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Analytical Applications of Aptamers

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Abstract

Aptamers are single stranded DNA or RNA ligands which can be selected for different targets starting from a library of molecules containing randomly created sequences. Aptamers have been selected to bind very different targets, from proteins to small organic dyes. Aptamers are proposed as alternatives to antibodies as biorecognition elements in analytical devices with ever increasing frequency. This in order to satisfy the demand for quick, cheap, simple and highly reproducible analytical devices, especially for protein detection in the medical field or for the detection of smaller molecules in environmental and food analysis. In our recent experience, DNA and RNA aptamers, specific for three different proteins (Tat, IgE and thrombin), have been exploited as bio-recognition elements to develop specific biosensors (aptasensors). These recognition elements have been coupled to piezoelectric quartz crystals and surface plasmon resonance (SPR) devices as transducers where the aptamers have been immobilized on the gold surface of the crystals electrodes or on SPR chips, respectively.

Keywords: Aptamers, Biosensors, Thrombin, IgE, HIV TAT protein.

INTRODUCTION

Aptamers are artificial nucleic acid ligands that can be generated against amino acids, drugs, proteins and other molecules. They are isolated from complex libraries of synthetic nucleic acids by an iterative process of adsorption, recovery and amplification called systematic evolution of ligands by exponential enrichment (SELEX) [1]. The SELEX process [2,3] involves iterative cycles of selection and amplification starting from a large library of oligonucleotides with different sequences (generally 10^{15} different structures). After the incubation with the specific target and the partitioning of the binding from the non-binding molecules, the oligo-nucleotides that are selected are amplified to create a new mixture enriched in those nucleic acid molecules having a higher affinity for the target. After several cycles of the selection process, the pool is enriched in the high affinity sequences at the expense of the low affinity binders.

Several reviews and research works on aptamers have appeared in literature. The potential application of aptamers as alternative ligands in diagnostic assays [4], biosensors [5-7] or other analytical techniques [8,9] have been presented.

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When an analytical device or assay requires a bio-molecular recognition event, antibody-based detection methodologies are still considered the standard assays in environmental, food and clinical analysis. These assays are well established and they have been demonstrated to reach the desired sensitivity and selectivity. However, the use of antibodies in multianalyte detection methods and in the analysis of very complex samples could encounter some limitations mainly deriving from the nature and synthesis of these protein receptors. In order to circumvent some of these drawbacks, other recognition molecules are being explored as alternatives.

In addition to the very important aspect of having an unlimited source of identical affinity recognition molecules available, aptamers can offer advantages over antibodies that make them very promising for analytical applications [5,6,9].

The main advantage is the overcoming of the use of animals or cell lines for the production of the molecules. Antibodies against molecules that are not immunogenic are difficult to generate. On the contrary, aptamers are isolated by *in vitro* methods that are independent of animals and an *in vitro* combinatorial library can be generated against any target.

Moreover, the aptamer selection process can be manipulated to obtain aptamers that bind a specific region of the target and with specific binding properties in different binding conditions. After selection, aptamers are produced by chemical synthesis and purified to a very high degree by eliminating the batch-to-batch variation found when using antibodies. By chemical synthesis, modifications in the aptamer can be introduced enhancing the stability, affinity and specificity of the molecules.

Another advantage over antibodies can be seen in the higher temperature stability of aptamers; in fact antibodies are large proteins sensitive to the temperature and they can undergo irreversible denaturation. On the contrary, aptamers are very stable and they can recover their native active conformation after denaturation.

The primary limitation on the use of aptamers (mainly RNA aptamers) in bioanalytical methods has been their nuclease sensitivity which is very critical for their use in ex vivo and in vivo applications [10]. However, it has been shown that the stability of such molecules can be improved by chemical modification of the ribose ring at the 2'-position [11].

With respect to their application, aptamers were selected in the past mainly for their use as therapeutic agents. In addition to the therapeutic field, aptamers have been then used in several analytical methodologies, such as affinity chromatography [12], capillary electrophoresis [13,14], mass spectrometry [15,16] or biosensors [7].

These aptamers-based methods have been mainly employed in the clinical area for the development of diagnostic assays, whereas to date only a limited number of studies have demonstrated the possibility of using aptamers in assays for the analysis of environmental or food samples.

