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ScienceDirect
Biosensors and Bioelectronics 23 (2008) 1602–1609
BIOSENSORS & BIOELECTRONICS
www.elsevier.com/locate/bios

Different approaches for the detection of thrombin by an electrochemical aptamer-based assay coupled to magnetic beads

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Received 30 November 2007; accepted 18 January 2008
Available online 29 January 2008

Abstract

Different assay formats based on the coupling of magnetic beads with electrochemical transduction were compared here for the detection of thrombin by using a thrombin specific aptamer. By using the thrombin-binding aptamer, a direct and an indirect competitive assay for thrombin have been developed by immobilising the aptamer or the protein, respectively. Moreover, another strategy was based on the direct measurement of the enzymatic product of thrombin captured by the immobilised aptamer. All the assays were developed by coupling the electrochemical transduction with the innovative and advantageous use of magnetic beads.

The assays based on the immobilisation of the protein were not successful since no binding was recorded between thrombin and its aptamer. With the direct competitive assay, when the aptamer was immobilised onto the magnetic beads, a detection limit of 430 nM for thrombin was achieved. A lower detection limit for the protein (175 nM) was instead obtained by detecting the product of the enzymatic reaction catalysed by thrombin. All these assays were finally compared with a sandwich assay which reached a detection limit of 0.45 nM of thrombin demonstrating the best analytical performances.

With this comparison the importance of a deep study on the different analytical approaches for thrombin detection to reach the performances of the best assay configuration has been demonstrated.

Keywords: Aptamer; Thrombin; Magnetic beads; Electrochemical detection

1. Introduction

The coupling of magnetic beads with electrochemical transduction for the detection of thrombin are discussed in the present paper. These established principles were here enriched by adding in the unique features of aptamers.

Aptamers are nucleic acid ligands that can be generated against amino acids, drugs, proteins and other molecules. They are isolated from a large random library of synthetic nucleic acids by an iterative process of binding, separation and amplification called Systematic Evolution of Ligands by EXponential enrichment (SELEX) (James, 2000). Several reviews on aptamers have appeared in literature in the last 10 years, after the first publications on their selection (Ellington and Szostak, 1990; Tuerk and Gold, 1990; Tombelli et al., 2004; Tombelli et al., 2007) and their possible use as biorecognition element in biosensors has been well evidenced. Despite the well known advantages presented by aptamers with respect to antibodies (O'Sullivan, 2002; Tombelli et al., 2004), practical applications of aptamer-based sensing and diagnostics are still not competitive with antibody-based tests, since well established operating conditions have not been created yet and each individual aptamer-based assay has to be carefully optimised in order to find the optimum operating protocol to obtain the best analytical performances.

The thrombin-binding aptamer (15-mer, 5′-GGTTGG-TGTGGTTGG-3′) was the first one selected *in vitro*, specific for a protein without nucleic acid-binding properties (Bock et al., 1992). The interaction between thrombin and the aptamer has been taken as a model system by many authors since the thrombin-binding aptamer G-quartet structure has been established (Macaya et al., 1993; Smirnov and Shafer, 2000) and the binding site has been identified (Paborsky et al., 1993). This aptamer has been coupled to different transduction principles to demonstrate the wide applicability of aptamers as bioreceptors in biosensors (Baldrich et al., 2004; Radi et al., 2005; Hianik et

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doi:10.1016/j.bios.2008.01.020

al., 2005; Ikebukuro et al., 2005; Gronewold et al., 2005; Bang et al., 2005; Cai et al., 2006; Le Floch et al., 2006; Mir et al., 2006; Yoshida et al., 2006; Radi et al., 2006; Zhang et al., 2006). The signal transduction approaches varied from the quartz crystal microbalance (Hianik et al., 2005; Bini et al., 2007), fluorescence measurements (Hamaguchi et al., 2001; Nagatoishi et al., 2007) or other optical methods (Pavlov et al., 2004). Aptamer-based biosensors with electrochemical transduction have appeared in literature only in 2005 (Cai et al., 2006; Radi et al., 2006; Bang et al., 2005, Ikebukuro et al., 2005; Mir et al., 2006). As evidenced by Baldrich in 2004 and Mir in 2006 and as emerging from the examination of all the published aptamer-based biosensor works, the conditions for the optimal binding of the protein to the specific aptamer can vary a lot depending on the format adopted for the assay and these conditions have to be optimised again each time the aptamer or the assay configuration are changed.

