsion between negative charges of the DNA aptamer backbone and the redox probe,  $[\text{Fe}(\text{CN})_6]^{3-}/[\text{Fe}(\text{CN})_6]^{4-}$ , which results in a barrier for the interfacial electron transfer. After incubation with thrombin

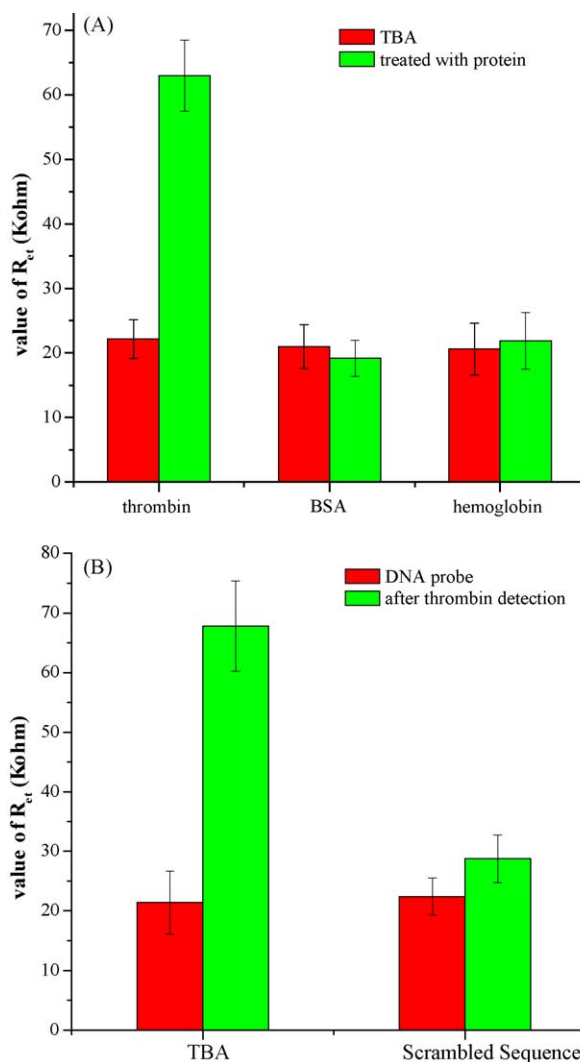


Fig. 3. Binding specificity of the aptamer to its protein target. (A) Comparison of the change of the electron-transfer resistances ( $R_{et}$ ) at the TBA-monolayer functionalized electrode upon exposure to thrombin, bovine serum albumin (BSA), and hemoglobin. (B) A scrambled sequence also tested for its interaction with thrombin. The impedance measurement conditions as in Fig. 2. Error bars show the errors of the response of three different electrodes at the same protein concentration.

analyte, the formation of the TBA–thrombin complex on the electrode surface contributes to the large increase of  $R_{et}$  to 63 k $\Omega$  (curve c). This is consistent with the fact that the resistive hydrophobic layer of protein molecules insulates the conductive support and perturbs the interfacial electron transfer between the electrode and the electroactive species in solution.

The binding affinity/specificity between the aptamer and the protein target determines the increase of  $R_{et}$ . As can be seen from Fig. 3A, the increase of  $R_{et}$  for the bound complex of human thrombin and its specific aptamer (TBA) is most obvious, while the change of resistance is minimal for other proteins (BSA or hemoglobin) and TBA. Similarly, the interaction between the thrombin and the DNA probe

