Available online at www.sciencedirect.com
ScienceDirect
Bioelectrochemistry 70 (2007) 127–133
Bioelectrochemistry
www.elsevier.com/locate/bioelechem

Influence of ionic strength, pH and aptamer configuration for binding affinity to thrombin

Tibor Hianik *, Veronika Ostatná, Michaela Sonlajtnerova, Igor Grman

Laboratory of Biophysics, Faculty of Mathematics, Physics and Computer Science, Comenius University, Mlynská dolina F1, 842 48 Bratislava, Slovakia

Received 12 July 2005
Available online 7 April 2006

Abstract

We used the methods of electrochemical indicators and the quartz crystal microbalance (QCM) for detection of thrombin–aptamer interactions. We analyzed how the method of immobilization of aptamer to a solid support, the aptamer configuration as well as variation in ionic strength and pH will affect the binding of thrombin to the aptamer. The immobilization of aptamer by means of avidin–biotin technology revealed best results in sensitivity in comparison with immobilization utilizing dendrimers of first generation and in comparison with chemisorption of aptamer to a gold surface. Linear and molecular beacon aptamers of similar structure of binding site revealed similar binding properties to thrombin. Increased concentration of NaCl resulted in weakening of the binding of thrombin to the aptamers, probably due to shielding effect of Na^+ ions. The binding of the thrombin to the aptamer depends on electrolyte pH, which is presumably connected with maintaining the three dimensional aptamer configuration, optimal for binding the protein.

Keywords: DNA aptamers; Thrombin; Electrochemical indicators; Methylene blue; QCM; Dendrimers

1. Introduction

There exists a growing interest to the applications of nucleic acids as recognition elements [1–3]. The implementation of nucleic acids (DNA and RNA) in the biosensor assembly makes it possible to create affinity biosensors with controlled selectivity and sensitivity toward various chemical and biological compounds, especially proteins. These DNA sensors can be employed for testing structural changes of proteins as well as for diagnosis of human diseases related to gene mutations, autoimmune syndrome and cancer.

The main feature of these DNA sensors is in fact that the oligonucleotides are directly enriched with the binding sites adapted to a specific analyte. One of the novel technology providing this in vitro selection is the SELEX (Systematic Evolution of Ligands by Exponential enrichment) [4]. The stability of the complexes is characterized by the apparent dissociation constants which are typically in 1–100 nM range for aptamer–protein complexes. This is near the affinity range of antibody–antigen complexes. Unbound DNA/RNA molecules are eluted from the column while the bound aptamers are isolated from the complex and then amplified by PCR.

The aptamer based approach makes it possible to create a wide variety of high affinity artificial receptors against proteins or small molecules and is considered as a real alternative to the conventional immunoassay techniques in the nearest future due to higher stability of aptamers in comparison with natural antibodies and due to their wider variety and specificity toward target molecules [3,5–8].

Although the first SELEX-related patent was filed in 1989 [9], the potentialities of the aptamer based biosensors have not been realized in a full scale due to the problems with aptamer stability during immobilization and signal registration. Several problems related to the practical application of aptamer based recognition are still under solutions, for example how immobilization of aptamers to the supported films and the their microenvironment will affect the aptamer structure and aptamer–ligand interactions. Problems are connected with application of aptamers in a complex biological systems, where interferences with other molecules could take place. Currently, radio labeled aptamers are used for quantification of protein kinase [10] and in vivo detection of

* Corresponding author. Tel.: +421 2 60295683; fax: +421 2 65426774.
E-mail address: hianik@fmph.uniba.sk (T. Hianik).

1567-5394/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.bioelechem.2006.03.012

clots [11]. However, to be widely employed in clinical practice, aptamers must be detected via a nonradioisotope method with a comparable sensitivity, e.g. aptamers can be covalently linked to an enzyme [12], or fluorescently labeled aptamers can be exploited [13]. However most reliable and cost effective would be the exploitation of the direct physical methods that do not require labeling of aptamers by additional chemical ligands. This highly promising direction route has not been exploited so far in sufficient details. It is highly advantageous to explore the possibility of immobilization of aptamers onto novel materials for biosensing, e.g. dendrimers. Among various aptamers used so far in laboratory experiments for thrombin are most explored [13,14]. Two types of aptamers were used — "linear" aptamer and aptamer beacon. Linear aptamer maintains in certain physico-chemical conditions (pH, ionic strength) typical three dimensional conformation with specific binding site for thrombin. Molecular beacon initially forms a loop that changes conformation following binding of the protein [13]. It is, however, not known whether these different forms of aptamers have similar or different binding properties. Additional problem consists in maintaining highest binding affinity of the aptamer. This affinity should depend on ionic strength and electrolyte pH.

