

Analytical Performances of Aptamer-Based Sensing for Thrombin Detection

Alessandra Bini, Maria Minunni, Sara Tombelli, Sonia Centi, and Marco Mascini*

Università degli Studi di Firenze, Via della Lastruccia 3, 50019 Sesto Fiorentino, Italy

Aptamer-based assays represent a modern and attractive approach in bioanalytical chemistry. The DNA thrombin aptamer has been extensively investigated, and the coupling of this aptamer to different transduction principles has demonstrated the wide applicability of aptamers as bioreceptors in bioanalytical assays. The goal of this work was to critically evaluate all the parameters that can influence the sensor performances by using the thrombin aptamer immobilized onto piezoelectric quartz crystals. The optimization of the immobilization and the binding protocol was of paramount importance, and improvements in analytical performances could be obtained by optimizing simple steps in immobilization and assay conditions. Moreover, the work demonstrated the possibility of using aptamer-based sensors in complex matrixes, opening the possibility of a real application to diagnostics or medical investigation.

Bioanalytical approaches based on aptamers as affinity ligands represent an interesting tool for protein detection,^{1,2} and homogeneous or heterogeneous assays have been proposed for the analysis of the relative target.^{3,4} A very interesting application is the exploitation of aptamers as biorecognition elements in biosensors,^{5–7} where most of the work refers to the thrombin-binding aptamer, with or without labels.^{8–18} However, even if aptamer-based sensing represents a modern and attractive ap-

proach, a critical evaluation of the key steps (immobilization, binding assay format, and conditions) in developing such devices is still needed.¹⁹ Moreover, most of the reported work deals with standard solutions of the analyte, but very important applications of aptasensing would interest real matrixes, which have been considered only in very few cases.^{6,20,21}

In this paper, a study conducted with the aptamer specific for thrombin coupled to label-free piezoelectric transduction is reported. We aimed at critically evaluating the importance of simple key steps in aptasensor development, which have significant implications on its analytical performances in terms of selectivity, linearity (R^2), reproducibility (coefficient of variation CV %), and stability (cycles). In particular, the influence of the aptamer immobilization procedure (chemistry, length, concentration, and pretreatment of the aptamer) as well as the binding conditions employed in the assay was studied.

The sensor is finally applied to the analysis of complex matrixes such as human serum and plasma to exploit its ability to also bind the target analyte in these complex samples.

The elucidation of critical parameters for optimal aptamer performance is of general applicability in aptasensing development, and also when other transduction principles and aptamers are employed. The final aim and the novelty of the paper is to demonstrate that simple, sometime neglected, steps are strategic for the true analytical applicability of the label-free device, having however in mind that each aptamer possess individual characteristics.

EXPERIMENTAL SECTION

Apparatus. The 9.5-MHz AT-cut quartz crystals (14 mm) with gold evaporated (42.6-mm² area) on both sides were purchased from International Crystal Manufacturing. The measurements were conducted in a methacrylate cell where only one side of the crystal was in contact with the solution. Experiments were carried out using the quartz crystal analyzer Liquid Oscillator Unit with

* To whom correspondence should be addressed. E-mail: Marco.mascini@unifi.it. Fax: +39 0554573384.

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the software QCMagic 0.3.1× by Elbitech (Marciana, Livorno, Italy). The resonance frequency was displayed in real time and recorded. All the measurements have been performed at room temperature ($T \approx 25\text{ }^{\circ}\text{C}$).

Reagents and Aptamers. The DNA thrombin-binding aptamer was purchased from MWG Biotech (Milan, Italy) with two different modifications: (a) 5'-biotin-TT TTT TTT TTT TTT TTT TTT GGT TGG TGT GGT TGG - 3'; (b) 5'-biotin-GGT TGG TGT GGT TGG - 3'; (c) 5'-SH-(CH₂)₆-GGTTGGTGTGGT TGG-3'.

The composition of the buffers used for the experiments is reported below: (a) immobilization buffer, 300 mM NaCl, 20 mM Na₂HPO₄, 0.1 mM EDTA pH 7.4; (b) binding buffer, 50 mM Tris-HCl, pH 7.4, 140 mM NaCl, 1 mM MgCl₂.

Human serum albumin (HSA) and all the reagents used for the buffer preparation were purchased from Sigma (Milan, Italy).