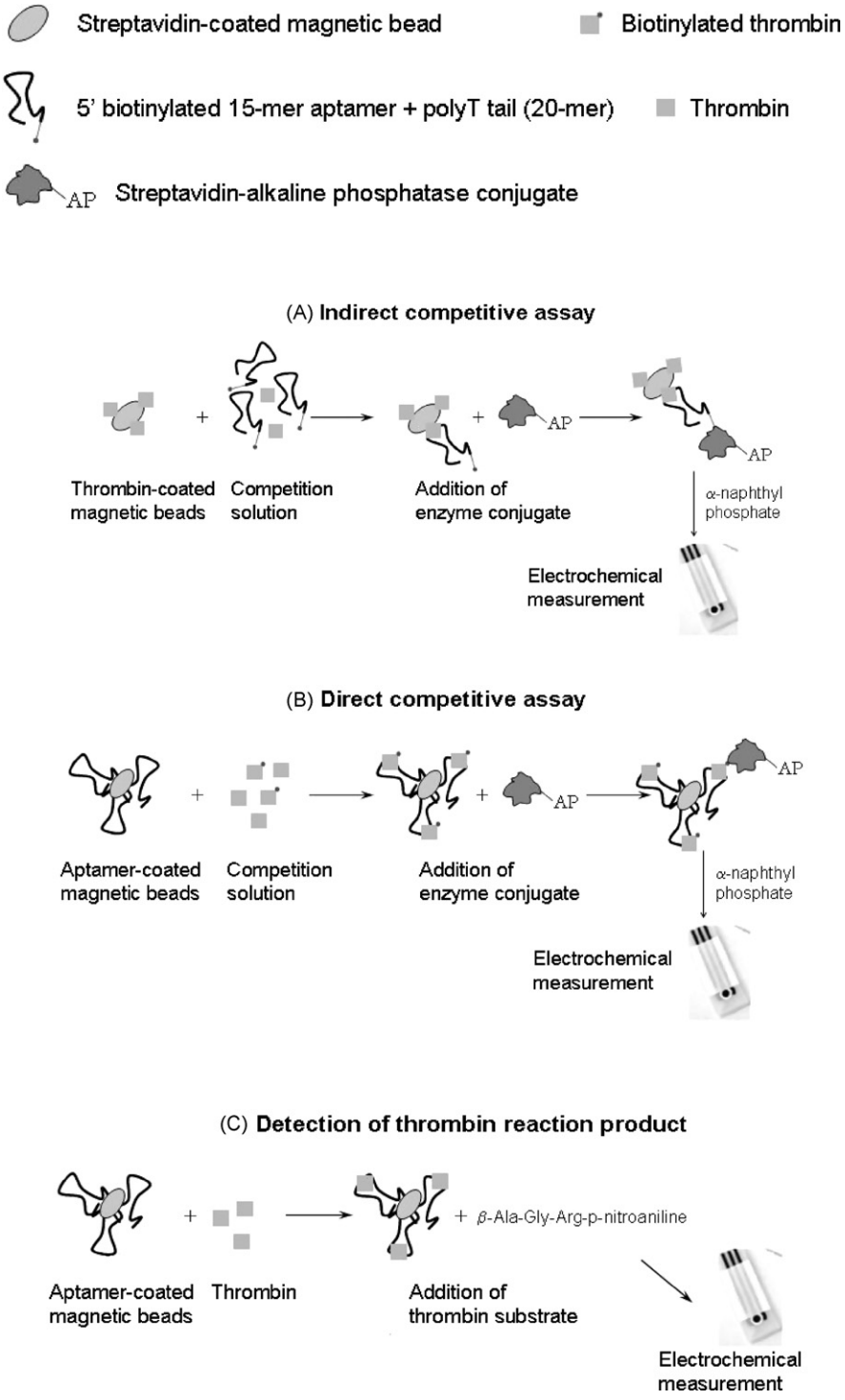

Fig. 1. Scheme of the direct competitive assay (A), indirect competitive assay (B) and of the assay based on the measurement of the thrombin reaction product (C).

In this work, several formats of an aptamer-based electrochemical assay were examined and optimised. The results demonstrated the variability of optimal assay conditions when using aptamers in different assay formats, in accordance with Baldrich in 2004 conclusions. The innovative aspect of all the proposed assays is the coupling of aptamers with magnetic beads for the aptamer or target immobilisation before the electrochemical transduction. Magnetic beads are known to be a powerful tool in a variety of bioassays (Rye and Nustad, 2001). Their use improves the performances of the affinity interaction for the faster assay kinetics achieved because the beads are in suspension and for the minimised matrix effect due to improved washing and separation. Moreover, they allow the analysis of complex samples without any pre-enrichment or purification steps (Zacco et al., 2006). Their application can also be automated in order to further decrease the assay time (Rashkovetsky et al., 1997).

In our recent paper (Centi et al., 2007b), an aptamer-based electrochemical sandwich assay coupled with magnetic beads was reported. The assay demonstrated a good reproducibility with a CV% of 8%, a high selectivity with a negligible signal obtained with HSA 72 μM and a DL of 0.45 nM. As it is well known in affinity assays, the sandwich format can be used to obtain a better sensitivity and specificity than that obtained with other formats. However, aptamer-based assays can be set up in a variety of formats (sandwich or competitive assays, direct or indirect assays). The main difference between the formats is the immobilised species (aptamer or target protein), the number of experimental steps involved, and in which order the different reagents are exposed to the surface. The choice of the format depends on the molecular size of the analyte, the availability of reagents and the cost. The advantages to use a competitive assay format (direct and indirect) with respect to the sandwich one are mainly related to the fact that only one aptamer is required (not for many proteins two different aptamers have been selected) and the time necessary for the assay is faster. So, when it is possible to perform different assay formats for the detection of the same target, it is useful to compare the analytical performances of each, in order to choose the approach that is the best compromise in terms of sensitivity, specificity, analysis time and costs.

