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Bioorganic & Medicinal Chemistry Letters 15 (2005) 291–295
Bioorganic & Medicinal Chemistry Letters

Detection of aptamer–protein interactions using QCM and electrochemical indicator methods

Tibor Hianik,[a,*] Veronika Ostatná,[a] Zuzana Zajacová,[a] Ekaterina Stoikova[b] and Gennady Evtugyn[b]

[a]*Faculty of Mathematics, Physics and Computer Sciences, Comenius University, 842 48 Bratislava, Slovakia*
[b]*Department of Chemistry, Kazan State University, 420008 Kazan, Russia*

Received 7 July 2004; revised 27 October 2004; accepted 28 October 2004
Available online 18 November 2004

Abstract—We report novel method of detection thrombin–aptamer interaction based on measurement the charge consumption from the electrode covered by DNA aptamers to an electrochemical indicator methylene blue (MB), that is bounded to a thrombin. The binding of thrombin to an aptamers has been detected also by QCM method in flow measuring cell. We showed that using MB it is possible to detect thrombin with high sensitivity and selectivity.

1. Introduction

DNA/RNA aptamers are single stranded nucleic acids with high affinity to proteins or to other low and macromolecular compounds, which is comparable with affinity of antibodies.[1,2] DNA/RNA aptamers can be chemically modified by thiol groups or by biotin, that allowing them to attach to the solid surface.[3] This system can be used as a biosensor for detection the thrombin,[4] antibodies[3] or other medically important molecules in complex biological liquids. RNA aptamers have been selected also for prions,[5] which open new possibilities for rapid diagnosis of transmissible spongiform encephalopaties (TSE). The detection of protein aptamer interaction has been performed by various methods, such are quartz crystal microbalance (QCM),[3] surface plasmon resonance (SPR)[6] or fluorescence method.[7] Considerable attention has been focused on detection the α-thrombin. This is connected with unique role of this serine protease in blood coagulation.[7,8]

In our recent paper we have shown that electrochemical indicator methylene blue (MB) could be used for the study of protein–DNA interactions.[9] It is known that MB is reduced to a leucomethylene blue (LB) at an electrode surface by accepting two electrons at certain reduction potential.[10,11] MB binds both to the DNA and to the proteins. This indicator can be therefore used also for detection of protein–aptamer interactions.

In this work we report novel method of detection thrombin–aptamer interaction based on measurement the charge consumption from the electrode covered by DNA aptamers to a MB, that is bounded to a thrombin. The binding of thrombin to an aptamers has been detected also by QCM method in flow measuring cell. We showed that using MB it is possible to detect thrombin with high sensitivity that is comparable with fluorescence detection method. The method based on MB is easier and do not require additional chemical modification of the DNA by fluorescence probe and by quencher as it does when aptamer beacons are used.[7]

2. Experimental section

2.1. DNA aptamer

We used 32-mer DNA aptamer modified by biotin at 3′ end of the following sequence: 3′-BIO-GGG TTT TCA CTT TTG TGG GTT GGA CGG GAT GG-5′. This aptamer has at its 5′ end typical motif with high affinity to the thrombin.[12] The aptamer has been synthesized by Generi Biotech, Czech Republic and used as obtained.

Keywords: Aptamer; Thrombin; Methylene blue; Cyclic voltammetry; Charge consumption.
* Corresponding author. Tel.: +421 2 60295683; fax: +421 2 65426774; e-mail: hianik@fmph.uniba.sk

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doi:10.1016/j.bmcl.2004.10.083

2.2. Electrode preparation, mass and charge transfer detection

For preparation of aptamer biosensor we used either gold electrode of a diameter of 2 mm or AT cut quartz of a fundamental frequency 9 MHz (CH Instruments Inc., USA). The electrodes were carefully cleaned as follows: first they were immersed in a chloroform (Merck) and for 3 min extensively cleaned in an ultrasound sonicator bath (Tesla). After washing in double distilled water, the gold surface was cleaned with a hot mixture of piranha solution (a 1:3 mixture of 30% (v/v) H_2O_2/conc. H_2SO_4) during 5 min and then washed in double distilled water. (The piranha solution represents potential hazard, therefore it has to be handled with special care.) Cyclic voltammetry (voltage range 0.5–1.4 V vs SCE, scan rate 170 mV/s) was applied for final electrochemical cleaning in 0.2 M H_2SO_4 until an oxidation peak at approximately +0.9 V appeared and remain unchanged. The DNA aptamer was immobilized to an electrode surface by avidin-biotin technology as described elsewhere.[3] For this purpose the gold surface was first modified by 3,3′-dithiopropionic acid-di(*N*-succinimidylester) (DSP) (Fluka) and then the avidin (Molecular Probes Inc.) was added in a concentration 2 mg/mL. The electrode was then kept at 4 °C overnight. After rinsing with a buffer the electrode was then incubated with 0.025% of bovine serum albumin (BSA) (Sigma) solution. Then the electrode was immersed into the buffer (20 mM TRIS, 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4) contained the aptamer in a concentration of 2 μM for 1 h at the temperature 20 °C. Proper folding of aptamer has been provided by heating the buffer contained aptamer to 60 °C for 3 min and then cooling by immersion into the ice bath.[3] In experiments we used thrombin (Fluka). For the study of the nonspecific binding we used human immunoglobuline (IgG) and human serum albumin (HSA) (Sigma). All proteins were dissolved in a buffer in a concentration 1 mg/mL, that served as a stock solution.

