

Full Paper

## Admittance Biosensor Based on Aptamer and Nano Composite Film for Determination of Thrombin using Coulometric Admittance Voltammetry

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**Abstract-** This work describes a novel thrombin admittance biosensor based on self-assembled anti-thrombin-aptamer. The biosensor was constructed by modification of glassy carbon electrode by using of MWCNT- 1-butyl-3-methylimidazolium hexafluorophosphate, electro-deposition of gold nanoparticles and finally loading DNA aptamer on the electrode surface. The electrochemical response of thrombin (in form of coulometric admittance) was obtained by coulometric FFT admittance voltammetry (CFFTAV). The analyte signal was established on the decrease of the admittance related to the redox couple of probe (Fe(CN)<sub>6</sub>]<sup>3-/4-</sup>). The influences of important parameters in the determination process were investigated, and optimized. The change in the analyte response was proportional to the concentrations in range of 0.03 to 18 nM, with a detection limit of 8×10<sup>-12</sup> M. Moreover, the proposed biosensor exhibited high sensitivity and long-term stability. The biosensor was successfully used to the determination of thrombin content in the biological samples.

**Keywords-** Thrombin, DNA, Coulometric Admittance Voltammetry, Biosensor, Au nanoparticles

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## 1. INTRODUCTION

Thrombin (TM) has been considered as a multifunctional serine protease and an important role player in formation blood clotting, and its concentration is essential in in medicine such as, the central nervous system injury, thromboembolic disease [1,2]. In fundamental researches, the detection and quantification of TM can be very important for clinical applications. In this direction, for early disease electrochemical methods for detection could be the best candidate for screening and diagnosis. Therefore, research on the obtaining a sensitive, reliable, and low-cost of TM biosensor for using in blood matrixes has gotten a considerable interests.

Among the biosensor, aptasensors with an aptamer as the biosensing elements has been proposed. Where, aptamers (RNA or DNA) that known as artificial nucleic acid ligands with a specific binding affinity for protein [3-6]. Due to their advantages, aptasensors provide a simple, sensitive and selective platform; therefore, they electrochemical have attracted particular attention [7-10]. In recent years, application of metal and carbon nanoparticle, in construction of such aptasensors, could be considered as an improvement in their sensitivity, due to enhancement of electrical and electrocatalytic activity properties [11-13].

For example, it has shown that in the design of many biosensor, modification with carbon nanotubes (CNTs) can cause an electrocatalytic activity for electro-oxidation of molecules [14,15]. Also, anti-fouling capability of electrode surfaces upon modification with CNTs, is the other important practical advantages for obtaining higher surface activity [16,17]. In this direction, it is clear that the high conductivity Ionic liquids (IL) also, can facilitate the electron transfer at the electrode solution interface [18]. In addition to its high ionic conductivity, good stability and well biocompatibility, IL was used as an electrode modified material in new works [19,20].

In this trend, the further improve of sensitivity in detection have gained by using metal NPs, various NPs have been exploited for loading onto carbon particles to amplify detection signal. In fact, carbon particles would be desirable matrixes to be decorated by metal NPs such AuNPs for constructing of bimolecular sensors, where the successful applications of NPs in the new works mainly depended on the controllable size and outstanding monodispersity [21,22]. AuNPs provide a natural environment for bimolecular immobilization and gained much more attention in electroanalytical studies because of its unique properties such as easy preparation, good biocompatibility and relatively large surface area [23-26].

This work introduces a new electrochemical technique for determination of TM based on coulometric FFT admittance voltammetry (CFFTAV) [27-31] combine with a new biosensor. The new designed biosensor was fabricated by MWCNT mixed with 1-butyl-3-methylimidazolium hexafluorophosphate (BMIMPF<sub>6</sub>) and Au NPs. Electrochemical Impedance spectroscopy (EIS) was used to characterize the surface of the biosensor. The presence of a DNA-aptamer in the biosensor provides an environment for enhancement the response of the analyte. The optimal conditions were find and the designed electrochemical technique exhibited a linear response to TM concentrations.