In this work we applied the method of electrochemical indicators to elucidate the effect of the method of aptamer immobilization, ionic strength and pH on the binding affinity as well as the mass detection method based on quartz crystal microbalance (QCM) to study the interaction of thrombin with DNA aptamer of two different configurations "linear" (APTA) and molecular beacon (LOOP).

2. Experimental

2.1. Materials and immobilization of aptamers

We used 32-mer DNA aptamer (APTA) modified by either thiol group or by biotin at 3′ end as well as molecular beacon aptamer (LOOP) modified at 3′ end by biotin. The sequence of APTA and the LOOP was as follows: 3′-GGG TTT TCA CTT TTG TGG GTT GGA CGG GAT GG-5′ (APTA) and 5′-GGT TGG TGT GGT TGG CAA CC-3′ (LOOP). Both aptamers have at its 5′ end typical motif with high affinity to the heparin (APTA) or fibrinogen (LOOP) binding sites of thrombin [13]. The aptamers were synthesized by Thermo Electron GmbH (Germany) and used as obtained.

For preparation of aptamer biosensor we used either gold electrode of 2 mm diameter 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 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) for 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 remained unchanged.

The DNA aptamer was immobilized to an electrode surface by avidin–biotin technology as described elsewhere [5]. For this purpose the gold surface was first modified by 3,3′-dithiopropionic acid-di(*N*-succinimidylester) (DSP) (Fluka) and incubated at room temperature for 15 min. The electrode was then washed three times with buffer (140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM TRIS, pH 7.4) and then immersed in buffer containing 2 mg/mL avidin (Molecular Probes Inc.) and incubated at 4 °C overnight. After protein incubation the electrode was washed 2 times with buffer and incubated for 1 h in buffer solution of 0.025% bovine serum albumin (BSA). This approach was used to block free binding site on the activated surface. The electrode was then immersed in 2 μM aptamer solution in buffer for 1 h at 20 °C. After this the electrode was used immediately or kept at 4 °C for less than 1 week.

Aptamers were immobilized also to a dendrimerized gold surface as follows. After cleaning the gold electrode was rinsed by deionised water, then by absolute ethanol and after this procedure it was immediately immersed into a mixture of hexadecanethiol (HDT) (Fluka) and G1 PAMAM dendrimer (G1) (Aldrich) dissolved in ethanol in the molar ratio 1:1.5 for 22 h. The concentration of HDT was 1 mM and that of G1 1.5 mM. Then the layer was rinsed by absolute ethanol, then by millipore water and finally by absolute ethanol and then dried under air at room temperature (approximately 20 °C). After drying the layers were stored in small containers in a refrigerator at 4 °C. G1 dendrimer served as a source of the amine-terminated groups for immobilization of avidin. This immobilization was performed as follows. 15 μL drop of avidin dissolved in a phosphate buffer (concentration 1 mg/mL) was added to an electrode surface covered by HDT-G1 layer. After the water was evaporated, the avidin molecules were crosslinked with glutaraldehyde. For this purpose the electrode was placed in a vacuum compartment (volume 10 mL) for 30 min (the pressure in a compartment corresponded to 30 mm of Hg). The compartment contained small amount (1 mL) of 5% glutaraldehyde in water. Electrode was then rinsed with deionised water and immersed into the buffer containing the 2 μM aptamer for 1 h at 20 °C.

Direct chemisorption of aptamer to a gold surface was performed as follows. After careful cleaning (see above) the gold electrode was immersed into the water solution of 5 μM aptamer for 30 min and then rinsed with deionised water and immersed in 1 mM ethanol solution of 6-merkapto-1-hexanol (MCH) (Fluka) for 30 min. MCH was used in order to remove aptamer molecules that were only physically adsorbed to a gold support.

Proper folding of aptamer in each case has been provided by heating the buffer containing aptamer to 95 °C for 3 min and then cooling by immersion into the ice bath [5]. The above mentioned immobilization procedures were used in experiments utilizing electrochemical indicator methylene blue (MB) (see below). This indicator was added into the buffer in which experiments were performed in a concentration 2 μM.