In piezoelectric microgravimetry, the changes of the resonant frequency of the quartz crystal resonator due to changes of its mass load are measured. In our experiments, a standard set-up, comprising a 74LS320 oscillator circuit of Analog Devices (Norwood MA, USA) was used. According to Sauerbrey,[13] the change of resonant frequency of the resonator, Δf, is opposite to its mass change, Δm,

$$\Delta f = -2.26 \times 10^{-6} f_0^2 \, \Delta m / A \qquad (1)$$

where A is the surface area of the working electrode, f_0 being fundamental frequency of the crystal (in our case, $A = 0.28\,\mathrm{cm}^2$, $f_0 = 9$ MHz) and Δm are expressed in g. Therefore, the changes of the resonance frequency of the crystal indicate the changes of its mass, for example, here caused by binding of thrombin molecules to the aptamers. The oscillation frequency changes were measured with the UZ 2400 frequency meter (Grundig, Germany) connected through an RS232 interface with an IBM Pentium computer. The frequency was measured with 1 Hz accuracy.

The crystal was mounted between two silicon rubber o-rings, in the flow-through cell such that, the analyte solution wetted only one side of the crystal. The effective detection volume of the flow-through cell was 100 μL. The analyte solution stream was introduced into the latter by means of an in-house computer-controlled syringe system with a flow rate 60 μL/min.

Charge consumption from electrode surface to the methylene blue has been measured by differential pulse voltammetry (DPV) and using also cyclic voltammetry (CV) by means of electrochemical analyzer CHI 410 (CH Instruments Inc., USA). The three electrode setup was used in experiments: the gold electrode served as a working electrode, saturated Ag/AgCl electrode was used as a reference and Pt wire as an auxiliary electrode (all electrodes were from CH Instruments Inc., USA). The working electrode with immobilized aptamers has been immersed into the buffer contained 2 μM of methylene blue. After addition of the analyte (thrombin, IgG or HSA) into the 5 mL of the buffer solution the CV was applied in a voltage range −0.5 to −0.1 V with a scan rate 0.1 V/s. After 20 cycles the DPV method was applied at the same voltage range with following parameters: pulse amplitude 50 mV; step potential 5 mV. All experiments have been performed at temperature 20 ± 1 °C.

3. Results and discussion

In the first series of experiments we studied the binding of thrombin to the aptamers using QCM. The experiments were performed as follows. First the thrombin at a certain concentration was added in a continuously flowed buffer during 10 min. Then only the buffer has been allowed to flow in order to remove unbounded protein molecules. The value of steady-state frequency corresponded to the binding of the thrombin at certain concentration. Addition of thrombin resulted in decrease of the oscillation frequency of the quartz crystal. The plot of the changes of the frequency as a function of thrombin concentrations is shown in Figure 1. The sharp changes of the frequency started already at rather low concentration of the thrombin (detection limit approx. 1 nM). With increased concentration of the throm-

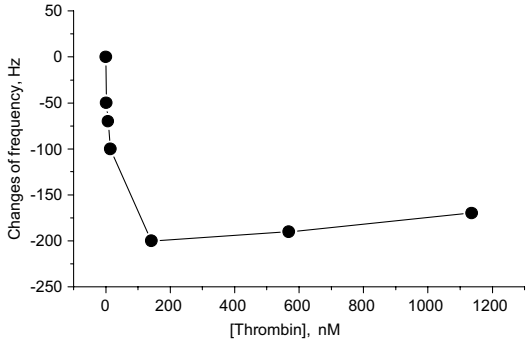

Figure 1. Representative plot of the frequency changes of the quartz crystal as a function of the concentration of the thrombin.

bin, however the saturation of the plot of frequency versus thrombin concentration took place at approx. 140 nM of the thrombin. A slight increase of the frequency at higher concentration of the thrombin could be connected with partial detachment of the aptamer from the sensor surface or with increased viscous forces. The saturation corresponds to the frequency changes of 200 Hz.