## 2. MATERIALS AND METHODS

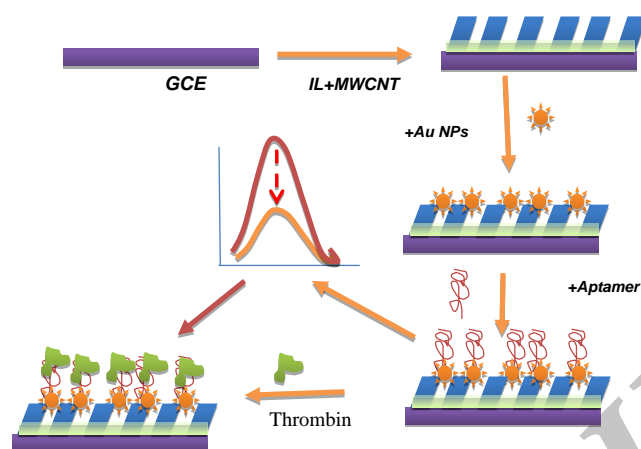
### 2.1. Reagents

All chemicals and solvents used were of analytical grade and used as received. Thrombin, BMIMPF<sub>6</sub> were obtained from Sigma Chemical Co. Shortened carboxylate MWCNT were supplied by Shenzhen Co, China. The thiolated anti-thrombin oligonucleotide: ssDNA(5'-(SH)-(CH<sub>2</sub>)<sub>6</sub>-CCA TCT CCA CTT GGT TGG TGT GGT TGG-3' (ATA) was purchased from TaKaRa (Dalian, China). 0.1 M phosphate buffered solutions (PBS, pH 7.0) containing 5 mM KCl and 2 mM MgCl<sub>2</sub> was employed to investigate the performance of electrodes and 0.05 M PBS (pH 7.4) was served as washing solution. The needed aqueous solutions were prepared in doubly distilled water which was obtained from a Milli-Q water purifying system. All other reagents were of analytical reagent grade and used without further purification.

### 2.2. The biosensor fabrication

A glassy carbon electrode (GCE, 3. mm in diameter) were polished, with 1.0, 0.3 and 0.05  $\mu\text{m}$  alumina slurry, and then it was washed thoroughly with doubly distilled water. The electrodes were successively sonicated in 1:1 nitric acid, acetone and doubly distilled water, and then allowed to dry at room temperature. The fabrication of MWCNT-IL on GCE involves three sequential stages. 10 mg of purified MWCNT were dispersed in 10 mL of dimethylformamide (DMF) with the aid of sonication for 2 h to give a 1.0 mg/mL homogeneous suspension. MWCNTs-BMIMPF<sub>6</sub> was prepared by adding 0.05 g of BMIMPF<sub>6</sub> into the suspension and sonication was used to homogenize the solution. Then, 10  $\mu\text{L}$  of the MWCNT-IL suspension was dropped on the GCE, and let it dry at room temperature for 3 h, to form a uniform membrane coated MWCNT-IL/GCE. For preparation of AuNPs/MWCNT-IL/GCE, Au NPs was deposited electrochemically in 0.2 M Na<sub>2</sub>SO<sub>4</sub> aqueous solution of HAuCl<sub>4</sub> (3.0 mM) at the potential of -0.2 V. Then, for fabrication of DNA-AuNPs/MWCNT-IL/GCE, the modified electrode was incubated in 20  $\mu\text{L}$  droplet of anti-thrombin aptamer (1  $\mu\text{M}$  in the buffer at pH 7.4) for 12 h. under such condition DNA was attached onto AuNPs surface through the thiol-linker. Finally, the electrode surface was

rinsed with deionized water, followed by drying under an N<sub>2</sub> atmosphere. A schematic representation of the biosensor fabrication steps and is illustrated in Fig. 1.



**Fig. 1.** Schematic figure of the preparation DNA-AuNPs/MWCNT-IL/GCE

For detection of TM, 25  $\mu$ L droplets, of different TM concentrations in the buffer, was placed on the biosensor and, to allow TM–aptamer binding, kept for 25 min. Then, by rinsing with deionized water, the non-binding thrombin was removed from the electrode surface. The binding of TM with aptamer arm producing a much less flexible stranded element. This, in turn, prevents the  $[\text{Fe}(\text{CN})_6]^{3-/4-}$  complex to reach to the electrode surface, and producing a smaller signal.