In the case of QCM experiments we used both type of aptamers (APTA and LOOP) modified by biotin. These aptamers were immobilized to a gold surface by means of neutravidin (Molecular

Probes Inc). Neutravidin was dissolved in deionised water (concentration 0.2 mg/mL). In contrast with avidin, neutravidin does not contain polysaccharides, therefore thiol groups at certain amino acids have better access to the gold. Thus, the neutravidin can be chemisorbed to the surface directly without additional chemical step. The solution of neutravidin was allowed to flow in a flow cell to one side of the crystal with a flow rate 35 μL/min for 15–25 min. This time was sufficient to reach saturation in coverage of the crystal surface. Then the deionised water was allowed to flow in order to remove physically adsorbed neutravidin. Finally 1 μM aptamer in buffer (140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM TRIS, pH 7.4) was allowed to flow in a flow cell. In experiments we used human thrombin (Fluka). The concentration of thrombin was determined spectrophotometrically using UV 1700 (Shimadzu, Japan).

2.2. Experimental methods

2.2.1. Electrochemical indicator

The method of detection of thrombin–aptamer interactions using methylene blue (MB) was described in our recent paper [15]. Briefly, MB can be reduced at the electrode surface by two electrons to a leucomethylene blue (LB). This reduction results in charge consumption, that can be determined, e.g. by cyclic voltametry (CV) or by differential pulse voltammetry (DPV). MB is positively charged, it will therefore bind both to proteins and to DNA. Higher concentration of MB at surface resulted in higher current between working and reference electrode. Because the electrode with aptamer was immersed in a buffer containing MB, the aptamer was saturated by this indicator. Addition of thrombin into the buffer resulted in a binding of MB to a protein surface as well as binding of protein to the aptamers. Increased concentration of thrombin at the electrode surface resulted in an increase of the current. The reduction of MB was observed at −0.28 V vs. Ag/AgCl electrode. The DPV was measured using potentiostat CHI 410 (CH Instruments, USA). A three-electrode configuration was used. Gold electrode with immobilized aptamers served as working electrode, while Ag/AgCl and Pt electrodes (CH Instruments, USA) were used as reference and counter electrodes, respectively. The DPV was measured in the following conditions: voltage range −0.5 to −0.1 V, pulse amplitude 50 mV; step potential 5 mV.

2.2.2. QCM

In piezoelectric microgravimetry, the changes of the resonant frequency of the quartz crystal 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 [16], the change of resonant frequency of the crystal, Δf, is related to its mass change, Δm,

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

where A is the surface area of the working electrode, f_0 being fundamental frequency of the crystal (in our case, $A = 0.28$ 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, e.g., 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 cell was constructed in University of Toronto, Canada and was generously gifted by Prof. M. Thompson. The analyte solution stream was introduced into the latter by means of Genie Programmable Syringe Pump (Kent Scientific, USA) with a flow rate 35 μL/min.

3. Results and discussion

3.1. Influence of the method of immobilization of the APTA for sensor sensitivity

In the first series of experiments we studied how the method of immobilization of the APTA will influence the sensor sensitivity. The experiments were performed by means of DPV using MB. We determined first the DPV for various concentrations of thrombin (see Ref. [15]) and then using the DPV records calculated the amount of charge transferred from the electrode surface to MB. This amount is proportional to the area under the DPV peak [17]. The plot of relative changes of charge transfer as a function of the thrombin concentration and for different methods of immobilization of aptamer is shown in Fig. 1. We can see that in all cases the charge transfer increases with the increasing concentration of thrombin. The sensitivity of the sensor is highest for the method of immobilization of aptamer to a gold surface covered by avidin, medium for that using dendrimer layers and minimal for aptamer chemisorbed directly to a gold. The minimal sensitivity in the later case can be probably due to higher density of aptamers on a surface that restrict the maintaining optimal configuration of aptamer binding site to the thrombin. The differences between immobilization of APTA to

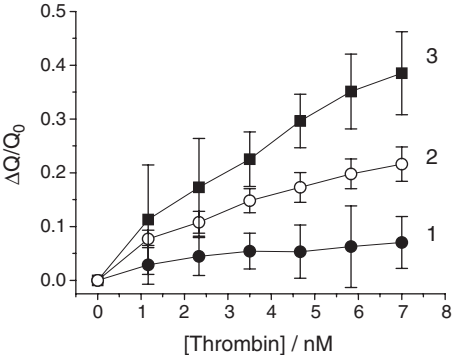

Fig. 1. The plot of the relative changes of charge transfer $\Delta Q/Q_0$ ($\Delta Q = Q - Q_0$, where Q_0 is the charge transfer without thrombin and Q at certain thrombin concentration) as a function of thrombin concentration and for different method of aptamer (APTA) immobilization: 1 — chemisorption of thiolated aptamer; 2 — dendrimer surface covered by avidin; 3 — surface covered by avidin. Results represent mean ± SD obtained in three experiments for each system.

