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ORIGINAL PAPER

Effect of the immobilisation of DNA aptamers on the detection of thrombin by means of surface plasmon resonance

Veronika Ostatná · Hana Vaisocherová · Jiří Homola · Tibor Hianik

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Abstract We report a multichannel surface plasmon resonance (SPR) sensor for detection of thrombin via DNA aptamers immobilized on the SPR sensor surface. A detailed investigation of the effect of the immobilisation method on the interaction between thrombin and DNA aptamers is presented. Three basic approaches to the immobilisation of aptamers on the surface of the SPR sensor are examined: (i) immobilisation based on chemisorption of aptamers modified with SH groups, (ii) immobilisation of biotin-tagged aptamers via previously immobilized avidin, neutravidin or streptavidin molecular linkers, and (iii) immobilisation employing dendrimers as a support layer for subsequent immobilisation of aptamers. A level of nonspecific binding of thrombin to immobilized human serum albumin (HSA) for each of the immobilisation methods is determined. Immobilisation of aptamers by means of the streptavidin–biotin system yields the best results both in terms of sensor specificity and sensitivity.

Keywords DNA aptamer · Thrombin · Dendrimers · Surface plasmon resonance

V. Ostatná · T. Hianik (✉)
Department of Nuclear Physics and Biophysics,
Comenius University,
Mlynská dolina F1,
842 48 Bratislava, Slovak Republic
e-mail: tibor.hianik@fmph.uniba.sk

H. Vaisocherová · J. Homola
Institute of Photonics and Electronics,
Academy of Sciences of the Czech Republic,
Chaberská 57,
182 51 Prague, Czech Republic

Introduction

Biosensors based on DNA or RNA aptamers (aptasensors) represent an interesting alternative to antibody-based biosensors. Aptamers are linear sequences of DNA or RNA that under certain conditions (ionic strength and composition, pH and temperature of environment) adopt three-dimensional structures with binding sites specific for certain proteins or even for low molecular weight compounds [1]. Aptamers with a high specificity for a large variety of compounds can be prepared by means of chemical synthesis, which makes aptamers very attractive candidates for the development of biosensors [2–6]. A novel technology providing in vitro selection capability is referred as to systematic evolution of ligands by exponential enrichment (SELEX) [7]. The stability of the aptamer–target complex is characterized by the apparent dissociation constant that is typically in the 1–100 nM range for aptamer–protein complexes.

Although the first SELEX-related patent was filed in 1989 [7], the potential of aptamer for biosensors has not been fully harnessed yet. Several aspects related to the practical application of aptamer-based recognition are still under investigation, including the influence of immobilisation of aptamers to solid supports and their microenvironment on the aptamer structure and aptamer–ligand interactions. This is particularly important with respect to the potential application of aptamers for detection of protein analytes in complex biological liquids, where interferences with other molecules could take place. The first sensing applications of aptamers involved quantification of protein kinase [8] and in vivo detection of clots [9] via radiolabelled aptamers. Later, to avoid radioactive labelling, aptamers were covalently linked to enzymes [10] or fluorescent labels [11]. In order to develop rapid, reliable and cost-effective aptasensors, combination of aptamers with label-free sensor technologies

has been receiving growing attention. Aptamers were successfully applied in electrochemical sensors [12–16]. So et al. [17] reported direct detection of thrombin by aptamers immobilized on a single-walled carbon nanotube field effect transistor (SWNT-FET). A new electrochemical method for monitoring the aptamer–protein interactions based on quantum dot semiconductor nanocrystals was developed by Hansen et al. [18]. Label-free measurement of ligand–aptamer interactions was also demonstrated by means of impedance spectroscopy [19, 20]. Mass and thickness shear mode methods were utilized for study of the protein–nucleic acid and protein–aptamer interactions [13, 21, 22]. A comparative study employing quartz crystal microbalance (QCM) and surface plasmon resonance (SPR) methods was reported by Tombelli et al. [23]. Sensitivity improvement by using nanoparticles was also demonstrated [24]. These detection methods provide limits of detection around 1–10 nM. Fourier transform infrared attenuated total reflection (FTIR-ATR) and DNA aptamer methods were applied for detection of thrombin by Liao et al. [25]. The DNA aptamer developed by Tasset et al. [26] was immobilized covalently on Si surface using a UV irradiation method. As a quantitative measure, the areas of N–H and CH_2 bands were used. This method allowed detection of thrombin down to 10 nM. Recently substantial improvement in the detection limit for aptamer-based biosensors has been reported. The sandwich-type detection of thrombin was realized in a potentiometric aptasensor based on a Cd^{2+}-selective microelecrode [27]. The analyte was first allowed to bind to the aptamer layer at the Au electrode. Then aptamers labelled with CdS nanoparticles and selectivity for other binding sites of thrombin were added. The Cd^{2+} was released from the sensor surface by H_2O_2 treatment and the concentration of the Cd^{2+} was measured by using an ion-selective electrode. Owing to the high sensitivity of the Cd^{2+}-selective electrode, the sandwich assay allowed detection of thrombin at concentrations down to 0.14 nM. A comprehensive review of recent advances in aptamer-based biosensing can be found elsewhere [28–30].