### 2.3. Instrumentation

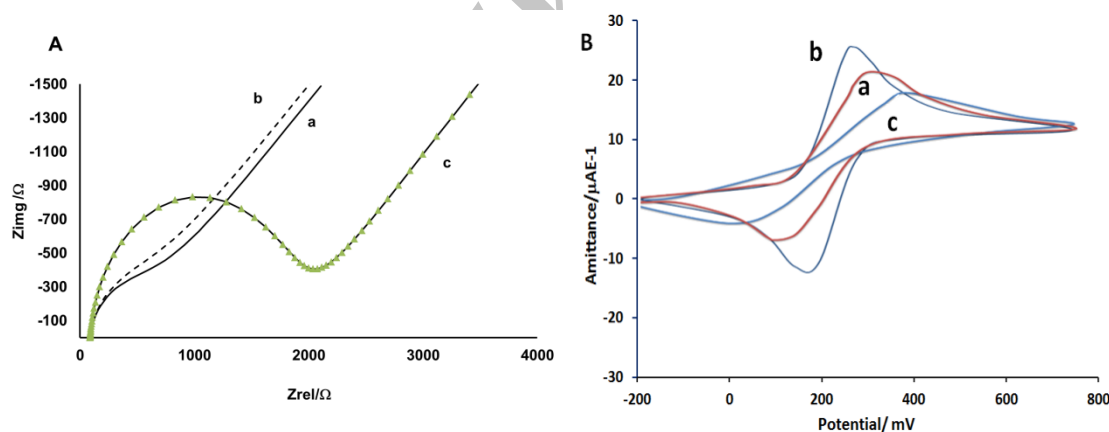
The electrochemical measurement system used, for admittance voltammetric measurements, was a homemade potentiostat, which was connected to a PC equipped via an analog to digital (A/D) data acquisition board (PCL-818H, Advantech Co.). In the measurements, the memory and CPU of the computer were used with highest priority by the A/D board for obtaining the best condition, where the potential waveform was repeatedly applied to the working electrode and then the data was acquired. In fact, all generating the analog potential waveform and acquiring and processing of data was done by the developed software in the lab. We able to process and store data or plot them in real time. EIS measurements were performed in 3 mM  $\text{K}_3\text{Fe}(\text{CN})_6$  in PBS at pH 7.4.

## 3. RESULTS AND DISCUSSION

EIS technique was used to characterize the construction layer by layer of the materials on the GC electrode, where a solution of  $\text{Fe}(\text{CN})_6^{4-/3-}$  3 mM was used as a redox probe for

collecting the data. The results of the measurements are shown in Fig. 2A. The curves are the results of faradic EIS of bare GCE (curve a), AuNPs/MWCNT-IL/GCE (curve b), and DNA-AuNPs/MWCNT-IL/GC (curve c). It can be seen that when the surface of the bare GC electrode was coated with AuNPs/MWCNT-IL the impedance of the electrode surface decreased, which is due to the surface enhancement and the easiness of  $\text{Fe}(\text{CN})_6^{4-/3-}$  diffusion toward electrode surface and charge transfer on the MWCNT film. In addition, depositions of the conductive Au NPs layer can cause more drop in the total impedance of the electrode. Contrarily, when the DNA was linked to the surface of AuNPs/MWCNT-IL/GCE the value of the total impedance increases, dramatically, increased (curve c), which is indication of formation of a well-packed DNA monolayer on the surface of AuNPs/MWCNT-IL/GCE. The obtained cyclic voltammetric results were in agreement with the obtained results from EIS.

Fig. 2B presents the cyclic voltammograms of  $[\text{Fe}(\text{CN})_6]^{3-/4-}$  on GCE (curve a) AuNPs/MWCNT-IL/GCE (curve b) and DAN-AuNPs/MWCNT-IL/GCE (curve c) in PB solution at pH 7.4. In curve a,  $[\text{Fe}(\text{CN})_6]^{3-/4-}$  shows two small redox peaks, at the bare GCE, which corresponds to a small rate kinetic reaction. Likewise, for AuNPs/MWCNT-IL/GC electrode, the recorded cyclic voltammetric has two well-defined peaks at potentials 198 and 290 mV. This could be attributed to the fact that AuNPs/MWCNT-IL has better conductivity and higher surface area, which could improve the reaction capacity of the electrode. Such condition could further enhance the electron transfer rate and, consequently, the reversibility of the redox processes.