G1 surface and to avidin surface could be connected with higher flexibility of aptamers immobilized to an avidin surface, that is in addition linked to the gold support through a flexible linker. In contrast, when G1 is used as a support, avidin is relatively rigidly attached to this surface by glutaraldehyde. Similar results were recently obtained by SPR method [18].

3.2. Influence of ionic strength and pH for sensor sensitivity

In order to check whether ionic strength and electrolyte pH affect the sensitivity of binding thrombin to the aptamer we used the method of electrochemical indicator to detect the binding of thrombin to aptamer in the electrolyte of different concentration of NaCl as well as for three different pH. Fig. 2 shows the plot of relative changes of charge transfer as a function of thrombin concentration for various concentrations of NaCl (X=0–500 mM) in a buffer. Aptamer was immobilized by avidin–biotin technology, that provided best sensitivity of sensor response (see Fig. 1, curve 3). In order of comparison, we included curve 3 from Fig. 1 corresponding to 140 mM NaCl also to Fig. 2. We can see that with increasing the concentration of NaCl the sensitivity of the sensor decreases. This effect could be connected with either the shielding of the negative charges at DNA aptamer as well as at protein surface or with changes of the conformation of the binding site of the aptamer. For more understanding of the mechanisms of the influence of Na^+ ions on the binding properties of the thrombin to the aptamer, we compared the effect of the ionic strength on charge transport between electrode with immobilized aptamer and MB without and with thrombin. Fig. 3 shows the dependence of the relative changes of charge transport as a function of the concentration of NaCl at presence of 2 μM MB without thrombin (curve 1) and at presence of 10 nM thrombin (curve 2). We can see that in both cases the charge transport decreases with increasing the ionic strength. It is likely, that the shielding effect is a dominant process that affect the charge transport. The shielding of negative charges of DNA aptamer and protein could result in a decrease of binding of positively charged MB to DNA or to protein molecules and consequently the charge transport should decrease. It has been shown, that cations Li^+, Na^+ and Cs^+ form weak complexes with antithrombin aptamer [19], and thus probably weakly influence the aptamer conformation. On the other hand it cannot be excluded that at higher concentration of Na^+ thrombin could aggregate, which may cause its lower affinity to the aptamer.

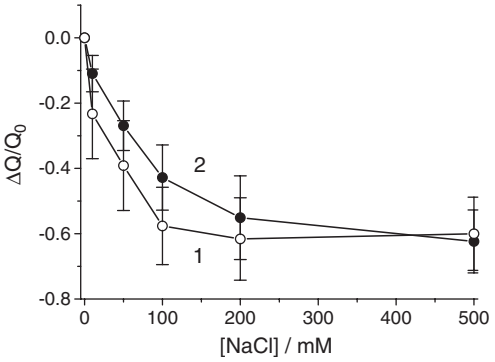

Fig. 3. The plot of the relative changes of charge transfer $\Delta Q/Q_0$ ($\Delta Q = Q - Q_0$, where Q_0 is the charge transfer at [NaCl]=0 and Q at certain NaCl concentration) as a function of NaCl concentration for electrode covered by aptamer and at presence of 2 μM MB: 1 — without; 2 — at presence of 10 nM thrombin. Aptamer (APTA) was immobilized to the gold surface covered by avidin.

The electrolyte pH influences substantially the sensor sensitivity. It is seen on Fig. 4 when the plot of relative changes of the charge transfer as a function of thrombin concentration is showed for three different electrolyte pH. The pH was adjusted by addition of small amount of 0.1 M NaOH or 0.1 M HCl to the electrolyte (140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$). We can see that maximal response of the sensor took place for pH 7.5, while for lower and higher pH the sensitivity was lower. It is likely, that pH affects the structure of aptamer binding site and is not connected with protonation or deprotonation of the protein. The isoelectric point of human thrombin is 7.0–7.6 [20]. Therefore, if protonation or deprotonation would be crucial for binding of the thrombin to the aptamer,