Among various aptamers used so far in laboratory experiments, aptamers against thrombin have been most explored [11, 26], since these aptamers were among the first developed and the detection of thrombin is of substantial interest for medical diagnostics. Aptamers against fibrinogen [31] and heparin [26] binding sites of thrombin differ in their structure and affinity for thrombin. Supporting oligonucleotide parts of an aptamer can be designed to allow various aptamer configurations. Two configurations of aptamers have been used so far, the so-called linear aptamer and aptamer beacon. Under certain physicochemical conditions (pH, ionic strength) a linear aptamer maintains a typical three-dimensional structure with a specific binding site for thrombin. A molecular beacon initially forms a loop that changes the conformation in response to the protein binding [11]. Recently it has been shown that both linear and molecular beacon aptamers have similar affinities for thrombin [13]. However, changes in aptamer structure, even if they do not occur directly in the binding site, could affect its affinity for the target protein.

In our previous work [13] we applied electrochemical indicators and QCM methods to study the thrombin–aptamer interactions at various conditions and using various methods of aptamer immobilisation. This paper investigates immobilisation of aptamers against thrombin by using a recently developed multichannel optical sensor based on surface plasmon resonance (SPR). This technique measures refractive index changes induced by the immobilisation of aptamers on the sensor surface and aptamer–thrombin interaction and thus provides a view of these processes complementary to QCM. Furthermore, ability to perform multiple measurements in different channels of a single sensor chip enhances reproducibility and reliability of the measurements. In this paper immobilisation methods based on neutravidin, avidin and streptavidin layers are compared and regenerability of the sensing surface is demonstrated.

Materials and methods

Reagents

Avidin was purchased from Sigma-Aldrich (St. Louis, MO, USA), neutravidin was from Molecular Probes Inc. (Eugene, OR, USA). The C_{11}-mercapto-1-undecanol ($C_{11}OH$) and C_{16}-mercaptohexadecanoic acid ($C_{16}COOH$), 6-mercapto-1-hexanol (MCH), hexadecanethiol (HDT), *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(*N*-succinimidyl)uronium tetrafluoroborate (TSTU) and poly(amidoamine) dendrimer (PAMAM) (generation G1) were purchased from Sigma-Aldrich (St Louis, MO, USA).

The following buffers were used: 1 mM sodium acetate, pH 5 (SA) or 20 mM Tris-HCl, 1 mM $CaCl_2$, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, pH 7.4 (Tris), phosphate buffer 0.75 M NaCl, 1.4 mM KH_2PO_4, 8 mM Na_2PO_4, 3 mM KCl, pH 7.4 (PBS), 0.1 mM citrate buffer pH 4 (CB).

We studied the interaction of thrombin with DNA aptamers of the following structure:

1. A 32-mer oligodeoxynucleotide modified by biotin at the 3′ end of the following sequence: 3′-GGG TTT TCA CTT TTG TGG GTT GGT GTG GTT GG-5′ (APTA). The sequence of the tail (not underlined) is identical to those of an aptamer used in our previous study [13]. This is a random sequence that avoids self-hybridisation of DNA.
2. A 19-mer oligodeoxynucleotide, modified by an –SH group at the 3′ end. The SH group was linked to DNA

through a short hydrocarbon linker: SH–$(CH_2)_6$–GGT TGG TGT GGT TGG-5′ (APTA-SH).

3. A 20-mer oligodeoxynucleotide modified by biotin at the 3′ end of the following sequence: 3′-CCA ACG GTT GGT GTG GTT GG-5′ (LOOP).

Aptamers were purchased from Thermo Electron Corporation (Ulm, Germany). These aptamers have a typical motif composed of two G quartets with a high affinity for fibrinogen of thrombin [31]. The sequence corresponding to this motif is underlined in the aptamer structures shown above. Human serum albumin (HSA) was purchased from Sigma-Aldrich, and human α-thrombin was purchased from Fluka (Buch, Switzerland).