For this reason, in this paper we deeply investigate the topic already reported in Centi et al. (2007b), developing different competitive assay schemes using aptamer as biorecognition element for thrombin detection. With this aim, a direct and an indirect competitive assay by immobilising either the aptamer or the protein were studied (Fig. 1A and B). All these assays were based on an electrochemical transduction and the aptamer or the target protein was immobilised onto magnetic beads. Different immobilisation approaches for the protein were tested onto tosyl-activated magnetic beads or streptavidin-coated magnetic beads. The aptamer or the protein in solution, were labelled with biotin and they were reacted with streptavidin-conjugated alkaline phosphatase. The detection of the product generated by the enzymatic reaction was achieved by differential pulse voltammetry (DPV).

Moreover, being thrombin a catalytic protein we also developed an assay based on the direct measurement of the enzymatic product of thrombin captured by the immobilised aptamer (Fig. 1C).

Some of the above mentioned formats were also reported by Mir in 2006 and by Baldrich in 2004. However, the main differences between this work and the works of these groups involve the use of magnetic beads as solid support on which the aptamer-based assay is performed. In some our recent papers we have demonstrated that the use of magnetic beads instead of the electrode surface as solid support increased greatly (about a factor of 100) the sensitivity of an electrochemical affinity assay and reduced the assay time (Centi et al., 2005; Centi et al., 2007a,c).

The experimental work was also supported by the use of the surface plasmon resonance device Biacore X^{TM}, through which different information on the tested assay formats have been obtained.

2. Materials and methods

2.1. Reagents and biomolecules

N-Hydroxysuccinimide (NHS), 1-ethyl-3-(dimethylaminopropyl) carbodiimide (EDAC) and streptavidin were purchased from Sigma–Aldrich (Milan, Italy). All the reagents for the buffers and EDTA were from Merck (Milan, Italy). Human α-thrombin, bovine and human serum albumin (BSA, HSA), biotin, streptavidin–alkaline phosphatase-conjugated, α-napthyl phosphate, *p*-nitrophenyl phosphate (PNPP), thrombin substrate β-Ala-Gly-Arg-*p*-nitroaniline were provided by Sigma (Milan, Italy). Biotinylated thrombin was provided by Novagen (Milan, Italy). Streptavidin-coated (MyOne^{TM} Streptavidin C_1) and tosyl-activated (M-280 tosyl-activated) magnetic beads were purchased from Dynal Biotech (Milan, Italy), monoclonal anti-thrombin IgG from Biodesign International (Milan, Italy).

The thrombin aptamers (MWG Biotech, Milan, Italy) used in this study, have the following sequences: biotinylated 15-mer aptamer with polyT(20) tail (5′-biotin-TTTTTTTTTTTT-TTTTTTTTTGGTTGGTGTGGTTGG-3′) and biotinylated 29-mer aptamer (5′-biotin-AGTCCGTGGTAGGGCAGGTTGG-GGTGACT-3′). The two aptamers (15-mer and 29-mer) were reported to recognise different sites of thrombin (K_d of 26 nM and 0.5 nM, respectively) (Tasset et al., 1997).

The buffers used for the experiments are reported in the Supporting Information.

2.2. Electrochemical instrumentation

The electrochemical cells were planar three electrode strips formed by a carbon working electrode, a carbon counter electrode and a silver pseudo-reference electrode. The electrodes were screen-printed using a DEK 248 screen-printing machine (DEK, Weymouth, UK). Silver-based (Electrodag PF-410) and graphite-based (Electrodag 423 SS) polymeric inks were obtained from Acheson (Milan, Italy); the insulating ink (Vinylfast 36–100) was from Argon (Lodi, Italy). A polyester flexible film (Autostat CT5), obtained from Autotype (Milan, Italy), was used as printing substrate.

Electrochemical measurements were performed using an μAutolab type II PGSTAT with a GPES 4.9 software package (Metrohm, Rome, Italy). All the measurements were carried out at room temperature by using differential pulse voltammetry (DPV).

The sample mixer with 12-tube mixing wheels and the magnets were purchased from Dynal Biotech (Milan, Italy).

2.3. SPR measurements

Surface plasmon resonance measurements were performed using the Biacore X^{TM} instrument (Biacore AB, Uppsala, Sweden). Carboxylated dextran-coated chips (CM5 chip, Biacore AB, Uppsala, Sweden) were used for the immobilisation of the biotinylated aptamers, biotinylated thrombin or thrombin. The immobilisation protocol both for thrombin and for the biotinylated aptamer is reported in the Supporting Information.

After the immobilisation, the interaction between the aptamer and thrombin was monitored with an association time of 15 min followed by a washing step with running buffer. Binding interactions were monitored at a constant flow rate of 5 μl/min at a temperature of 25 °C.

The binding and running buffer was Tris–HCl 50 mM pH 7.4 containing NaCl 140 mM and $MgCl_2$ 1 mM.

2.4. Assays with thrombin immobilised onto magnetic beads

The scheme of the different tested assays is reported in Fig. 1. The detailed description of the assays can be found in the Supporting Information.

2.4.1. Electrochemical indirect competitive assay (tosyl-activated magnetic beads)

The scheme of the assay is reported in Fig. 1A.

2.4.2. Electrochemical indirect competitive assay (streptavidin-coated magnetic beads)

The scheme of the assay is reported in Fig. 1A.

2.4.3. ELISA measurements

The detailed description of the protocol for the ELISA assay is reported in the Supporting Information.