Using Eq. 1 and the know molecular mass of the α-thrombin (chains L + H[13]) (M_w = 35,422) it is possible to calculate the number of thrombin molecules attached to the aptamer layer: $N_{thrombin} = (\Delta m/M_w) \times N_A$, where N_A is Avogadro's number (6.02×10^{23} molecules/mol). $N_{thrombin} = (3.06 \times 10^{-7}/35{,}422) \times 6.02 \times 10^{23} = 5.18 \times 10^{12}$. If we considering the viscosity effect, which result in apparently higher value of Δf (approx. two-fold,[14]) then approx. 2.6×10^{12} molecules of thrombin is bounded to an aptamer layer. On the other hand, on the base of crystal structure of the thrombin[15] one can estimate the cross-sectional area of this molecule, which is approx. 20 nm^2. Therefore approx. 1.4×10^{12} thrombin molecules are required for covering the electrode surface. This value is lower then that obtained by QCM method. The reason can be connected with possible aggregation of thrombin molecules at the electrode surface and formation of certain multilayer structure. Another reason can be connected with uncertainty of determination of the number of thrombin molecules at the surface due to the influence of viscosity. Using the Scatchard plot it is possible to analyze the binding constant K_b. According to Scatchard:[16]

$$\Delta f/(f_0 c) = K_b N - K_b \Delta f/f_0 \quad (2)$$

In the case of not cooperative binding, the plot of $\Delta f/(f_0 c)$ as a function of $\Delta f/f_0$ should be the straight line (Δf are the frequency changes following addition of the thrombin, f_0 is the frequency of the oscillation of the crystal prior addition of the thrombin). From the slope of the curve it is possible to determine the binding constant K_b. Performing this procedure we obtained: $K_b = 0.17 \pm 0.06\,nM^{-1}$.

Method based on electrochemical indicator MB is schematically illustrated in Figure 2. Methylene blue binds both to DNA and to the protein. For charge transfer from electrode to the MB, that is, for MB reduction it is important that MB should be close to the electrode surface.

Therefore, the charge transfer from the electrode to MB should be more intensive to the proteins that specifically bind to the aptamer in comparison with that in a solution. The charge consumption can be measured by CV or by DPV methods, by determination of the area under the reduction peak.[17] The DPV method has revealed more sensitivity in comparison with CV. However, CV was useful for control all steps of sensor fabrication. Therefore, first the cyclic voltammetry method has been applied at the presence of MB for obtaining the information about the biosensor properties. The voltage was changed in an interval (−0.5 to 0.5 V) with a scan rate 50 mV/s. The typical CV is showed in Figure 3 for

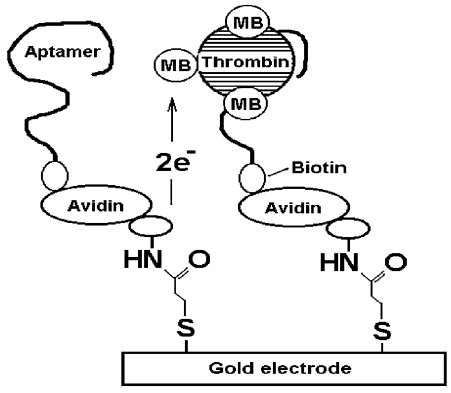

Figure 2. Schematic representation of the biotinylated aptamers immobilized on a gold electrode through avidin and the charge transfer from the electrode to the electrochemical indicator methylene blue (MB).

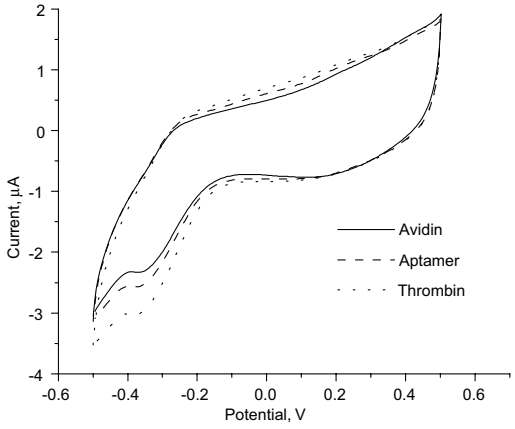

Figure 3. The cyclic voltammogram for the gold electrode covered with avidin only, or aptamer attached to the avidin layer, or thrombin bounded to the aptamer (see legend) at the presence of 2 μM MB. Concentration of thrombin was 57 nM. Scan rate: 50 mV/s.

several steps of sensor preparation. It is seen that in the region of reduction of MB (between −0.4 and −0.2 V) the most dramatic changes of CV take place.