Experimental apparatus

We used a recently developed eight-channel surface plasmon resonance (SPR) sensor platform based on spectral modulation and wavelength division multiplexing (WDM) [32]. This sensor is based on a special multireflection element in which a collimated beam of polychromatic light is made incident on the SPR sensor surface under two slightly different angles of incidence. Upon the first incidence on the surface of the sensor (channel A), light excites a surface plasmon at the outer metal surface. The excitation of surface plasmons produces a sharp absorption dip in the spectrum of optical wave centred at the wavelength λ_A. The reflected light is redirected inside the element and made incident on the metal film in another region (channel B) under a different angle of incidence. At this angle of incidence, the incident optical wave excites a surface plasmon at a different wavelength λ_B, generating a second dip in the spectrum of the optical wave centred at wavelength λ_B. Consequently, the wavelength spectrum of the transmitted light exhibits two SPR dips corresponding to SPRs in two distinct areas of the metal film, which form two independent sensing channels. Response of each channel is encoded into a shift in the position of the respective SPR dip. The WDMSPR sensor used herein combines the wavelength division multiplexing of pairs of sensing channel with four parallel light beams to provide a total of eight sensing channels. The scheme of an eight-channel SPR sensor is presented in Fig. 1. A more detailed description of the system can be found elsewhere [32]. The WDMSPR sensor chips were prepared on a SF14 glass slide by deposition of an adhesion-promoting titanium layer (thickness less than 2 nm) and SPR-active gold layer (thickness ca. 55 nm) using the e-beam evaporation in vacuum. An eight-channel flow-cell with independent inlets and outlets was used to confine liquid samples in the eight sensing channels. The flow-cell consisted of an acrylic substrate and a gasket made of a 50-μm-thick polyurethane sheet forming eight flow-cell chambers. The volume of each flow-cell chamber was 2 μL. Input flow-cell ports were connected via tubing (Upchurch Scientific, USA) with two four-channel peristaltic pumps Reglo Digital (Ismatec, Switzerland), which controlled the flow of liquid samples through the flow-cell. This multichannel sensor enables simultaneous monitoring of the binding of thrombin to aptamers immobilized on various supports (avidin, streptavidin, neutravidin or by means of chemisorption) as well as monitoring of nonspecific interactions.

Immobilisation of aptamers

Preparation of streptavidin, avidin or neutravidin layers

In order to prepare the surface of the SPR chip for immobilisation of aptamers, the chip was soaked in piranha solution (1:3 mixture of 30% hydrogen peroxide and sulfuric

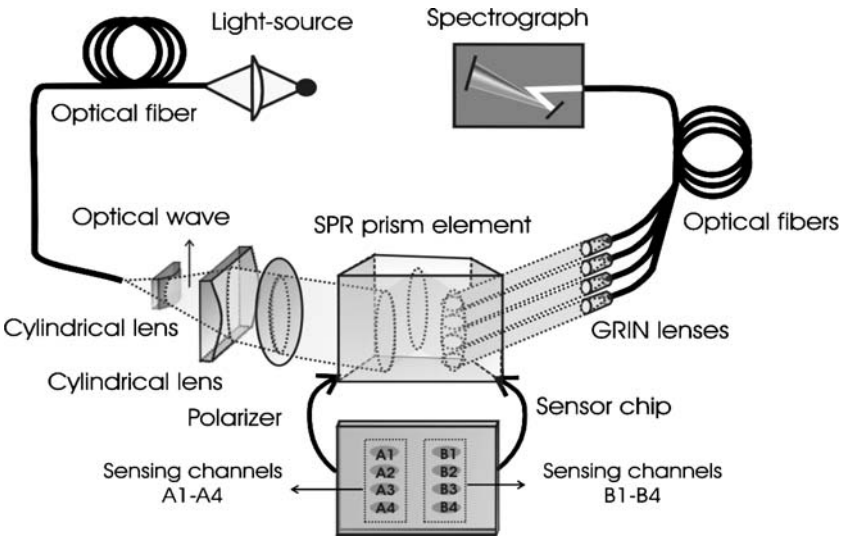

Fig. 1 An eight-channel SPR sensor combining parallel architecture with the wavelength division multiplexing of serially ordered channels

acid) for 3 min, then washed with deionised water and dried under a nitrogen stream. A 7:3 mixture of alkanethiols $C_{11}OH$ and $C_{16}COOH$ was dissolved in absolute ethanol at 1 mM ($C_{16}COOH$ was used for immobilisation of streptavidin (MW 60 kDa), avidin (MW 68 kDa) or neutravidin (MW 53 kDa), whereas $C_{11}OH$ provided a stable non-fouling background). Sensor chips were immersed in alkanethiol solution and stored in a dark place at a room temperature for 12 h. The chips were then rinsed with ethanol, dried with nitrogen, rinsed with deionised water and dried with nitrogen again. The carboxylic terminal groups on the sensor surface were activated by TSTU dissolved in 1 mg mL^{-1} dimethylformamide for 4 h [33]. After activation, the chips were rinsed with water, dried with nitrogen and mounted in the SPR instrument. Streptavidin, avidin or neutravidin in SA buffer (concentration ca. 50 μg mL^{-1}) were flowed over the preactivated surface at flow rate of 30 μL min^{-1} for 20 min. The chip was then washed with blank SA buffer and subsequently with PBS. This high ionic strength solution was used to remove nonspecifically adsorbed streptavidin, avidin or neutravidin. The sensor was again washed with SA buffer and then with Tris buffer. After the layer of streptavidin, avidin or neutravidin was formed, Tris buffer containing 10 μM biotinylated DNA aptamer was flowed through the flow-cell at a flow rate of 30 μL min^{-1} for 30 min. This step completed the formation of sensing surface.