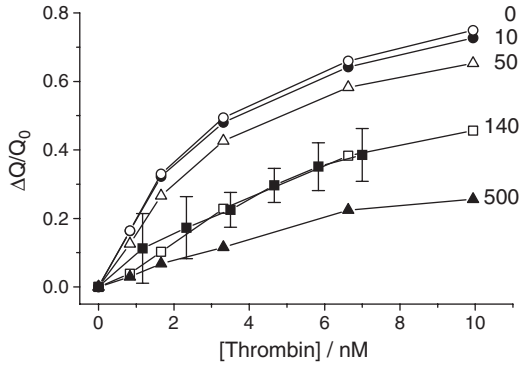

Fig. 2. The plot of the relative changes of charge transfer $\Delta Q/Q_0$ as a function of thrombin concentration and for various concentration of NaCl (X=0–500 mM) in a buffer (X NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM TRIS, pH 7.4). The concentration of NaCl in mM is showed at the curves. Curve with symbols ■ is curve 3 from Fig. 1 for 140 mM NaCl (for immobilization of aptamer by avidin–biotin method). Aptamer (APTA) was immobilized to the god surface covered by avidin.

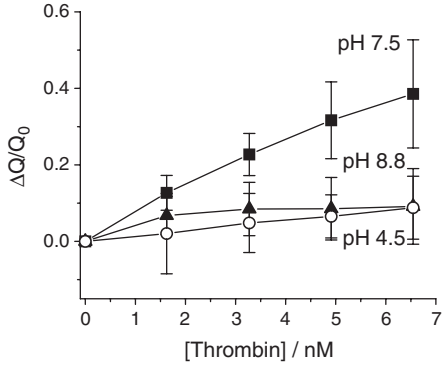

Fig. 4. The plot of the relative changes of charge transfer $\Delta Q/Q_0$ as a function of thrombin concentration and for various electrolyte pH. The pH was adjusted by addition of small amount of 0.1 M NaOH or 0.1 M HCl into the electrolyte: 140 mM NaCl+5 mM KCl+1 mM $CaCl_2$+1 mM $MgCl_2$. The pH values are showed at the curves. Aptamer (APTA) was immobilized to the gold surface covered by avidin. Results represent mean±SD obtained in three experiments for each pH.

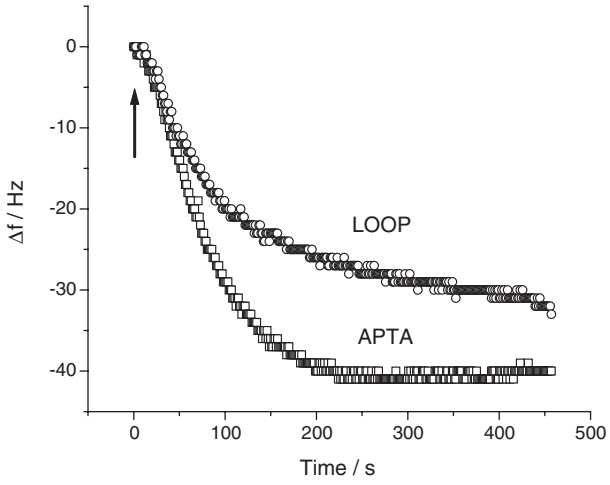

Fig. 5. The kinetics of changes of the frequency following addition of the APTA or LOOP aptamers to a measuring cell covered by neutravidin. Arrow indicates starting of the flow of the buffer with aptamer.

then the sensitivity of the sensor should be different for relatively low and high pH, which was not the case.

Thus, ionic strength and pH have substantial influence on the binding effectivity of the thrombin to the aptamer binding site and should be taken into account in practical application of the sensor.

3.3. QCM study of the binding the thrombin to aptamers of different configurations

In order to check how important is the configuration of aptamer for binding the thrombin, we used the QCM method and analyzed the binding of the thrombin to the linear aptamer (APTA) and to the molecular beacon aptamer (LOOP). Both aptamers have been immobilized to a surface of AT-cut crystal covered by neutravidin. Flow of the neutravidin through the cell in a concentration of 0.2 mg/mL resulted in substantial decrease of the frequency of the crystal oscillations. The changes of frequency at saturation (i.e. after approximately 25 min from starting the flow) were 150±19 Hz (13 independent experiments). The decrease of resonance frequency following addition of neu-