Modification of the Sensor Surface and Biotinylated Aptamer Immobilization. The gold surface of the quartz crystal was modified by immobilizing the thrombin-binding aptamer. The crystal was washed with a 1:1:5 solution of H₂O₂ (30%), NH₃ (30%), and MilliQ water for 10 min and then rinsed with MilliQ water. The biotinylated aptamer was immobilized via biotin-streptavidin interaction on the gold sensor surface previously modified with a layer of thiol/dextran/streptavidin as reported by Tombelli et al.²²

Thiolated Aptamer Immobilization. The thiolated aptamer is anchored to the sensor surface by chemisorption based on the formation of gold-thiol bonds. Several crystals were modified with thiolated aptamer following the procedure previously reported.²³

Binding Measurements. Once the receptor was immobilized on the gold surface, the binding step with thrombin in binding buffer was realized by adding 100 μL of the protein solution at different concentrations in the range 50–200 nM. The reaction was monitored for 20 min, the solution was then removed, and the surface was washed with the same binding buffer to eliminate the unbound protein. The analytical signal, reported as a frequency shift (Hz), was given by the difference between the value recorded before the binding and after the washing (baseline), once the binding has occurred; both values are taken when the crystal is in contact with the same solution (binding buffer).

After each cycle of binding, the crystal surface was regenerated by a 1-min treatment with 2 M NaCl.¹⁹ With this treatment, the sensor-bound analyte is released at increased ionic strength, which unfolds the three-dimensional structure of the aptamer without damaging the oligomer structure, and the baseline is reached again, allowing the multiuse of the sensor.

Spiked Samples. Standard solutions of thrombin were added to serum or plasma to test the performance of the sensor in complex matrixes. Since serum does not contain coagulation factors, the addition of thrombin does not affect the samples. On the contrary, the addition of thrombin to plasma, which contains all the proteins involved in the coagulation cascade including fibrinogen, leads to the formation of fibrin and to rapid sample clotting. To avoid this phenomenon, fibrinogen was precipitated from plasma before the addition of thrombin in the preparation of spiked samples.

Table 1. Comparison between the Results Obtained with the Sensor When Immobilizing the Biotinylated or the Thiolated Aptamer

immobilized aptamer	immobilization shift (Hz) and density (molecules/cm ²)	thrombin 100 nM (Hz)	thrombin 200 nM (Hz)
biotinylated aptamer (with tail)	-225 ± 36 ; 2.4×10^{13}	-16 ± 9	-48 ± 10
thiolated aptamer	-138 ± 30 ; 3.4×10^{13}	-12 ± 13	-23 ± 13

Such selective precipitation is based on the use of ammonium sulfate as precipitant: 250 μL of plasma was treated with 1250 μL of 2 M ammonium sulfate and 1000 μL of 0.1 M sodium chloride. The solution was mixed for 3–4 min, then centrifuged, and the supernatant was eluted in a NAP column for rapid desalting and buffer exchange. The protein amount of the raw plasma and of the eluted solution was evaluated by spectrophotometric measurements at $\lambda = 280\text{ nm}$, and a loss of protein content ($\sim 40\%$) was detected after precipitation of fibrinogen.

RESULTS AND DISCUSSION

The developed aptasensor consists of the 15-mer thrombin aptamer (5'-GGTTGGTGTGGTTGG-3') immobilized on the gold surface of 9.5-MHz piezoelectric crystals and the analyte (thrombin) added in solution. The signal is recorded in real time without the use of any label.

The binding buffer for the dilution of thrombin has been previously²⁴ optimized by using optical sensing with the Biacore X device and then transferred to the piezoelectric system. The best performances in terms of sensitivity and reproducibility were obtained with thrombin diluted in 50 mM Tris-HCl, pH 7.4, 140 mM NaCl, 1 mM MgCl₂, buffer very similar to the one used for aptamer selection.²⁵

The second step was the investigation of the influence of the aptamer immobilization method on its binding with thrombin. The aptamer has been immobilized on gold when modified with a thiol group or on streptavidin when functionalized with biotin at its 5'-end with the insertion of a polyT (20-mer) tail. After the immobilization, the binding between the immobilized aptamer and 100 and 200 nM thrombin was studied with an interaction time of 20 min. The immobilization results for the biotinylated and the thiolated aptamer are reported in Table 1. From the shifts resulting from the immobilization of the two different aptamers, a similar density of immobilized molecules can be estimated considering $1\text{ Hz} = 2\text{ ng/cm}^2$. The tested aptamers were compared in terms of signal amplitude, reproducibility, and selectivity. The results indicated that the best performances were obtained with the 15-mer biotinylated aptamer carrying the polyT(20) tail.