Preparation of mixed films of thiolated aptamers

The gold surface of the chip was cleaned and rinsed thoroughly with deionised water as described above and dried with nitrogen. Self-assembled monolayers of thiolated DNA aptamers were prepared in situ by flowing Tris buffer containing 0.1 μM aptamer along cleaned gold-coated substrates for 30 min. Then, 0.1 mM mixed water/ethanol solution (1:100 v/v) of MCH was flowed along the chip surface for 15 min to remove physically adsorbed APTA-SH. MCH molecules chemically adsorb to a gold surface and remove physically adsorbed APTA-SH. This results in formation of a mixed aptamer–MCH monolayer.

Immobilisation of aptamers onto dendrimer films

A self-assembled monolayer containing G1 dendrimers was formed on the gold surface as follows: cleaned gold surface (for cleaning procedure, see Preparation of streptavidin, avidin or neutravidin layers) was immersed in the mixture of 1 mM HDT and 0.15 mM G1 in ethanol for 22 h at a room temperature. The layer was then rinsed several times with ethanol and deionised water [34] and mounted in the SPR instrument. HSA dissolved in Tris buffer (concentration ca. 50 μg mL^{-1}) was flowed through the flow-cell for 15 min followed by Tris buffer with 1 mg mL^{-1} dextran sulfate for 10 min. Finally, SA buffer containing streptavidin (concentration ca. 50 μg mL^{-1}) was flowed through the flow-cell for 20 min. Subsequently, a solution of 0.5% glutaraldehyde in CB buffer was used for immobilisation of streptavidin. The next step of the sensing layer preparation was the same as described in Preparation of streptavidin, avidin or neutravidin layers. All experiments were performed at a flow rate of 30 μL min^{-1} and a room temperature (25±1 °C).

We should note that the immobilisation procedures based on interaction of biotinylated oligonucleotides and other biopolymers with neutravidin, avidin or streptavidin as well as those based on interaction of thiolated biopolymers with gold surface are well established and the efficiency of these immobilisation methods was demonstrated by various techniques such as SPR [35], TSM [21], QCM [13] and electrochemical methods [36].

Thrombin detection

The study of the interaction of thrombin with the aptamer-coated sensor surface was performed as follows. A solution of thrombin in buffer was flowed through the sensor flow-cell for 30 min. After the sensor signal reached a steady-state level, buffer (without thrombin) was flowed through the flow-cell to remove physically adsorbed thrombin. The kinetics of the sensor response (resonant wavelength) was recorded. In all of the performed measurements, we compared the signal corresponding to the binding of analyte with the reference signal i.e. signal from the sensor surface coated with aptamers but not in contact with solution containing thrombin (the sensor comprised a total of four measuring and four reference surfaces).

Results and discussion

In the first series of experiments, we measured the sensor response to the binding of thrombin to the biotinylated aptamer (APTA). This aptamer was immobilized on the sensor surface coated with neutravidin, avidin and streptavidin, respectively. Figure 2a shows the shift of the resonant wavelength (in relative units, RU) to the binding of neutravidin or avidin or streptavidin (dissolved in SA buffer) to a surface with NHS-ester-terminated alkanethiols that were formed from carboxyls activated by carbodiimide chemistry. The resulting surface coverages were estimated to be 172 ng cm^{-2} for neutravidin, 83 ng cm^{-2} for avidin and 68 ng cm^{-2} for streptavidin. The reproducibility of the results was rather high and the surface coverage by the abovementioned proteins in separately prepared sensor surfaces did not differ by more than 10%.