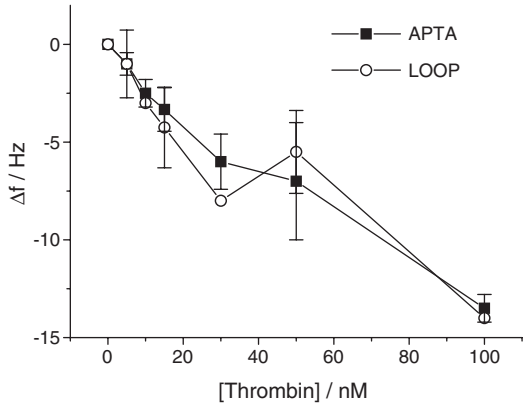

Fig. 6. The plot of the changes of the frequency as a function of thrombin concentration for aptamer sensors based on APTA and LOOP, respectively (see legend). Results represent mean±SD obtained in three experiments.

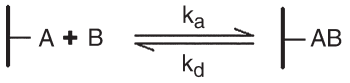

Fig. 7. The scheme of the binding of ligand B (thrombin) to its receptor A (aptamer), that is immobilized at the solid support. k_a and k_d are association and dissociation constants, respectively.

travidin is a known phenomena [1] and is connected with increasing of the mass of the crystal due to chemisorption of neutravidin. According to Sauerbrey [16] (see Eq. (1)) from changes of Δf it is possible to calculate the changes of mass, Δm, and knowing the molecular weight of neutravidin (60 kD) it is possible to estimate the number of neutravidin molecules chemisorbed at a gold surface of the crystal. Thus, according to Eq (1) the changes of Δf=150 Hz correspond to the changes of mass Δm=229 ng. This means that number of neutravidin molecules at the surface is 2.3×10^{12}. We should, however, note that changes of the frequency could not be directly transformed into the mass changes. This is connected with the peculiarity of Sauerbrey equation, which is valid for dry crystal. In the solution, a contribution of viscoelasticity between the layer adsorbed at the crystal and surrounding electrolyte should be taken into account. The effect of viscoelasticity certainly exists as it follows from complex changes of motional resistance R_m (see Ref. [1]). In addition, water molecules that entrap into the layer could also contribute to the increase of the mass. One simple solution would be the detection of oscillation frequency of dry crystal. However, drying of the crystal with neutravidin layer resulted its denaturation, which is not desirable. It has been however established that correction factor approximately 2 could be applied to correct changes of frequency connected with viscoelasticity [21]. Thus, considering this correction, the number of neutravidin molecules at the surface will be approximately 1.15×10^{12}. After the frequency was stabilized, the biotinylated APTA or LOOP aptamers dissolved in deionised water were allowed to flow through the cell. Due to strong affinity of biotin to the neutravidin the frequency of the crystal decreased. The result of this experiment is shown in Fig. 5. We can see that kinetic changes of the frequency for APTA and LOOP are different. The changes of the frequency for APTA are faster than that for LOOP. This may be connected with more bulky structure of the LOOP. Also the steady state value of frequency changes for APTA are higher than for LOOP. This is however connected with higher molecular weight of APTA (10576 D) in comparison with LOOP (6791 D). Using analogical approach like above, including correction factor from the obtained frequency changes for APTA and LOOP (40±8.6 and 30±5.6 Hz, respectively), we obtained that approximately 1.74×10^{12} molecules of APTA and

Table 1
The kinetics constants of association, k_a, and dissociation, k_d, as well as equilibrium constants of association, K_A, and dissociation, K_D, for the interaction of the thrombin with the aptamers of different configuration: APTA and LOOP

Aptamer	k_a, $nM^{-1}\ s^{-1}$	k_d, s^{-1}	K_A, nM^{-1}	K_D, nM
APTA	0.97±0.45	86±73	0.011±0.006	88±52
LOOP	3.27±1.22	127±100	0.026±0.018	39±27

Results obtained from three independent experiments.

2.03×10^{12} molecules of LOOP were adsorbed to a neutravidin surface. Both values are higher than the number of neutravidin molecules at the surface. The neutravidin, like avidin, is a tetramer. Each monomer contains one binding site for biotin [22]. Because the neutravidin is a symmetric molecule we can assume that due to its immobilization to a solid support at least 2 biotin binding sites should be exposed to the solution. Thus, the above obtained number of aptamer molecules is evident that the surface of the crystal is covered by APTA and LOOP on approximately 76% and 88%, respectively.

Addition of thrombin to a sensor surface resulted in decrease of the resonance frequency for both APTA and LOOP based sensors. The plot of the dependence of the changes of the frequency as a function of thrombin concentration is shown in Fig. 6. We can see that this plot is similar for both APTA and LOOP. In the case of SPR method, however, the sensor based on APTA revealed approximately 1.5 times higher sensitivity in comparison with LOOP [18].