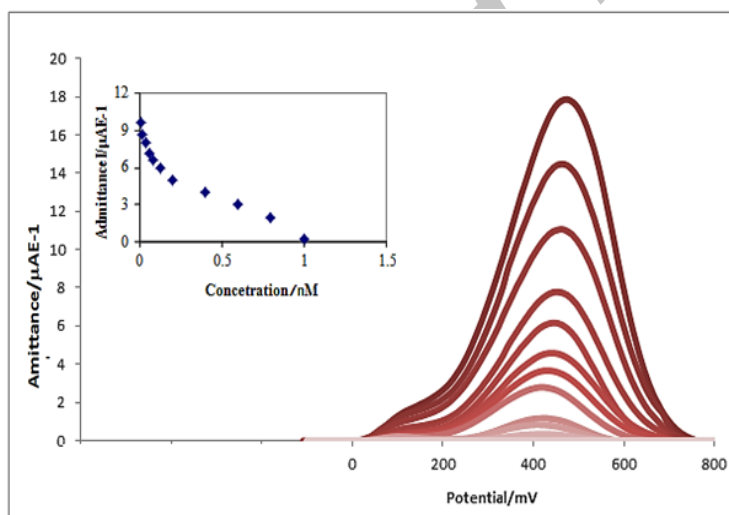
**Fig. 2.** A) EIS plots of modified electrode in 3 mM  $\text{K}_3\text{Fe}(\text{CN})_6$  with : (a) bare GCE, (b) AuNPs/MWCNT-IL/GCE and (c) DNA-AuNPs/MWCNT-IL/GCE. and B) Cyclic voltammograms of 3 mM  $\text{K}_3\text{Fe}(\text{CN})_6$  in buffer on (a) bare GCE, (b) AuNPs/MWCNT-IL/GCE and (c) DNA-AuNPs/MWCNT-IL/GCE

On the other hand, the DNA-AuNPs/MWCNT-IL/GCE demonstrations decline in the current of redox peaks, and changes the anodic and cathodic peak potentials to 130 mV and 390 mV, respectively. This could be due to the established kinetics barrier between

$[\text{Fe}(\text{CN})_6]^{3-/4-}$  and the new modified electrode. As calculated from the value of charge under the peak in CV, biosensors with  $[\text{Fe}(\text{CN})_6]^{3-/4-}$  surface density over  $6 \times 10^{11}$  molecules/cm<sup>2</sup>.

### 3.1. Determination method

Fig. 3 shows CFFTA and the changes in voltammetric response of the TM-DNA-AuNPs/MWCNT-IL/GC electrode in the potential range of 0 to 800 mV with different concentrations of TM in the range of 0.1 to  $1.5 \times 10^{-9}$  M in the buffer solution at pH 7.4. The admittance peaks at the potential of 370 mV, is the result of redox reaction of  $[\text{Fe}(\text{CN})_6]^{3-/4-}$  at the electrode surface [32-34]. As mentioned above, the accumulation of the TM at the electrode, which form TM-DNA-AuNPs/MWCNT-IL/GCE, can hinder the direct electron transfer between the  $[\text{Fe}(\text{CN})_6]^{3-/4-}$  and the active sites of the surface biosensor. Also, it can be say that the signal decrease could be likely due to the conformational effects of DNA interact with TM, which can more restrict the three-dimensional diffusion of the probe to the electrode surface. The consequence of this is correlation between the admittance peak decreases with concentration of TM.