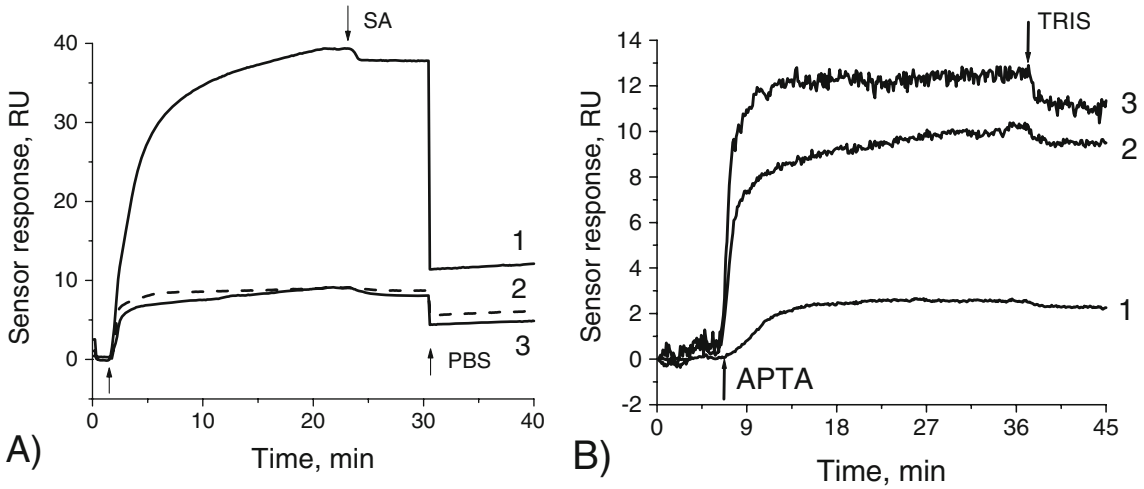

Fig. 2 **a** Sensor response (RU) following addition of *1* neutravidin, *2* avidin or *3* streptavidin to a surface covered by $C_{16}COOH$ with activated carboxylic groups and after surface washing by SA and/or PBS buffer. **b** Sensor response following addition of biotinylated aptamer (APTA) to a surface covered by *1* neutravidin, *2* avidin or *3* streptavidin and following the washing by Tris buffer. The *arrows* indicate of the injection of the SA buffer with proteins and of blank SA, PBS or Tris buffers

The highest amount of the immobilized avidin-type proteins was observed for neutravidin (curve 1). As follows from Fig. 2a, shortly after the injection of solution containing the protein, a large shift in the resonant wavelength took place; the sensor response reached equilibrium in about 30 min. After the steady-state condition has been reached, SA buffer without protein was flowed through the flow-cell. Only a minor decrease of the resonant wavelength was observed. This decrease is associated with the removal of proteins nonspecifically adsorbed to the sensor surface. It is also observed that the steady-state condition was reached rather rapidly, in 3–4 min after the injection of the SA buffer. The PBS buffer of higher ionic strength was then flowed through the flow-cell. The sensor responses in PBS buffer are about the same for avidin or streptavidin and about twice as high for neutravidin. The decrease in SPR signal following the flow of blank SA buffer is associated with removal of the weakly adsorbed proteins. The decrease of the SPR signal following the flow of PBS is probably due to further removal of physically adsorbed proteins from the surface due to substantially higher ionic strength of this buffer, which can disturb electrostatic interactions between adsorbed proteins. The results suggest that a similar degree of the coverage of the surface by neutravidin, avidin or streptavidin was achieved. Subsequent injection of the biotinylated aptamer (APTA) resulted in an increase in the sensor response (Fig. 2b). Clearly, the sensor response is significantly higher for streptavidin-coated surface compared with those for neutravidin and avidin. This effect may be due to a higher affinity of biotin for the streptavidin compared with neutravidin and avidin [30]. We also compared responses of the SPR sensor to the binding of APTA and LOOP to a streptavidin-coated sensor surface. The kinetics of the binding of LOOP and APTA were similar; the equilibrium response for LOOP was 11% higher than that for APTA. The resulting surface coverage was estimated to be 157 ng cm^{-2} for APTA and 177 ng cm^{-2} for LOOP. This corresponds to 9×10^{12} and 1.6×10^{13} molecules cm^{-2}, respectively, and correlates well with those obtained in our QCM experiments [13] and SPR experiments reported elsewhere [35] for 29-mer biotinylated aptamer immobilized on a streptavidin layer. Binding of APTA to a dendrimer-coated surface produced a sensor response about 25% higher than the binding of APTA to streptavidin. The resulting surface coverage for APTA on dendrimers was estimated to be 191 ng cm^{-2}, which corresponds to 1.1×10^{13} molecules cm^{-2}.

Figure 3 shows the sensor response to a sequence of injections of thrombin or HSA with increasing concentration. As follows from Fig. 3, the sensor response for surfaces formed by aptamer attached to the neutravidin and avidin are very similar. However, a substantially higher signal is observed when streptavidin is used as a molecular linker. This increased sensitivity is probably mainly due to the larger number of aptamers immobilized on the surface as discussed above. It can be deduced from Fig. 3 that the nonspecific interaction of HSA with the surface of the sensor is very low—practically no sensor response was observed when HSA was injected at (increasing) concentrations similar to those of thrombin. This suggests that the detection of thrombin is specific. It should be noted that physiological concentrations of HSA (ca. 32–35 mg mL^{-1}) are higher than those used in this work. However, the most recent results suggest that even at much higher HSA concentrations (3 mg mL^{-1}) and in 10 times diluted human blood serum the response of aptasensor following addition of thrombin is specific [37]. Moreover, owing to high sensitivity of recently reported aptasensors, it is necessary