2.5. Assays with the thrombin-specific aptamer immobilised onto magnetic beads

The scheme of the different tested assays is reported in Fig. 1. The detailed description of the assays can be found in the Supporting Information.

2.5.1. Electrochemical direct competitive assay

The scheme of the assay is reported in Fig. 1B.

2.5.2. Electrochemical detection of thrombin reaction product

The scheme of the assay is reported in Fig. 1C.

3. Results

3.1. Assays with thrombin immobilised onto magnetic beads

3.1.1. Electrochemical indirect competitive assay

Tosyl-activated and streptavidin-coated magnetic beads were used for the covalent coupling of thrombin or for the binding of biotinylated thrombin, respectively.

In the first format, the formation of covalent bonds between the magnetic beads and the protein analyte needs time: the manufacturer recommends 16–24 h if the immobilisation occurs at 37 °C. In our case, in order to avoid the possible denaturation of the protein, the immobilisation was carried out at room temperature and therefore a longer time (48 h) was used.

Different amounts of thrombin in the range 1–5 μg were tested to modify the beads. A solution of biotinylated 15-mer aptamer with polyT(20) tail 1 μM was then added to the modified beads and, after the addition of the enzyme-conjugate, the electrochemical detection was performed.

The electrochemical signal increased with the thrombin amount, until it reached a plateau corresponding to 3 μg of thrombin, which saturated the magnetic beads (9 ± 3 μA, 18 ± 3 μA, and 21 ± 2 μA using 1 μg, 3 μg and 5 μg of thrombin, respectively). This amount of thrombin was chosen for the protein immobilisation.

Experiments were performed using modified and not modified magnetic beads, in order to compare the non-specific binding of the aptamer and of the enzyme on the non-modified beads. Thrombin-modified and non-modified beads were incubated for 15 min with a solution of the 15-mer aptamer with polyT tail in the concentration range 0.1–3 μM. After magnetic separation and washing, the beads were re-suspended in 500 μl of enzyme-conjugate for 10 min and then the electrochemical measurement was carried out. Specific and non-specific signals measured in the aptamer concentration range 0.1–3 μM were compared. The extent of the non-specific signal, due to the adsorption of the enzyme-conjugate on the beads, was comparable with the signal obtained on thrombin-modified beads (specific signal).

Due to the low discrimination, the competitive assay with thrombin in solution was not carried out.

With streptavidin-coated magnetic beads, in order to optimise the concentration of aptamer to be used in the competition, magnetic beads were modified with biotinylated thrombin and then incubated for 15 min with different concentrations of biotinylated aptamer in the range 0.1–3 μM, after blocking the remaining sites on the beads surface with biotin. The beads were then incubated with the enzyme-conjugate solution and the electrochemical measurements were performed. The concentration 1 μM, corresponding to the saturation of the signal, was chosen to perform the competitive assay.

Using this concentration of biotinylated aptamer, in order to evaluate the possible presence of non-specific effects, electro-

chemical measurements with the enzyme were carried out in absence of biotinylated thrombin on the beads and in absence of thrombin or aptamer in the competition solution. Similar signals ($\sim 16 \pm 2\ \mu A$) were obtained in all the three cases, demonstrating the non-specificity of the interactions.

The same control was performed by an ELISA assay which confirmed the non-specific adsorption of the enzyme on the solid surface without any increase in signal due to the specific interaction between the immobilised thrombin and the aptamer (data not shown).

In these conditions the competitive assay was not performed due to the non-discrimination between a specific and a non-specific signal.

To understand if the failure of the two assays was due to the poor binding of the aptamer to the immobilised thrombin with the consequent "non-amplification" of the specific signal with respect to mere adsorption, SPR measurements were carried out.

At this purpose, thrombin was directly immobilised on a dextran-modified chip, to mimic its immobilisation onto tosyl-activated magnetic beads and the binding with the aptamer in solution was monitored. Moreover, biotinylated thrombin was immobilised on a streptavidin-modified CM5 chip and the binding of the aptamer was monitored as well.

The sensorgram recorded during the injection of the aptamer onto immobilised thrombin (direct immobilisation onto dextran) evidences the absence of binding between thrombin immobilised on the chip and the aptamer in solution and confirms the poor results obtained also on the magnetic beads. The same was observed when biotinylated thrombin was immobilised (data not shown).

These results are in accordance with published experiments (Baldrich et al., 2004) where an enzyme-labelled aptamer was tested onto immobilised thrombin without a resulting binding. The authors assumed that the labelling of the aptamer with a large molecule such as an enzyme prevents its folding into the quadruplex structure necessary for the binding to thrombin. In the case of our studies the aptamer is labelled with a small molecule (biotin) which cannot prevent its correct folding, but the binding is probably hindered by the immobilisation of thrombin.

To prove whether immobilisation on the chip caused degradation or modification of thrombin, anti-thrombin IgG (40 ppm) was injected and the binding with the immobilised protein was monitored. Very strong interaction was observed (Fig. 2) (ΔRU buffer(A) − buffer(B) = 600 RU) indicating that thrombin on the chip was still able to bind the antibody after the immobilisation.