Several examples of biosensors developed by immobilizing aptamers specific for three different proteins (thrombin, Tat, and IgE) will be here reported. DNA or RNA aptamers have been immobilized onto the gold surface of piezoelectric quartz crystals, surface plasmon resonance (SPR) chips or magnetic beads with electrochemical transduction and the interaction with the target protein has been examined to evaluate the analytical performances of the biosensors.

An electrochemical assay for the detection of thrombin was developed by immobilizing onto magnetic beads the DNA aptamer specific for thrombin (thrombin binding aptamer, TBA). Thrombin is not a DNA-binding protein but nevertheless, thrombin-specific aptamers have been selected using the SELEX technology [17]. The thrombin-binding aptamer (TBA) was the first in vitro selected aptamer targeted toward a protein [17].

Two biosensors have been constructed using an RNA aptamer specific for HIV-1 Tat (Trans-activator of transcription) protein. Yamamoto et al. [18,19] was the first to develop an aptamer that yielded an efficient binding specificity against Tat, but not for other cellular factors.

A DNA aptamer specific for IgE has been also used immobilized onto the gold surface of SPR chips to study its interaction with the specific target protein, human IgE [20].

The results demonstrate the applicability of aptamer-based assays to the detection of different proteins of clinical interest present at low concentrations, with very good selectivity.

APTASENSORS FOR THROMBIN DETECTION

Thrombin (factor IIa) is the last enzyme protease involved in the coagulation cascade and it converts fibrinogen to insoluble fibrin that forms the fibrin gel either in physiological conditions or in a pathological thrombus [21].

Thrombin is formed by two peptide chains of 36 and 259 amino acids, which are linked by disulfide bonds. The known molecular weight of α-thrombin is 35422 Da. The concentration of thrombin in blood can vary considerably: thrombin, not present in blood under normal conditions, can reach low μM concentrations during the coagulation process but low levels (low nM) of thrombin generated early in hemostasis are also important to the overall process [22].

The thrombin-binding aptamer (15-mer, 5'-GGTTGGTGTGGTTGG-3') has been the first DNA aptamer selected in vitro, specific for a protein without nucleic acids-binding properties and it has been studied as an anticlotting therapeutic tool.

This aptamer has been used as a model system, coupled to different transduction principles to demonstrate the wide applicability of aptamers as bioreceptors in biosensors [23-26]. The thrombin-binding aptamer has been extensively investigated: its G-quartet structure has been established [27] and the binding site has been studied and determined [28].

We report here an example of an aptamer-based assay for the detection of thrombin directly into human plasma [29].

The assay is based on electrochemical transduction coupled to magnetic particles, modified with the aptamer. With the addition of a second aptamer after the interaction of the analyte with the modified beads, similarly to immunoassays, this approach can be thought as a sandwich method (Figure 1).

The work aims to couple a simple target capturing step, by employing magnetic beads, to the high sensitivity requested for thrombin analysis, assured by the electrochemical detection with Differential Pulse Voltammetry (DPV) on screen printed electrodes.

A sandwich assay format is chosen following an approach similar to the one reported by Ikebukuro et al. [25] who employed an electrochemical detection using a conventional gold electrode and limited to

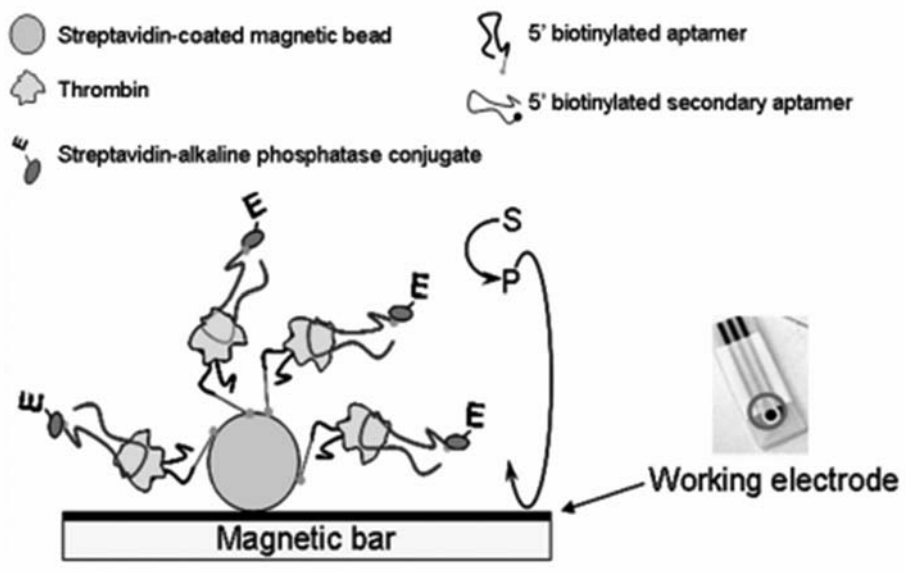

Figure 1. Scheme of the electrochemical sandwich assay coupled to magnetic beads.