This aptamer showed a good reproducibility (average CV for the two concentrations, 13%) and a very good selectivity as demonstrated by the low signal ($\Delta F < 3\text{ Hz}$) obtained with a high concentration of HSA (77 μM), used as negative control. The higher sensitivity obtained with this aptamer is probably due to the presence of the spacer, which maintains the aptamer far from

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Table 2. Comparison between the Analytical Characteristics of the Sensor Obtained When Immobilizing the Biotinylated Aptamer with a PolyT Tail

immobilized aptamer concn	binding time (min)	linear regression	R^2	CV (%)	cycles ^a
1 μ M	20	$y = -0.22x + 1.94$	0.933	35	14
1 μ M thermal treatment	20	$y = -0.23x + 0.42$	0.977	21	18
0.5 μ M thermal treatment	20	$y = -0.23x + 0.68$	0.988	14	18
0.5 μ M thermal treatment	30	$y = -0.22x$	0.998	10	22

^a Binding cycles performed without loosing in sensitivity

the sensor surface, allowing the proper conformation for molecular recognition. When the biotinylated aptamer (without polyT tail) was also immobilized, very low sensitivity (ΔF for 200 nM thrombin, <20 Hz) was found, even if comparable surface density occurred.

Further investigation has been conducted with the biotinylated aptamer with the polyT tail by studying the different parameters influencing the aptamer immobilization and consequently its binding to the protein. In particular, the influences of a thermal treatment of the aptamer (heating at 95 °C for 1 min and then cooling in ice for 10 min) and its concentration (varied from 0.1 to 1 μ M) have been investigated. Moreover, in the binding conditions optimization, the interaction time has been increased from 20 to 30 min. The results are reported in Table 2, which shows the improvements in terms of sensitivity, linearity, reproducibility, and reusability, by varying these parameters.

The thermal treatment unfolds the aptamer making the biotin label at the 5'-end available for interaction with streptavidin on the crystal surface.²⁶ Even if the thermal treatment has no effect on the aptamer surface density (2.5×10^{13} molecules/cm²), the linearity in the thrombin range 0–200 nM ($R^2 = 0.977$), and the reproducibility of the binding step (CV = 21%) significantly improved. This result confirms that the thermal treatment ensures correct intramolecular folding. Moreover, the results indicate that the surface capacity was not affected by the dilution from 1 to 0.5 μ M of the aptamer. On the contrary, a further dilution to 0.1 μ M dramatically reduced the sensor sensitivity (data not shown).

Finally, the increase in the interaction time from 20 to 30 min resulted in improved linearity and reproducibility. The sensorgram showing a typical binding curve of the aptamer and 50 nM thrombin, obtained with the optimized conditions, is displayed in Figure 1.

To check the specificity of the sensor, HSA at a concentration of 77 μ M, in a 1400-fold excess with respect to thrombin, was used. The interaction did not result in a measurable frequency decrease ($\Delta F < 3$ Hz), demonstrating the high specificity of the sensor.

Finally, the ability of the aptasensor to detect the analyte in complex matrixes was tested. Thrombin is the last enzyme protease involved in the coagulation cascade and it converts fibrinogen to insoluble fibrin that forms the fibrin gel.²⁷ Since the

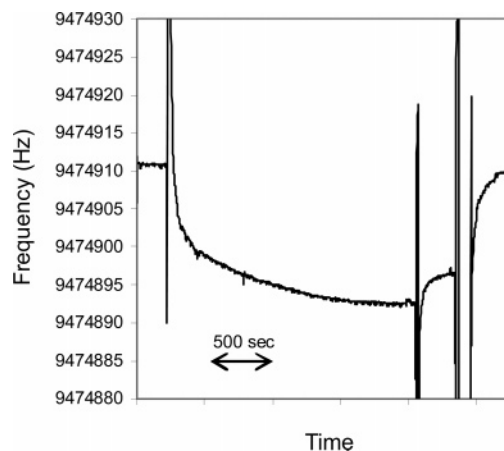


Figure 1. Typical binding curve (30 min) obtained with 50 nM thrombin interacting with the immobilized biotinylated aptamer.