In order to analyze quantitatively the kinetics of the binding of thrombin to the aptamers of different configuration (APTA and LOOP) we determined the kinetic constants of the binding reaction. The analysis was based on known peculiarities of binding of the ligand to the receptor [23]. The binding of the ligand (thrombin) to the receptor (aptamer) immobilized at the surface is schematically shown in Fig. 7 and is characterized by association k_a and dissociation k_d constants, respectively. Having these constants, it is possible to determine the equilibrium constants of association and dissociation, respectively: $K_A = k_a / k_d$, $K_D = 1 / K_A$. For determination of the kinetic constants we used an approach already published elsewhere [23]. Briefly, the rate of the formation of the complex AB can be expressed by the equation:

$$\mathrm{d}\frac{[AB]}{\mathrm{d}t} = k_a[A][B] - k_d[AB] \tag{2}$$

The formation of the complexes is accompanied by changes of the resonance frequency $f(t)$. The binding capacity of the surface is proportional to the changes of the signal corresponding to the occupation of all binding sites. During binding the concentration of free binding sites will be proportional to the difference $(f_{max} - f)$, where f_{max} is the binding capacity of the crystal. The initial signal f_0, (prior binding) is usually taking into as 0. The Eq. (2) is then:

$$\frac{\mathrm{d}f}{\mathrm{d}t} = k_a c(f_{max} - f) - k_d f \tag{3}$$

and after transformation:

$$\frac{\mathrm{d}f}{\mathrm{d}t} = k_a c f_{max} - f(k_a c + k_d) \tag{4}$$

where c is the concentration of free ligand in solution. By integration of the Eq. (4) we obtain:

$$f = \frac{k_a c f_{max}}{k_a c + k_d}\left(1 - e^{-(k_a c + k_d)t}\right) = f_{eq}\left(1 - e^{-k_{obs}t}\right) \tag{5}$$

where $k_{obs} = k_a c + k_d$. The binding curve can be fitted by Eq. (5), so the parameters f_{eq} and k_{obs} can be obtained. By measuring the binding curves for different concentrations of ligand it is possible to construct plot of k_{obs} as a function of concentration. This is the straight line, from which the kinetic constants k_a and k_d can be determined. By removing ligand B from the solution (e.g. by flowing the buffer through the cell), it is possible to observe dissociation of the complexes:

$$\frac{\mathrm{d}f}{\mathrm{d}t} = -k_d f \tag{6}$$

This approach allows to determine independently the dissociation constant k_d. If the initial number of complexes at the sensor surface is characterized by signal f_0, then by integration of the Eq. (6) we obtain:

$$f = f_0 e^{-k_d t} \tag{7}$$

The kinetics constants k_a and k_d can be obtained from association reaction, however for $k_d < 10^{-4}\ s^{-1}$ this approach resulted to an inaccuracy. Therefore it is more correct to determine k_d from dissociation curve. Kinetics constants as well as equilibrium constants determined for APTA and LOOP using the procedure described above for 5 different concentrations of thrombin are presented in Table 1. The kinetics and equilibrium constants in average differ for APTA and LOOP, however these differences are statistically not significant. The obtained results are in good agreement with that obtained by SPR method [18]. Thus, the kinetics of the binding of the thrombin to the aptamers of different configuration is similar.

4. Conclusion

The method of the immobilization of aptamer to a solid support affects the sensitivity of the aptamer for the proteins. Best results were obtained by immobilization of aptamer to a gold support by means of avidin–biotin technology. The configuration of the aptamer-linear or molecular beacon revealed similar properties in respect to sensitivity and kinetics of binding of the thrombin. Increased ionic strength resulted in a decrease of the sensitivity of the aptamer to the thrombin. This is probably connected with shielding effect of Na^+ ions on the aptamer binding sites. Electrolyte pH has also significant effect on the aptamer sensitivity. Best sensitivity was obtained for pH 7.4–7.5, while increase or decrease of pH resulted in decrease of sensitivity of the aptamer sensor. This effect can be connected with maintaining optimal structure of binding site of the aptamer.

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).

References

[1] N. Tassew, M. Thompson, Kinetic characterization of TAT RNA-Tat peptide and neomycin interactions by acoustic wave biosensor, Biophys. Chem. 106 (2003) 241–252.