standard solutions of the analyte down to a concentration of 10 nM. In both approaches, two selected aptamers binding thrombin in two different, non-overlapping, sites are used. The protein captured by the first aptamer is detected after the addition of the second biotinylated aptamer and of streptavidin labeled with an enzyme (alkaline-phosphatase). Detection of the product generated by the enzymatic reaction was achieved by differential pulse voltammetry onto screen printed electrodes. This approach based on the use of two different aptamers overcomes some of the drawbacks related to the use of antibodies.

Using this novel design, an electrochemical biosensor recognizing thrombin with high affinity, sensitivity and specificity was obtained, opening the possibility of a real application to diagnostics or medical investigation.

The optimized conditions resulted in an immobilized biotinylated aptamer, named primary aptamer, with a polyT tail to ensure a certain flexibility in the binding (1 μM 15-mer : 5'- biotin - TT TTT TTT TTT TTT TTT TTT GGT TGG TGT GGT TGG - 3') and a biotinylated 29-mer as secondary aptamer to be used in the sandwich (5' – biotin – AGTCCGTGGTA GGGCAGGTTGGGGTGACT–3') (0.1 μM, incubation time 15').

The polyT tail (20-mer) was chosen considering the absence of adenine in the aptamer sequence: in this case the thymines of the tail do not influence the aptamer secondary structure.

A dose-response curve for thrombin is reported in Figure 2. The height of the peaks obtained by DPV measurements for different concentrations of thrombin increases with the increase of thrombin concentration showing a typical behavior of a sandwich assay. In the Figure, the signals are reported as peak current against log of thrombin concentration.

A signal increase was observed for thrombin concentrations greater than 0.1 nM and the highest

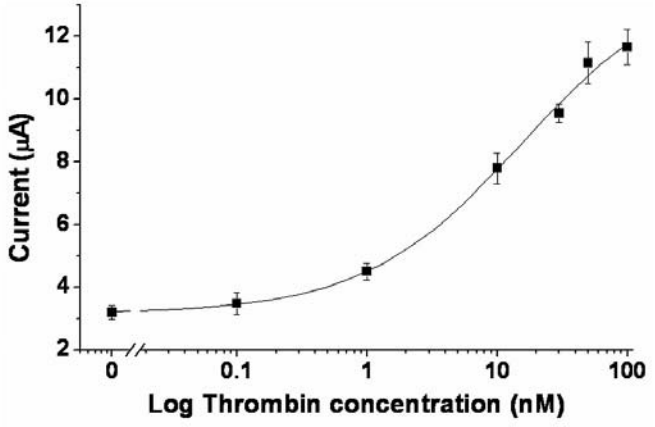

Figure 2. Electrochemical assay for thrombin coupled to magnetic beads: dose-response curve for thrombin.

current was measured at a protein concentration of 100 nM. The reproducibility, expressed as the average coefficient of variation (CV) is 8% (n=5 in the range 0-100 nM). The detection limit (DL) is 0.45 nM.

Human serum albumin at a concentration of 72 μM (5000 ppm) was used to test the specificity of the aptamer, which was found to be very high since a negligible signal in presence of HSA or of the blank solution was observed. The applicability of the assay to plasma samples was evaluated by investigating the presence of any matrix effect.

Plasma, after fibrinogen precipitation, diluted 10 times was tested alone or spiked with thrombin (in the concentration range 0-100 nM), and the results were compared with thrombin standard buffer solutions.

Figure 3 shows that comparable responses were found for buffer, and plasma: addition of thrombin to the sample resulted in protein concentration-dependent signal. The samples were incubated with the beads (coated with primary aptamer) and then the assay was carried out by adding the secondary aptamer and the conjugate.