The interaction of the thrombin as well as IgG and HSA with the aptamer has been studied by DPV method. Figure 4 shows the plot of DPV for aptamer without thrombin and at increased thrombin concentrations at the potential range where the reduction of MB take place. The DPV curves represent the function with maximum around −0.28 V, which correspond to the potential of MB reduction. We can see that with increased concentration of the thrombin the amplitude of the maximum increases. As we mentioned in the Section 2.2, the area under the DPV peak corresponds to the amount of the charge transferred from electrode to MB. The plot of relative changes of charge consumption as a function of thrombin, IgG or HSA concentrations is presented in Figure 5. We can see that with increasing thrombin concentration the charge transfer increased significantly (curve 1). Lower changes of charge

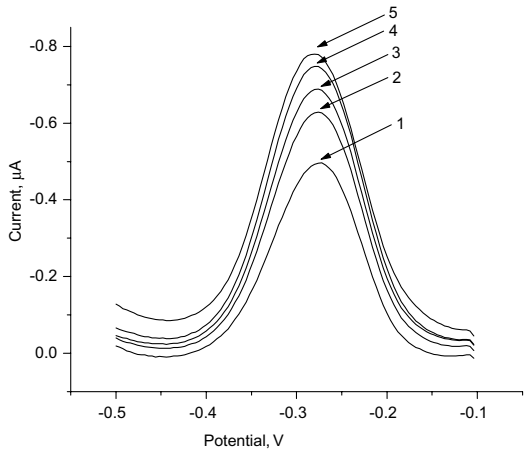

Figure 4. Differential pulse voltammogram for 1—aptamer without thrombin and for aptamer at different thrombin concentrations: 2—28.5; 3—57; 4—114; 5—684 nM.

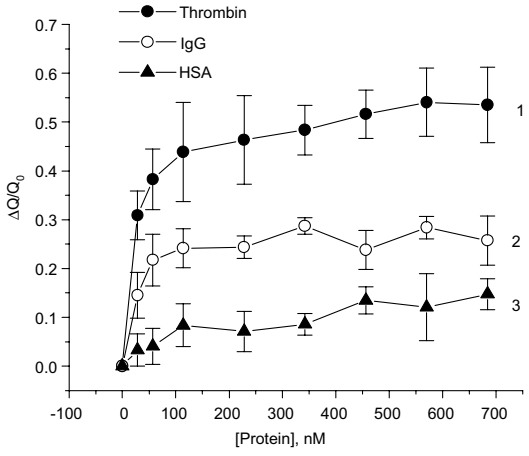

Figure 5. The plot of the relative changes of charge consumption during reduction of MB as a function of the concentration of the 1-thrombin, 2-IgG or 3-HSA ($\Delta Q/Q_0 = (Q - Q_0)/Q_0$, where Q_0 is charge consumption without analyte and Q that at certain thrombin, IgG or HSA concentration). Results represent mean (±SD) obtained for three independent experiments.

consumption have been observed in the case of nonspecific interactions with IgG (curve 2) and HSA (curve 3). Significant changes of charge transfer have been obtained already at approx. 10 nM of thrombin, that can be considered as a detection limit of this method. Similar results have been obtained in a three independent experiments. The values of charge transfer for thrombin differ significantly with that for IgG and HSA according to Student's *t*-test at least with $p < 0.1$.

Using the Scatchard plot we determined the binding constant for this system as well as for the method based on aptamer beacons utilizing the fluorescence measurements.[7] The values of binding constants are showed in Table 1. We can see that while for QCM and fluorescence method the binding constants do not differ significantly, approx. four times lower binding constant has been obtained in the case of method based on MB in comparison with QCM. One of the possible reason of this effect could be connected with changes of the charge of the thrombin molecules at the presence of MB, which adsorb to the thrombin. This may result in changes of binding affinity of thrombin to DNA aptamer. This effect, however requires further analysis.

Table 1. Binding constant, K_b, for thrombin obtained by various methods

Method	K_b (nM^{-1})
QCM	0.17 (±0.06)
Electrochemical indicators	0.04 (±0.01)
Fluorescence	0.09 (±0.01)[7]

The results represent mean (±SD) obtained by linear regression method using Origin, version 5.0.

Thus, the electrochemical indicator method allowing us to determine the protein concentration with relatively high sensitivity (detection limit 10 nM). The detection limit of QCM method was lower (1 nM), but the detection limit of fluorescence method was comparable with the method utilizing electrochemical indicator (10 nM). However considering easy to use protocol of the determination of the thrombin concentration by means of MB, it seems that the method of electrochemical indicators is rather perspective for practical applications. This method does not require additional modification of the aptamers by quencher and fluorescence probes as it is necessary for fluorescence detection method. However, further effort is necessary for improvement the selectivity of detection, for example, by the application of other electrochemical indicators. These works are in progress.

Acknowledgements

This work was supported by European Commission 6 FP program NoE NeuroPrion (Contract No. FOOD-CT-2004-506579) and by the Slovak Grant Agency (Project No. 1/1015/04). We thank to V. A. Spiridonova, A. M. Kopylov and T. S. Oretskaya for stimulating discussions and to M. Sonlajtnerova for technical assistance.

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