The experiments conducted following the two reported assay formats confirmed the difficulties of using thrombin immobilised onto solid supports for its binding to the aptamer in solution. The degradation or modification of the protein was excluded by testing its interaction with the specific antibody, evidencing that the found difficulties were probably due to steric hindrance related to the protein immobilisation. This could prevent the correct folding of the aptamer "around" the binding site on thrombin, with the resulting absence of specific signal.

Further experiments will be devoted to clarify if the presence of a spacer between thrombin and the solid phase can improve the binding with the specific aptamer, moreover, also the thrombin catalytic activity can be evaluated for this purpose.

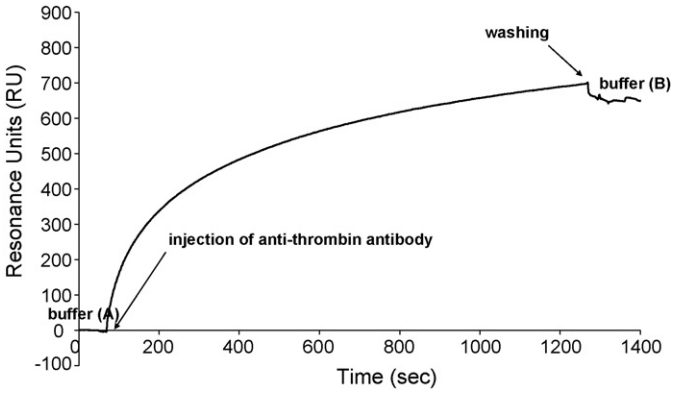

Fig. 2. Binding curve (15 min) obtained with IgG anti-thrombin (40 ppm) interacting with the immobilised biotinylated thrombin. A strong interaction was observed (ΔRU buffer(A) − buffer(B) = 600 RU) indicating that thrombin on the chip was able to bind the antibody after the immobilisation.

3.2. Assays with the thrombin-specific aptamer immobilised onto magnetic beads

3.2.1. Electrochemical direct competitive assay

The biotinylated 15-mer aptamer with polyT tail was immobilised on streptavidin-coated magnetic beads via streptavidin–biotin recognition. The concentration of the aptamer to bind to the beads (1 μM) was optimised in a previous work (Centi et al., 2007b).

To evaluate the extent of the non-specific binding, the beads without the aptamer were first incubated with biotin to saturate the streptavidin on the surface and then directly incubated with the enzyme. The non-specific signal was found to be around 40% of the specific one when the aptamer was immobilised and biotinylated thrombin was used.

To decrease the extent of non-specific binding, the effect of a further blocking agent in the solution of the enzyme-conjugate was evaluated. The experiments were performed incubating the aptamer-modified beads with a solution of biotinylated thrombin diluted 1:1000. After the magnetic separation and washing steps, the beads were added to the enzyme-conjugate solution containing 1% of BSA as blocking agent. The responses were then compared with those obtained in absence of aptamer immobilised on the magnetic beads. The specific signal, due to the aptamer-thrombin-binding, resulted to be very similar in presence and in absence of BSA, whereas non-specific signal, without the aptamer, resulted significantly lower (20%) when BSA (1%) was added to the solution of the enzyme-conjugate. For this reason, BSA (1%) was used as blocking agent in the enzyme-conjugate solution for the next experiments (Farabullini et al., 2007; Palchetti et al., 2007).

Since in a direct competitive assay it is important to optimise the concentration of the tracer, experiments were carried out to find the best dilution of biotinylated thrombin. Aptamer-coated magnetic beads were incubated for 30 min with different dilutions of tracer in the range 1:1000–1:50,000. The beads were

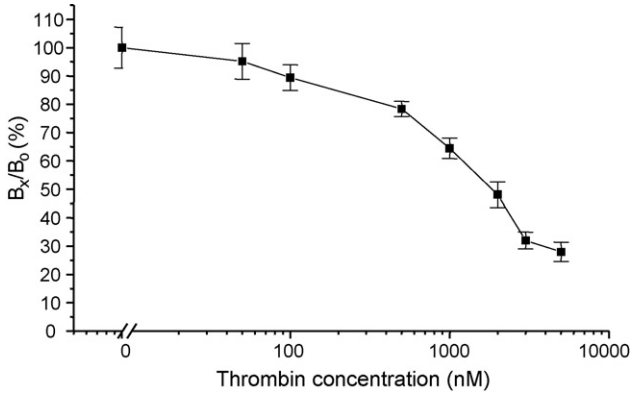

Fig. 3. Dose–response curve for thrombin (direct competitive assay). Aptamer-coated magnetic beads were incubated with a solution containing biotinylated thrombin (1:5000) and thrombin (concentration range: 0–2000 nM). The signal is expressed in percentage (relative) units as B_x/B_o (i.e. measured signal-to-blank signal ratio) and plotted vs. thrombin concentration. The average CV, calculated as mean of all the concentrations, is 6% and DL is 430 nM.