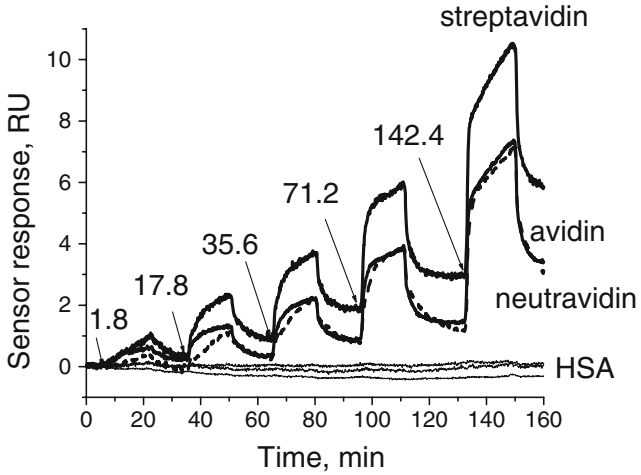

Fig. 3 Sensor response as a function of addition thrombin or HSA to a surface covered by aptamer (APTA) immobilized at neutravidin (*dashed line*), avidin or streptavidin (*solid lines*) layers with increasing concentration. We used the same concentration range for thrombin and HSA. *Arrows* indicate the injection of thrombin or HSA at respective concentrations shown at the arrows in nM

to work in substantially diluted solutions (100–1,000 times) which also eliminate the interferences with other compounds.

Figure 4 shows the sensor response as a function of the concentration of thrombin for different aptamers and immobilisation methods. A higher response was observed for the sensor composed of APTA immobilized on a streptavidin layer. When aptamer beacon (LOOP) was immobilized on the streptavidin layer, the response was significantly lower. The dependencies presented in Fig. 4 were fitted well by Langmuir isotherms, which mean that the binding process was not cooperative. The nonlinear Langmuir fit allowed determination of the binding constants, K_C, of the thrombin to the aptamer [38]. The results of determination K_C are presented in Table 1 in which it is seen that the highest binding constant was observed for the biosensor based on APTA immobilized on streptavidin layer. This is in agreement with the highest sensitivity of this sensor. The binding constants for aptamers immobilised on streptavidin or avidin layers, however, did not differ significantly, but were substantially lower for aptamers immobilized by chemisorption or on a surface of dendrimers. At the same time, the surface density of APTA was lower compared with LOOP as revealed from the estimation based on SPR sensor response for injection of the aptamers on a sensor surface presented above and also from our recent QCM experiments [13]. We assume that the observed effect on lower response of the sensor based on LOOP aptamers can be explained as follows. The interaction of thrombin should result in a change of conformation of LOOP and formation of the receptor binding site. However, this process can prevent other proteins from binding to the sensor surface. Thus, the sensor response should be lower than with linear aptamer.

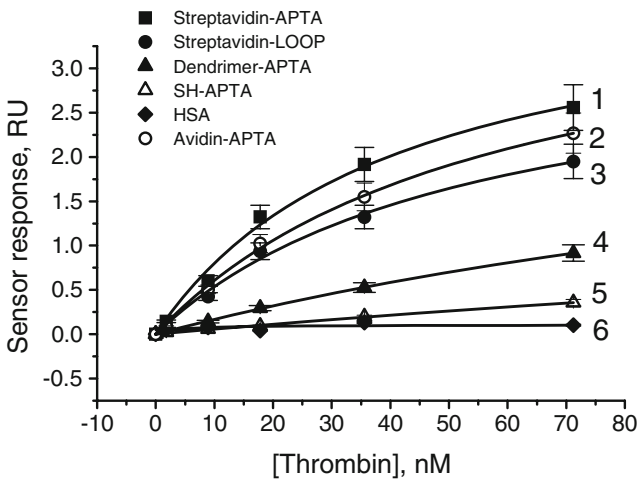

Fig. 4 Sensor response as a function of thrombin concentration for different types of aptamer immobilisation and different types of the aptamer: APTA immobilized on a *1* streptavidin layer, *2* avidin, *4* dendrimers covered by avidin; *3* LOOP immobilized on a streptavidin layer, *5* SH-APTA. For comparison, nonspecific interaction of HSA with a sensor surface created by APTA immobilized on a streptavidin (*6*). Results represent mean ± SD obtained in three independent experiments in each series. The curves were constructed by nonlinear Langmuir fit using Origin 7.5 (Microcal Software Inc)

As follows from Fig. 4, the sensor response to thrombin was highest when using biotinylated aptamers and a molecular linker, moderate for aptamers immobilized on a dendrimer layer, and lowest for chemisorbed SH-aptamers. Therefore, for detection purposes, the immobilisation of biotinylated aptamer to a molecular linker (streptavidin avidin, neutravidin) layer is clearly a better approach. A similar conclusion was made recently [39] in a study of thrombin–DNA aptamer interactions by means of the QCM method. It has also been shown that the buffer composition in which the binding of thrombin to aptamer takes place is very important [35]. The best sensitivity of SPR response was obtained in Tris buffer of composition similar to those used in this work [35]. The limit of detection for thrombin and SPR sensor functionalised with aptamer sensitive to fibrinogen binding sites of thrombin was estimated to be about 5 nM. This limit of detection is comparable or better than the limits of detection achieved using QCM [12, 13]

Table 1 Binding constant (K_C) determined from nonlinear Langmuir fit for various aptamers immobilized to different supporting layers