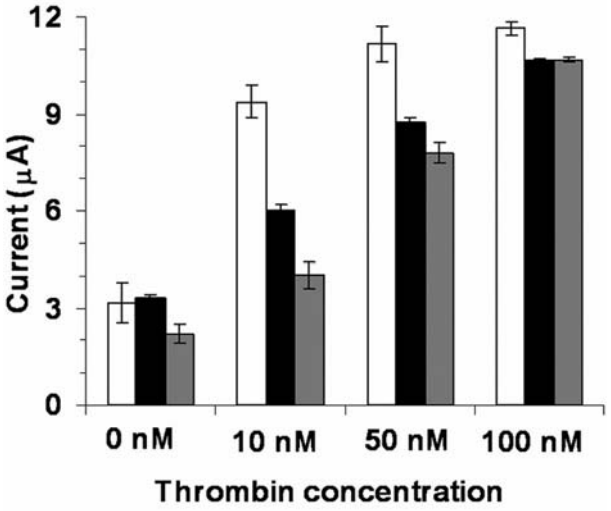

Figure 3. Results obtained with serum samples spiked with thrombin (black histograms) and comparison with the same concentrations tested in buffer (white histograms) and in plasma after fibrinogen precipitation (grey histograms).

For plasma a weak matrix effect was found considering the lower currents (average decrease ~ 80%) measured with respect to buffer. Probably this decrease is due to a reduced concentration of thrombin available for the binding caused, by the interaction with some matrix components.

APTASENSOR FOR HIV-1 TAT DETECTION

Tat is a protein of 86 to 101 amino acids controlling the early phase of the human immunodeficiency virus type 1 (HIV-1) replication cycle [30,31]. An RNA aptamer for Tat has been selected and characterized in 2000 [18,19].

Both for the piezoelectric and the SPR biosensor for Tat, the immobilization chemistry adopted, followed the approach described in Tombelli et al. [32]. The biotinylated aptamer (1 μM) (5' ACGAAGCUU GAUCCCGUUUGCCGGUCGAUCGCUUCGA 3') was immobilized on the streptavidin layer through biotin-streptavidin interaction on both the piezoelectric and the optical device. Onto piezoelectric crystals, an average value of (63±10) Hz was obtained (CV=16%) for the immobilization step, confirming the efficiency of the immobilization process.

The interaction between the immobilized aptamer and Tat (0.25-2.5 ppm) has been studied and the calibration curve is reported in Figure 4. A linearity region in the concentration range 0-1.25 ppm, and an experimental limit of detection of 0.25 ppm, can be observed.

The selectivity of the sensor was studied testing Rev protein (Regulator of Expression of Viral proteins), as non-specific molecule, which can be considered as the major interfering protein having the same molecular weight and being a basic protein as Tat. Two different concentrations of Rev, 0.65 and 2.5 ppm, were tested. The signals recorded were (5 ±

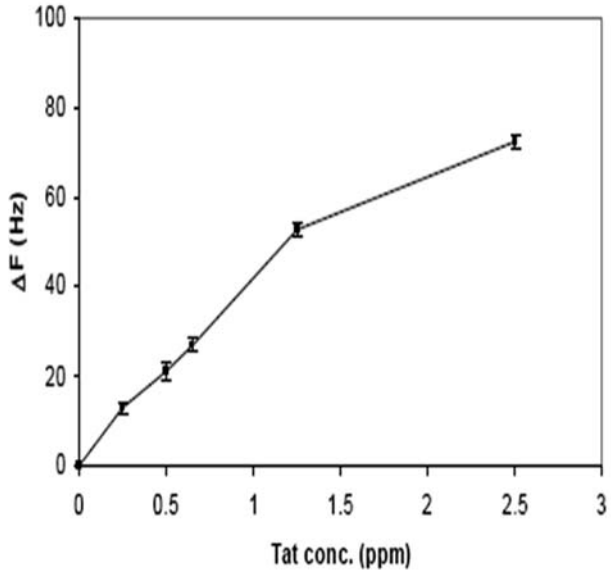

Figure 4. Calibration curve obtained from the interaction of Tat (0-2.5 ppm) with the immobilized biotinylated aptamer. Interaction time: 15 minutes.