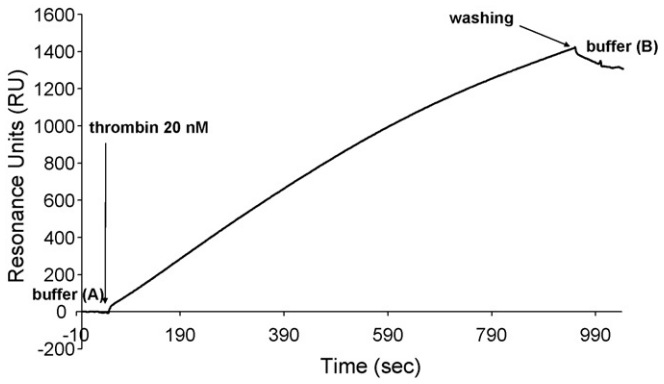

Fig. 4. The sensorgram recorded during the interaction of thrombin 20 nM with the immobilised biotinylated aptamer. The aptamer was immobilised on streptavidin (previously immobilised onto a CM5 chip) and the binding of thrombin was monitored. After 15 min of interaction, the chip was washed with the running buffer. As evidenced by the difference in the signal between the buffer before the injection of thrombin and the one of the same buffer at the end of the injection (ΔRU = 1300 RU), the aptamer immobilised on the chip was able to recognise thrombin.

then incubated with the enzyme-streptavidin solution and the electrochemical measurements were carried out. As expected, the signal increased with the increase of tracer concentration, until it reached a plateau corresponding to the dilution of biotinylated thrombin (1:5000) necessary to saturate the aptamer molecules (data not shown). A dilution of 1:5000 with respect to the stock was chosen to perform the competitive assay.

Under the optimised conditions, the electrochemical assay was applied to the detection of thrombin in the concentration range 0–2000 nM.

The corresponding calibration plot is shown in Fig. 3. The measured signal is expressed in percentage (relative) units as B_x/B_o (i.e. measured signal-to-blank signal ratio) and plotted vs. thrombin concentration. The current response decreased increasing thrombin concentration.

The assay was replicated in order to evaluate the reproducibility; to this purpose, three repetitions of each standard solution were carried out. The average coefficient of variation (CV) was 6%, calculated as mean of all the concentrations. The detection limit (DL) of the assay was evaluated as minimum detectable concentration, which is the lowest concentration of analyte which can be distinguished at a stated level of probability from a sample not containing the analyte. This concentration value was calculated by the evaluation of the average response of the blank minus three times the standard deviation. In this case, a DL of 430 nM was calculated.

The specificity of the 15-mer aptamer with polyT tail was evaluated against human serum albumin (HSA). This protein is present in blood at high concentration while thrombin is almost absent in healthy subjects. The electrochemical competitive assay was performed using HSA 72 μM (5000 ppm) and the resulting signal was compared with that obtained in absence of competition. These experiments demonstrated the high specificity of the assay since the signal in presence of HSA was very similar to that obtained in absence of analyte (19 μA and 21 μA, respectively).

To prove that the aptamer immobilised onto the sensor surface could correctly bind to thrombin, SPR measurements were carried. At this purpose, the aptamer was immobilised on streptavidin (previously immobilised onto a carboxymethylated chip) and the binding of thrombin was monitored. After 15 min of interaction, the chip was washed with the running buffer. The sensorgram recorded during the binding of thrombin is shown in Fig. 4. As evidenced by the difference in the signal between the buffer before the injection of thrombin (buffer (A)) and the one of the same buffer (buffer (B)) at the end of the injection (ΔRU = 1300 RU), the aptamer immobilised on the chip was able to recognise thrombin.

3.2.2. Electrochemical detection of thrombin reaction product

With the aim of completing the examination of the wide variety of possible assay formats for the electrochemical detection of thrombin coupled to magnetic beads, a further assay was developed by capturing the protein on the aptamer-modified beads and detecting the product of the catalytic reaction of thrombin with a suitable substrate.

50 μl of streptavidin magnetic beads modified with the aptamer were incubated with 200 μl of different concentrations of thrombin (100–600 nM) for 30 min. Bound thrombin was detected by re-suspending the beads in 200 μl of thrombin substrate β-Ala-Gly-Arg-*p*-nitroaniline (200 μM) for 30 min, at 37 °C. This substrate concentration was chosen, according to Mir et al. (2006), to be sure that the concentration of substrate was not the limiting factor of the enzymatic reaction.

The non-deareated solution containing the thrombin reaction product was deposited onto the surface of the working screen-printed graphite electrode, without any stirring. The thrombin reaction product was determined by DPV, since both the substrate and the *p*-nitroaniline released during hydrolysis have different redox potentials as shown in Fig. 5. The DPV peak potential of β-Ala-Gly-Arg-*p*-nitroaniline is −730 mV vs. Ag/AgCl pseudo-reference electrode, whereas the released