**Fig. 3.** CFFTA voltammograms of the TM-DNA-AuNPs/MWCNT-IL/GCE biosensor in with different concentrations of 0.1.0 to  $1.5 \times 10^{-9}$  M TM in the buffer solution at pH 7.4 The potential range was 0 to 800 mV at frequency 700 Hz and amplitude 20 mV

For determination of TM by CFFTAV technique, the admittance of the biosensor was measured during the continuous potentials cans, and then, coulometric response ( $\Delta Q$ ) was calculated by integrating of net admittance changes over the selected scanned potential range [33-35]. This procedure was carried out by numerical calculated of Q as follows;

$$Q(s\tau) = \Delta t \left[ \sum_{E=E_1}^{E=E_2} |A(s, E).E| \right] \quad (1)$$

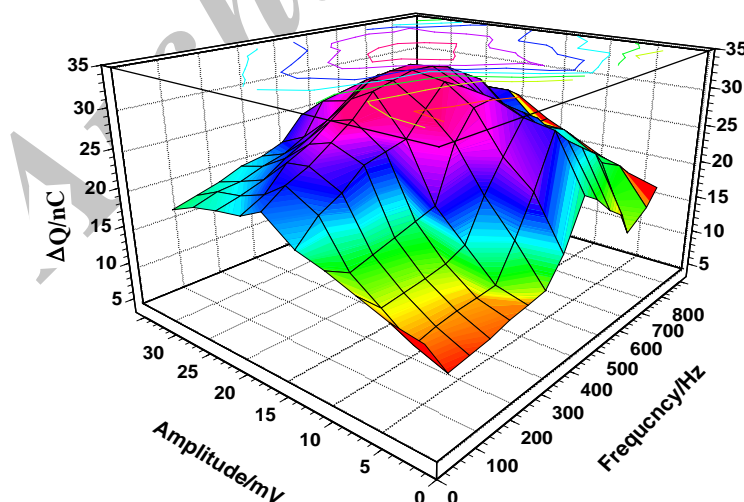
And then, biosensor response (the change in the charge under the peak,  $\Delta Q$ ) was calculated by this equation;

$$|\Delta Q| = |Q - Q_0| \quad (2)$$

Where  $s$  is the sweep number,  $\tau$  is the time period between subsequent sweeps,  $\Delta t$  is the time difference between two subsequent points on the FFTCAV curves,  $A(s, E)$  represents the admittance of the FFTCAV curve recorded during the  $s^{\text{th}}$  scan.  $Q$  is the electrical charge obtained by integration of the admittance (by converting it to the corresponding current) in potential range of 0 and 800 mV, and  $Q_0$  represents the value of  $Q$  in the absence of the analyte. Moreover, the results indicate that with increasing the concentration of TM in the spiked sample, absolute value of  $\Delta Q$ , in range of 0 to 800 mV, increases proportionally. The match of admittance signal with CV response confirms that the electrochemical response is derived from the binding of TM with aptamer and the resulted internal-attachment, where the value of  $\Delta Q$  decrease with increasing the concentration of TM. However, the maximum sensitivity of the measurement system could, when the most important parameters of the system, such as, SW frequency, amplitude and pH are optimized.

### 3.2. Optimization of SW amplitude and frequency

In any SW voltammetric technique, the value of signal, background noise and even more the peak shape of the analyte depends on frequencies and amplitude, which are used for the excitation potential waveform.