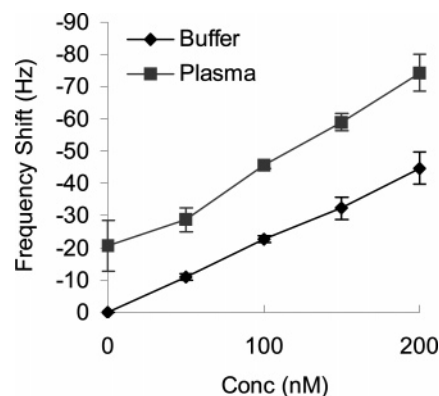


Figure 2. Calibration plot obtained with different concentrations of thrombin in buffer and plasma.

detection of thrombin in plasma or blood is clinically relevant, an aptamer-based sensor as alternative diagnostic tool for thrombin analysis or blood coagulation investigation could gain a great interest in the clinical and diagnostic area. Standard solutions of thrombin were added to serum and plasma to test the performances of the aptasensor in these complex matrixes. Since serum does not contain coagulation factors, the addition of thrombin does not affect the samples. On the contrary, the addition of thrombin to plasma, which contains all the proteins involved in the coagulation cascade including fibrinogen, leads to the formation of fibrin and to rapid sample clotting. To avoid this phenomenon, fibrinogen was precipitated from plasma before the addition of thrombin in the preparation of spiked samples. Thrombin was detected in serum diluted 1:100 spiked with thrombin in a concentration range 0–200 nM, with a blank value of –32 Hz (data not shown). The recorded signals increased with the concentration of added thrombin, demonstrating that the aptasensor was able to operate in this complex matrix. However, since serum does not contain the proteins involved in coagulation, further experiments have been carried out with plasma, which is the matrix where thrombin is normally detected. Figure 2 shows the signals obtained in spiked plasma without fibrinogen (final dilution 1:100). Reproducibility is good (CV = 13%), and the response is linear in the tested concentration range, 0–200 nM, ($R^2 = 0.990$). In this

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range, no saturation of the surface was observed. The matrix effect with a blank signal of -21 Hz is present but, despite the high complexity of the matrix, the increase in thrombin concentration could be detected. Due to the high dilution factor, the reached sensitivity is not sufficient for a direct application of the aptasensor to the detection of thrombin in real samples. This could be successfully performed by the use of amplification methods such as the use of a secondary affinity interaction or of enzymatic reactions.^{24,28} The goal of this work was to critically evaluate all the parameters that can influence the sensor performances by using thrombin as the model target, and the ability to detect the analyte in complex matrixes demonstrates the potentialities of aptamer-based sensing for potential medical investigations.

Moreover, a simple 2 M NaCl treatment was applied as a sensor regeneration step (1 min), allowing its reuse up to 20 times without losing in sensitivity.

Other label-free reported aptasensors are based on the quartz crystal microbalance (QCM),^{15,16} surface acoustic waves (SAW),^{29,30} or electrochemical transduction such as impedance¹⁴ or field-effect transistors.¹⁸ In the case of QCM,¹⁶ a detection limit for thrombin of 1 nM in buffer is reported, even if this datum is not supported by the study of other important analytical parameters such as specificity, reproducibility, or matrix effect. For SAW^{29,30} devices, the reported sensitivity is in the same order of magnitude

(submicromolar) of the proposed approach, but also in this case, the analysis is limited to standard solutions. By using impedance,¹⁴ significant improvement in the sensitivity, at least in buffer, was achieved, but no evaluation of real samples is given. To date, however, a rational evaluation of the different important key steps in aptasensor development has been reported only for an ELONA assay.¹⁹ The appealing aspect of this paper is represented exactly by the consideration of the different parameters influencing the analytical performances of this label-free, portable, and reusable sensor. Microarrays could represent an interesting tool in aptamer-based methods, improving the number of interactions that can be detected simultaneously and decreasing the amount of capture molecules immobilized on the chip with a reduction of the cost of analysis.

CONCLUSIONS

The proposed study can be considered as reference approach in aptasensing development. In particular, it should be stressed how little variations in simple steps in the assay development could lead to much improved analytical performances. In addition, the proposed study demonstrates the ability of label-free aptasensing to operate in complex matrixes. This is of paramount importance for a real applicability of these new, interesting, and attractive devices to clinical diagnostic, environmental, and food analysis.

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