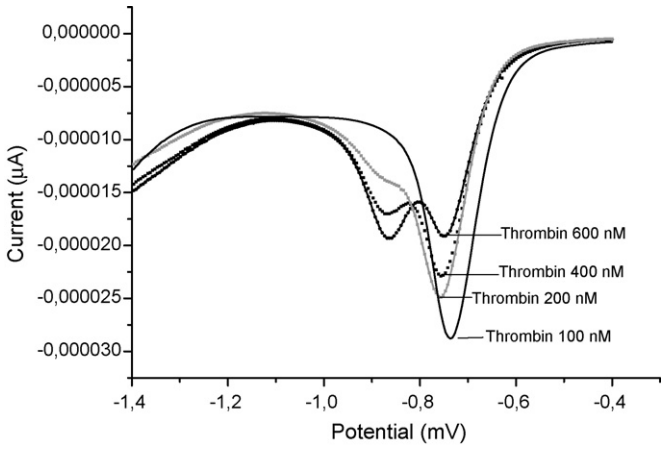

Fig. 5. DPV scans of thrombin substrate (β-Ala-Gly-Arg-*p*-nitroaniline); both the substrate and the *p*-nitroaniline released during hydrolysis have different redox potentials (the DPV peak potential of β-Ala-Gly-Arg-*p*-nitroaniline is −730 mV vs. Ag/AgCl pseudo-reference electrode, whereas the released *p*-nitroaniline peak potential is −870 mV vs. Ag/AgCl pseudo-reference electrode). Different concentrations of thrombin in the concentration range 100–600 nM were tested, whereas the substrate concentration was unvaried (200 μM).

p-nitroaniline peak potential is −870 mV vs. Ag/AgCl pseudo-reference electrode.

The aptamer-bound thrombin was detected by quantification of *p*-nitroaniline produced from the thrombin catalysed reaction. The DPV measurements showed a decrease of the peak at −730 mV and the appearance of a new peak at −870 mV, indicating the formation of *p*-nitroaniline (Fig. 5). The same measurements were carried out in absence of thrombin, and only a reproducible peak at −730 mV was observed (16.1 ± 0.4 μA).

A linear increase of *p*-nitroaniline peak current was observed in the studied concentration range of thrombin ($y = 0.0092x - 0.77$, $R^2 = 0.993$). On the contrary, a linear decrease in thrombin substrate was observed increasing the thrombin concentration ($y = -0.012x + 17$, $R^2 = 0.995$). This last equation was used for calculating the detection limit (DL), considering as blank the signal measured in absence of thrombin and subtracting 3σ to the mean value. Thus the calculated DL was 175 nM.

4. Discussion

Several assays based on an electrochemical transduction and the aptamer or thrombin immobilised onto magnetic beads have been examined. Different immobilisation approaches for the protein were tested onto tosyl-activated magnetic beads or streptavidin-coated magnetic beads. With the electrochemical indirect competitive assay based on tosyl-activated magnetic beads it was not possible to build up a dose–response curve since no discrimination was observed among specific and non-specific signal. Moreover, the immobilisation process of thrombin on these beads was extremely long requiring more than 48 h. As demonstrated by the SPR experiments the main problem of this method was the absence of binding between the aptamer in solution and immobilised thrombin.

Since the results reported above demonstrated that tosyl-activated beads were not suitable for this application, streptavidin-coated beads were tested. Thus, an electrochemical direct competitive assay as well as an indirect competitive assay were developed by immobilising the aptamer and thrombin, respectively.

In the direct competitive assay the competition between thrombin and biotinylated thrombin was observed and a detection limit of 430 nM for thrombin was obtained. The specificity of the assay was confirmed by using HSA 72 μM. SPR measurements demonstrated that the immobilised aptamer can correctly bind thrombin in solution.

On the contrary, in the indirect assay, where biotinylated thrombin was immobilised onto the beads, the binding was not observed. These findings were confirmed by SPR measurements which demonstrated that the aptamer in solution could not recognise the immobilised protein. However, a significant modification of the protein due to immobilisation can be excluded since an important binding of anti-thrombin IgG was observed.

To test all the different formats to detect thrombin via the electrochemical transduction coupled to magnetic beads, an assay based on the thrombin reaction product detection was carried out. In this case a detection limit of 175 nM for thrombin was observed.

By comparing the analytical performances of all the assays, the best results are obtained with the last method based on the detection of thrombin enzymatic product. The results obtained following the indirect competitive approach, when thrombin, biotinylated or not, was immobilised onto the beads, demonstrated that the assay could not be performed since the aptamer did not correctly bind to the immobilised protein. The direct competitive approach with the aptamer immobilised onto the beads was the only assay which gave successful results, with a sensitivity which was anyway too low compared with previous aptamer-based sensors published in literature (Centi et al., 2007b) or for a direct application of the assay to the detection of thrombin in real samples.

When dealing with thrombin as analyte, two different aptamers, selected to bind two distinct sites of the protein (Tasset et al., 1997), can be used as primary and secondary receptor in order to develop a sandwich aptamer-based assay. The increase in sensitivity and the high selectivity has been demonstrated recently (Centi et al., 2007b) by developing an aptamer-based sandwich assay coupled to magnetic beads. The assay demonstrated a good reproducibility with a CV% of 8%, a high selectivity with a negligible signal obtained with HSA 72 μM and a DL of 0.45 nM.

With this comparison we have demonstrated that to reach the performances of the best assay configuration (sandwich assay) a deep study on the different analytical approaches for thrombin detection should be performed. This study is thus necessary to define the best binding conditions among the different assays.

5. Conclusions

Different strategies were here described for the detection of thrombin by an electrochemical assay coupled to magnetic

beads. The different approaches were all based on the use of the thrombin specific aptamer immobilised onto magnetic beads.

The different assays were optimised and investigated in terms of the main analytical characteristics. The comparison between the tested strategies demonstrated that a careful study of the different binding or working conditions should be always performed to obtain the best analytical performances.

The problem related to each assay was verified by the use of SPR to deeply study the binding conditions by changing the parameters of the different assays.