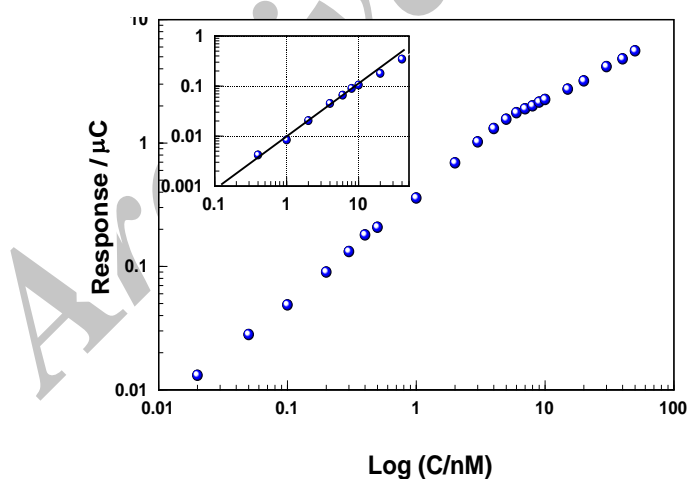


**Fig. 4.** The effect of frequency and amplitude on the response of DNA-AuNPs/MWCNT-IL/GCE for solution of 8 nM of TM

Likewise, in the CFPTAV measurement, which basically is modified form of SW voltammetric measurements, the response or sensitivity of the analyte depends on those parameters. Consequently, in order to have maximum sensitivity for the detection system, the analyte response ( $\Delta Q$ ) was measured in the SW frequencies range of 100 to 800 Hz and amplitudes of 2 to 40 mV were studied. Fig. 4 displays the dependence of  $\Delta Q$  to SW frequency and amplitude for solution of 8 nM of TM at pH 7.4. As it can be seen, the admittance of DNA-AuNPs/MWCNT-IL/GC electrode in the solution the increase with the enhancement of the SW frequency up to 700 Hz, as well as with the amplitude up to 20 mV. This may due to enhancement in value of the electron transfer.

### 3.4. Calibration curve and Analytical procedures

A stock solution of 1 mM TM was firstly prepared, and then an aliquot was diluted to the appropriate concentrations. Before each FFTCAV measurement, the three-electrode system was installed in a blank solution, and the admittance voltammetry scan from 0 to 800 mV. As mentioned above the electrode response is expressed in absolute value of the admittance peak area ( $|\Delta Q|$ , in form of C). Therefore, in this techniques the magnitude of the biosensor response depends on the choice of the integration range. Fig. 5 illustrates a typical  $|\Delta Q|$  response of the DNA-AuNPs/MWCNT-IL/GCE on a standard solution of TM (from 0.05 to 120.0 nM in the buffer solution, pH 7.4).



**Fig. 5.** The calibration curve for TM standard solutions in pH 7.4. The potential range of 0 to 800 mV at frequency 700 Hz and amplitude 20 mV. Integration potential range for the admittance is 0 to 800 mV

In order to find the best detection limits for the biosensor, each data point in the graph is averaged of the measured response for 3 consecutive additions of the TM standard solution, where the experimental parameters were set at optimum values. Under such conditions,



logarithm of the biosensor response showed a linear relation with logarithm of TM concentration in dynamic range of 0.05 to 18 nM, with a correlation coefficient of  $R=0.998$  and with %R.S.D. values 2.3%. The calculated detection limit, based on signal to noise ratio ( $S/N=3$ ), was be  $8\pm 2$  pM.

### 3.5. Pretreatment of real blood sample

In order to examine the applicability of this biosensor in real samples, control experiments were carried out in both the standard solution and the serum sample. In brief, serum was treated for the sake of converting prothrombin to TM, 5  $\mu$ L of 0.25% pancreatic enzyme was added to 40  $\mu$ L plasma, and then, 2  $\mu$ L of 20 mM calcium ion solution was added to further promote of the conversion of prothrombin to TM. During this process by adding TM into diluted healthy human serum samples five series of samples were prepared. As shown in Table 1, the recovery was acceptable between 95.1 and 109.0%. Such results, the proposed electrochemical detection system can provide a new area application of aptasensor in a real biological sample.

**Table 1.** Assay results of TM in real plasma sample ( $n=5$ )

Sample	Added amount (nM)	Amount found (nM)	Recovery (%)
1	10	10.9 $\pm$ 1	108.0
2	20	19.8 $\pm$ 1	99.0
3	30	27.7 $\pm$ 1	95.1

### 3.6. Stability and reproducibility of the Biosensor

The long-term storage stability of the DNA-AuNPs/MWCNT-IL/GC biosensor was tested. It was found that up to 45 days the sensitivity of the biosensor retained  $92.5\pm 0.2\%$  % of the initial value, and after that it gradually decreases afterwards (might be due to the DNA damage). This is an indication the biosensor was fairly stable and good reproducibility for an extensive period of time.

## 4. CONCLUSIONS

In this work, a novel and efficient electrochemical technique combined with DNA-AuNPs/MWCNT-IL biosensor has been accompanied for determination of TM. Under optimal conditions, the designed sensor exhibited a wide linear response to TM concentration, good sensitivity, a fast response time, repeatability (R.S.D value of 2.7%) and long term stability up to 45 days. It also demonstrates a highly producible sensitivity with

detection limit of 8 pM. Meanwhile, the archived detection strategy also proved to be able to distinguish the target TM in real sample. The electrochemical detection system has shown a unique features such as acceptable sensitivity and accuracy for detection of biomolecule, which can be used in measurement of such compounds in the biological samples.

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