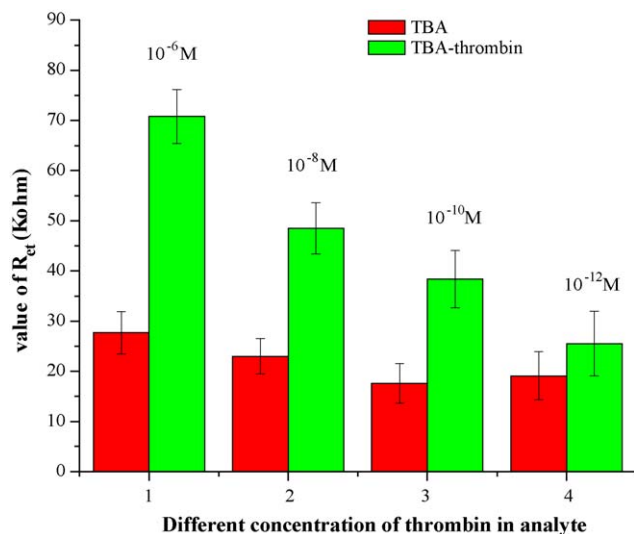


Fig. 4. Change of the electron-transfer resistances ( $R_{et}$ ) at the TBA-monolayer functionalized electrode upon the analysis of different concentration of the thrombin.  $R_{et}$  data are extracted from the corresponding Nyquist diagrams. Other conditions as in Fig. 2. Error bars represent the standard deviation of three measurements for each concentration of thrombin.

with scrambled sequences (5'-GGT GGT TGT TGT GGT) leads to a minimal change of  $R_{et}$  (Fig. 3B). This suggests that the unique quadruplex structure formed by the thrombin-specific aptamer sequence is required for the highly affiliative thrombin binding, as previously revealed by NMR and X-ray diffraction studies [23,25,28–30].

To ensure this detection platform can be used for detecting diluted protein samples, impedance responses with different thrombin concentrations (1.0  $\mu$ M–1.0 pM) are studied. As the concentration of the thrombin increases, the resultant electron transfer resistance is enhanced (Fig. 4). This impedance and aptamer-based assay shows a reliable and sensitive quantification of thrombin with a detection range of four orders of magnitude and a detection limit of 0.1 nM using 3S.D. of the blank solution ( $n = 7$ ).

#### 4. Conclusions

For the proteome and diagnostics applications, there are a large number of the proteins to be detected and it would be very useful to rely on a detection platform without the need to label specific protein targets. In this work, the introduction of impedance-based detection addresses the labeling issue and the use of nucleic acid aptamers offers higher stability (compared to antibody) and ease of sensor surface regeneration. The label-free protein detection assay demonstrated in the work shows novel means to sense aptamer–protein interaction at the transducer interface by monitoring the change of  $R_{et}$  obtained from electrochemical impedance spectroscopy. With more and more aptamer structures being identified by SELEX for specific proteins, the aptamer-based approach demonstrated in this work promises a sensitive and reliable

protein biosensor that out-rivals the counterpart utilizing the antigen and antibody.

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## Biographies

**Hong Cai** studied analytical chemistry at the East China Normal University, Shanghai, China. For her diploma, she worked with Professor Yuzhi Fang at the Department of Chemistry and she obtained her doctorate in 2003. During PhD study, her research works are mainly focus on the nano-material based biosensors and sequence-specific electrochemical detection of DNA hybridization. After that, she joined Dr. I-Ming Hsing's research group for postdoctoral training in Department of Chemical Engineering at Hong Kong University of Science and Technology. Her current research aims at the development of aptamer-based biosensors resulting in biosensor arrays and the integrated bioanalytical microsystem for DNA/protein analysis.

**Thomas Ming-Hung Lee** obtained his BEng, MPhil and PhD in chemical engineering at the Hong Kong University of Science and Technology in 1998, 2000 and 2003, respectively. During his graduate studies, he had been working on micro-PCR and electrochemical DNA sensors. In 2004, he joined Prof. Joseph Wang in New Mexico State University (currently in Arizona State University) as a postdoctoral fellow and had worked on nanocrystal-based bioelectronic coding of SNP as well as aptamer-based protein detection. Now, he returned to Prof. Hsing's group and is working on integrated bioanalytical microsystems.

**I-Ming Hsing** obtained his BS (1990) in Department of Chemical Engineering at the National Taiwan University and his MSCEP (1994) and PhD (1997) degrees from the Massachusetts Institute of Technology (MIT), USA. His group has been leading the research effort at the Hong Kong University of Science and Technology to develop novel microsystems for biological applications. His research interest in this area is to develop a point-of-use bioanalytical microsystem with emphasis on the fundamental understanding of biological reactions in a microenvironment as well as the realization of novel, microfabrication-compatible techniques, enabling quantitative analyses of biological events in the microscale. He has published 40 plus peer review papers and gave 30 plus invited talks.