Difficulties were found when thrombin was immobilised either on the magnetic beads or the SPR chips. Better performances were instead obtained with thrombin in solution and the aptamer immobilised onto the beads. The best results were reached by using a sandwich assay format.

Acknowledgement

The authors thank Dr. Serena Laschi for the faithful discussion overall the experimental work.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2008.01.020.

References

Baldrich, E., Restrepo, A., O'Sullivan, C.K., 2004. Anal. Chem. 76, 7053–7063.
Bang, G.S., Cho, S., Kim, B.-G., 2005. Biosens. Bioelectron. 21, 863–870.
Bini, A., Minunni, M., Tombelli, A., Centi, S., Mascini, M., 2007. Anal. Chem. 79, 3016–3019.
Bock, L.C., Griffin, L.C., Latham, J.A., Vermaas, E.H., Toole, J.J., 1992. Nature 355, 564–566.
Cai, H., Ming-Hung Lee, T., Hsing, I.-M., 2006. Sens. Actuators B: Chem. 114, 433–437.
Centi, S., Laschi, S., Fránek, M., Mascini, M., 2005. Anal. Chim. Acta 538, 205–212.
Centi, S., Silva, E., Laschi, S., Palchetti, I., Mascini, M., 2007a. Anal. Chim. Acta 594, 9–16.
Centi, S., Tombelli, S., Minunni, M., Mascini, M., 2007b. Anal. Chem. 79, 1466–1473.
Centi, S., Laschi, S., Mascini, M., 2007c. Talanta 73, 394–399.
Ellington, A.D., Szostak, J.W., 1990. Nature 346, 818.
Farabullini, F., Lucarelli, F., Palchetti, I., Marrazza, G., Mascini, M., 2007. Biosens. Bioelectron. 22, 1544–1549.
Gronewold, T.M.A., Glass, S., Quandt, E., Famulok, M., 2005. Biosens. Bioelectron. 20, 2044–2052.
Hamaguchi, N., Ellington, A., Stanton, M., 2001. Anal. Biochem. 294, 126–131.
Hianik, T., Ostatná, V., Zajacová, Z., Stoikova, E., Evtugyn, G., 2005. Bioorg. Med. Chem. Lett. 15, 291–295.
Ikebukuro, K., Kiyohara, C., Sode, K., 2005. Biosens. Bioelectron. 20, 2168–2172.
James, W., 2000. In: Mayers, R.A. (Ed.), Aptamers. Encyclopaedia of Analytical Chemistry, vol. 484. John Wiley and Sons, LTD, London.
Le Floch, F., Ho, H.A., Leclerc, M., 2006. Anal. Chem. 78, 4727–4731.
Macaya, R.F., Schultze, P., Smith, F., Roe, J.A., Feigon, J., 1993. Proc. Natl. Acad. Sci. U.S.A. 90, 3745–3749.
Mir, M., Vreeke, M., Katakis, I., 2006. Electrochem. Commun. 8, 505–511.
Nagatoishi, S., Nojima, T., Galezowska, E., Gluszynska, A., Juskowiak, B., Takenaka, S., 2007. Anal. Chim. Acta 581, 125–131.
O'Sullivan, C.K., 2002. Anal. Bioanal. Chem. 372, 44–48.
Paborsky, L.R., McCurdy, S.N., Griffin, L.C., Toole, J.J., Leung, L.L., 1993. J. Biol. Chem. 268, 20808–20811.
Palchetti, I., Laschi, S., Marrazza, G., Mascini, M., 2007. Anal. Chem. 79 (18), 7206–7213.
Pavlov, V., Xiao, Y., Shlyahovsky, B., Willner, I., 2004. J. Am. Chem. Soc. 126, 11768–11769.
Radi, A.-E., Acero Sanchez, J.L., Baldrich, E., O'Sullivan, C.K., 2005. Anal. Chem. 77, 6320–6323.
Radi, A.-E., Acero Sanchez, J.L., Baldrich, E., O'Sullivan, C.K., 2006. J. Am. Chem. Soc. 128, 117–124.
Rashkovetsky, L.G., Lyubarskaya, Y.V., Foret, F., Hughes, D.E., Karger, B.L., 1997. J. Chromatogr. A 781, 197–204.
Rye, P.D., Nustad, K., 2001. BioTechniques 30, 290–295.
Smirnov, I., Shafer, R.H., 2000. Biochemistry 39, 1462–1468.
Tasset, D.M., Kubik, M.F., Steiner, W., 1997. J. Mol. Biol. 272, 688–698.
Tombelli, S., Minunni, M., Mascini, M., 2004. Biosens. Bioelectron. 20, 224.
Tombelli, S., Minunni, M., Mascini, M., 2007. Biomol. Eng. 24, 191–200.
Tuerk, C., Gold, L., 1990. Science 249, 505.
Yoshida, W., Sode, K., Ikebukuro, K., 2006. Biochem. Biophys. Res. Commun. 348, 245–252.
Zacco, E., Pividori, M.I., Alegret, S., Galve, R., Marco, M.-P., 2006. Anal. Chem. 78, 1780–1788.
Zhang, H., Wang, Z., Li, X.-F., Le, X.C., 2006. Angew. Chem. Int. Ed. 45, 1576–1580.