Type of aptamer and supporting layer	K_C (μM^{-1})
APTA–streptavidin	24.5±4.0
APTA–avidin	17.4±1.9
LOOP–streptavidin	18.7±3.4
APTA–dendrimer	5.3±0.7
APTA–SH-gold	2.6±1.3

Results represent mean ± SD

and fluorescence [40]. Substantial improvement of thrombin detection was achieved by electrochemical impedance spectroscopy. The measured signal of electronic transfer resistance was amplified by using guanidine hydrochloride to denature the captured thrombin and to increase its hydrated radius, consequently blocking the electron transfer from solution to electrode. Using this approach, thrombin was detected at subpicomolar levels [41]. The application of the chemiluminiscence approach via target protein-induced strand displacement allowed further improvement of detection limits down to 1 fM [42]. A more detailed comparison is given in Table 2 in which the binding constants, K_C, for several methods of detection of thrombin by DNA aptamers are also presented. It is seen that there are certain differences between these constants. Especially remarkable differences were found between K_C determined by QCM and SPR methods. However, the K_C value substantially depends on the experimental conditions that affect the affinity of thrombin to the aptamer. In addition, the K_C value in the case of QCM method was determined based on the changes of the frequency that are not exclusively connected only with the mass changes, but also with surface viscosity. The contribution of surface viscosity could alter kinetic response of QCM sensor yielding inaccurate parameters of the aptamer–thrombin interaction [43].

Interaction between thrombin and DNA aptamer is associated with the existence of specific binding site that is, under certain physicochemical conditions, created by an oligodeoxynucleotide (ODN). Both ODN and protein are charged molecules. While ODN is negatively charged due to the sugar–phosphate backbone, thrombin charge depends strongly on pH. The isoelectric point of human thrombin is 7.0–7.6 [46] and thrombin is dominantly negatively (positively) charged when pH is higher (lower) than 7.0–7.6. Study of interaction between polyanion and bovine serum albumin [47, 48] suggests that the nonspecific interaction of proteins with polyanions exists even when both molecules are negatively charged. This effect has been explained by inhomogeneous distribution of charge of the protein and existence of areas of positive charge at the protein surface. A similar situation can be also expected in the case of aptamer–thrombin complexes. Therefore, the interaction of thrombin with aptamer should depend on electrolyte pH. In order to investigate this effect, sensor response at different pH values was measured. First, thrombin was introduced into the flow-cell containing APTA immobilized on a streptavidin layer (concentration of 35.6 nM). The pH of the buffer was 7.5. After the saturation was reached, the aptamer layer was washed with buffer of a different pH. Temporal sensor responses determined for different pH values are shown in Fig. 5. For the quantitative analysis of the effect of pH, we fitted the kinetic data shown in Fig. 5 to determine the dissociation constant K_d, given as:

$$R = R_0 \exp(-K_d.t) \quad (1)$$

where R is the sensor response in relative units [49]. The determined K_d values were 1.47±0.02, 1.02±0.07, 0.55±0.04 s^{-1} for pH 3.4, 6.5 and 7.4, respectively. Clearly, the dissociation constant increases with decreasing pH. Although it is likely that the binding of thrombin to aptamer depends on the protein charge, it should be noted that at low pH, the aptamer is protonated, which can affect the three-dimensional structure of the aptamer. In our recent work [13], the maximum response of the aptasensor (APTA) for thrombin was observed at pH 7.5; for lower and higher pH values, the sensor response was lower.

It was demonstrated that the reported aptasensor was regenerable using 2.5 mM HCl (Fig. 6). In the regeneration experiments, thrombin (concentration of 17.8 nM) was injected into the flow-cell, followed by the injection of blank buffer, which was replaced with 2.5 mM HCl. This detection/regeneration procedure was repeated several times. As follows from Fig. 6, incubation of buffer with

Table 2 Binding constant, K_C, for thrombin to DNA aptamers sensitive to fibrinogen binding site obtained by various methods

Method	Detection limit (nM)	K_C (μM^{-1})	Reference
QCM	5	170±60	[13]
EQCM, conducting polymer support	10	–	[44]
Electrochemical indicators	10	40±10	[12]
Fluorescence	10	90±10	[40]
SPR	5	25.5±4.0	
Immunochemistry	0.5	–	[45]
Enzyme-linked electrochemical assay coupled to magnetic beads	0.45	–	[35]
Potentiometry, Cd^{2+} nanoparticles	0.14		[27]
Electrochemical impedance spectroscopy	10^{-5}		[41]
Chemiluminiscence	10^{-6}		[42]