[2] M.I. Pividori, A. Merkoci, S. Alegret, Electrochemical genosensor design: immobilization of oligonucleotides onto transducer surfaces and detection methods, Biosens. Bioelectron. 15 (2000) 291–303.
[3] S. Tombelli, M. Minunni, E. Luzi, M. Mascini, Aptamer based biosensors for detection of HIV-1 TAT protein, Bioelectrochemistry 67 (2005) 135–141.
[4] A.D. Elington, J.W. Szostak, In vitro selection of RNA molecules that bind specific ligands, Nature 346 (1990) 818–822.
[5] M. Liss, B. Petersen, H. Wolf, E. Prohaska, An aptamer-based quartz crystal protein biosensor, Anal. Chem. 74 (2002) 4488–4495.
[6] A. Spiridonova, T. Rassokhin, A. Golovin, E. Petrova, T. Rozhdestvensky, Y. Pakhomova, A. Kopylov, A comparative thermodynamic study for both natural and artificial RNA/DNA-protein binary complexes, Bioelectrochemistry 56 (2002) 95–97.
[7] C.K. O'Sullivan, Aptasensors — The future of biosensing? Anal. Bioanal. Chem. 372 (2002) 44–48.
[8] F.W. Scheller, U. Wollenberg, A. Warsinke, F. Lisdat, Research and development in biosensors, Curr. Opin. Biotechnol. 12 (2001) 15–40.
[9] C. Tuerk, L. Gold, Systematic evolution of ligands by exponential enrichment, Science 249 (1990) 505–510.
[10] R. Conrad, A.D. Ellington, Detecting immobilized protein kinase C isozymes with RNA aptamers, Anal. Biochem. 242 (1996) 261–265.
[11] H. Dougan, J.B. Hobbs, J.I. Weitz, D.M. Lyster, Synthesis and radioiodination of a stannyl oligodeoxyribonucleotide, Nucleic Acids Res. 25 (1997) 2897–2901.
[12] S.E. Osborne, I. Matsumura, A.D. Elington, Aptamers as therapeutic and diagnsotic reagents: problems and prospects, Curr. Opin. Chem. Biol. 1 (1997) 5–9.
[13] G.T. McCauley, N. Hamaguchi, M. Stanton, Aptamer-based biosensor arrays for detection and quantification of biological molecules, Anal. Biochem. 319 (2003) 244–250.
[14] D.M. Tasset, M.F. Kubik, W.J. Steiner, Oligonucleotide inhibitors of human thrombin that bind distinct epitopes, J. Mol. Biol. 272 (1997) 688–699.
[15] T. Hianik, V. Ostatná, Z. Zajacová, E. Stoikova, G. Evtugyn, Detection of aptamer-protein interactions using QCM and electrochemical indicator methods, Bioorg. Med. Chem. Lett. 15 (2005) 291–295.
[16] G. Sauerbrey, The use of oscillator for weighing thin layers and for microweighing, Z. Phys. 155 (1959) 206–210.
[17] C.M.A. Brett, A.M.O. Brett, Electrochemistry. Principles, Methods and Applications, Oxford University Press, Oxford, 1993.
[18] V. Ostatná, H. Vaisocherová, J. Homola, T. Hianik, Biosensor for thrombin based on DNA aptamers. A comparative analysis of the methods of aptamer immobilization (in preparation).
[19] B.I. Kankia, L.A. Marky, Folding of the thrombin aptamer into a G-quadruplex with Sr^{2+}: stability, heat, and hydratation, J. Am. Chem. Soc. 123 (2001) 10799–10804.
[20] W. Berg, B. Hillvarn, H. Arwin, M. Stenberg, I. Lundstrom, The isoelectric point of thrombin and its behaviour compared to prothrombin at some solid surfaces, Thromb. Haemost. 42 (1979) 972–982.
[21] X.C. Zhou, L.Q. Huang, S.F.Y. Li, Microgravimetric DNA sensor based on quartz crystal microbalance: comparison of oligonucelotide immobilization methods and the application in genetic diagnosis, Biosens. Bioelectron. 16 (2001) 85–95.
[22] O. Livnah, E.A. Bayer, M. Wilchek, J.L. Sussman, Three-dimensional structures of avidin and the avidin–biotin complex, Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 5076–5080.
[23] P. Skladal, Piezoelectric quartz crystal sensor applied for bioanalytical assay and characterization of affinity interactions, J. Braz. Chem. Soc. 14 (2003) 491–502.