Results represent mean ± SD

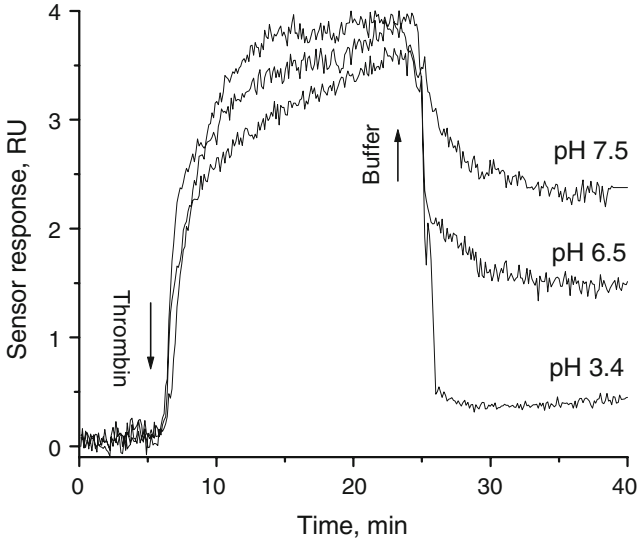

Fig. 5 Plot of the sensor response as a function of time and following addition of the thrombin at concentration 35.6 nM to a cell containing APTA immobilized on a streptavidin layer and following changes of the pH. The thrombin was added in a buffer of pH 7.5 and after the signal reached saturation the sensor surface was washed with a blank buffer of different pH. The moment of addition of the thrombin and moment of washing sensor with buffer of certain pH is indicated by *arrows*

thrombin with a regenerated sensor surface resulted in a sensor response similar to that obtained using the fresh aptamer-sensing surface. The sensor was regenerated up to five times without observing significant changes in the sensor response. Similar results were obtained using 2 M NaCl [19, 28, 39]. Application of 2 M NaCl is gentler and has a smaller effect on binding properties of the aptamer layer. The studies on regeneration suggest that the stability of the streptavidin linker layer is rather high. Moreover, it

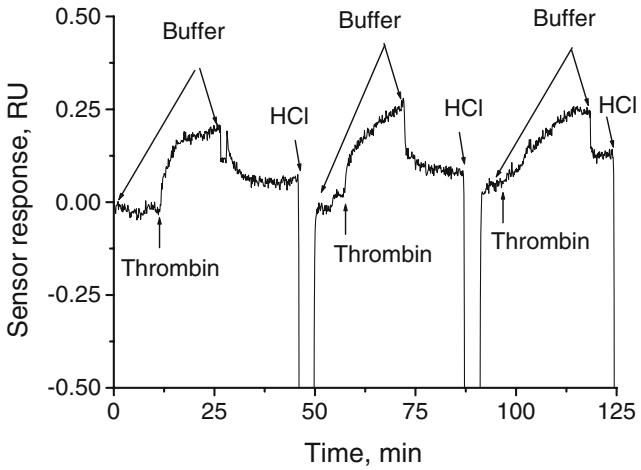

Fig. 6 Sensor response to the binding of thrombin (at a concentration of 17.8 nM) to an aptamer APTA immobilized on a layer composed of streptavidin and following washing steps when either blank buffer or 2.5 mM HCl was flowed through the cell. The injection of thrombin, buffer and HCl is indicated by *arrows*

was demonstrated that the sensor with immobilized aptamer could be stored in a refrigerator at 4 °C for at least 1 month without a significant loss of the sensor sensitivity.

While the work presented here focuses on the investigation of aptamer–thrombin interactions in a buffer, future work will advance this work towards the application of aptasensors for detection of proteins in complex biological liquids, which presents challenges in terms of nonspecific effects and sample preparation. Under normal conditions, thrombin is not present in blood, but reaches low micromolar concentrations during the coagulation process. Special care is therefore required for detection of thrombin in spiked blood plasma, which unlike serum contains all proteins involved in the coagulation process, and the addition of thrombin results in rapid sample clotting. Recently these issues were addressed in the work of Centi et al. [35], who reported detection of thrombin in spiked serum and blood plasma using an electrochemical method and enzyme-labelled aptamer. Considering the high sensitivity of several methods (with detection limits down to 1 fM), it should be possible to use highly diluted biological samples and thus reduce the effects caused by the nonspecific adsorption of other proteins.

Conclusions

We have combined DNA aptamers with a multichannel SPR sensor to detect thrombin. Three different methods for immobilisation of DNA aptamers on the SPR sensor surface were evaluated. They include immobilisation based on adsorption of thiol-terminated aptamers directly on the gold surface, immobilisation of biotinylated aptamers via neutravidin, avidin and streptavidin molecular linker layers, and immobilisation though a layer of dendrimers. It was demonstrated that the response of the aptamer biosensor to thrombin depends substantially on the method of immobilisation used. The best sensor sensitivity was obtained when the (biotinylated) aptamer was immobilized via a streptavidin layer. The analysis of interaction between thrombin and aptamer suggest that this interaction is not cooperative and that the binding constants depend on the method of immobilisation of aptamers. It was also demonstrated that electrolyte pH has substantial effect on the stability of the thrombin–aptamer complex. At pH lower than 7.5, the complex becomes unstable and therefore the sensor can be easily regenerated at low pH.

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