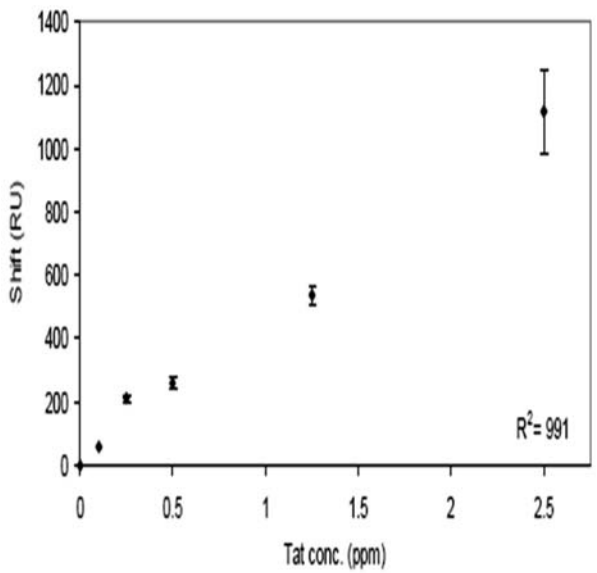

Figure 5. Calibration plot obtained with the SPR-based biosensor. Injections of Tat at different concentrations (0.12-2.5 ppm). Flow rate: 5 ml/min, T: 25°C.

1) and (12 ± 1) Hz, respectively, indicating a high selectivity of the sensor.

After each measurement the surface was regenerated in order to allow a further interaction between the immobilized aptamer and the protein. A solution 12 mM NaOH with 1.2 % EtOH allowed a complete regeneration with two steps of 30 sec and, in general, on the same crystal surface 15 measurements could be performed without loss in sensitivity (data not shown).

The binding between Tat protein and its specific aptamer has been also studied on the SPR instrument Biacore XTM. For this purpose dextran-modified chips have been used and modified with streptavidin and the biotinylated aptamer. An average value of (5410±94) RU (CV=2%) for the immobilization of streptavidin led to an aptamer immobilization value of 1639±75 RU (CV=5%), proving a high reproducibility of the immobilization method.

The interaction between the immobilized aptamer and Tat at different concentrations has been evaluated also with the SPR biosensor. As in the case of the piezoelectric biosensor, a concentration range of 0.12-2.5 ppm has been tested and the calibration graph is reported in Figure 5. A linear range can be observed up to 2.5 ppm and the average CV for all the concentrations tested in triplicate was 7%.

The specificity of the system has been evaluated testing different concentrations of BSA (0.1 ppm and 0.5 ppm) and the only binding buffer (which contains 0.1% BSA). No change in resonance units was obtained from the injection of these solutions. Moreover, the solution in which the protein is diluted by the supplier (0.2 M KCl and 5 mM glutathione) has been injected with the same operating conditions to verify the absence of non-specific effects due to its components. Also in this case the system appeared to be very specific since no signal could be observed.

As in the case of the piezoelectric biosensor, Rev protein has been used as negative control and injected into the instrument at different concentrations (0.125 and 1.25 ppm). A negligible signal (ΔRU<2 RU) has been obtained with the concentration 0.125 ppm, but from the injection of REV 1.25 ppm, a signal of 146 RU could be recorded.

Also in this case the surface of the chip can be regenerated to remove the protein and obtain the aptamer free again for another binding measurement. This step has been conducted using the same regenerating solution as for the piezoelectric system (12 mM NaOH with 1.2 % EtOH) and more than 50 cycles could be performed on the same chip without loss in sensitivity.

The comparison of the developed aptasensor with other Tat detection methods is very difficult since most of the published papers on this subject deal with Tat-derived peptides and not with the full length protein. Moreover, the biosensor revealed not to be sensitive enough for the detection of Tat in real samples, such as sera or cell culture supernatants, but the work suggests that the use of aptamers as bio-recognition element can be an interesting approach for the detection of proteins.

ALLERGY DETECTION BY USING APTASENSORS

The other model system based on an aptamer specific for IgE was examined coupled with an SPR transduction. Human IgE is a monomeric immunoglobulin of approximately 190000 Da. Of similar structure to the other immunoglobulins (IgG, IgM, IgD and IgA), it constitutes about 0.0005% of total serum immunoglobulins in adults [33]. IgE is considered the “gatekeeper” of immediate type I hypersensitivity [22]. A DNA aptamer specific for the Fc domain of IgE has been selected [34] and it has been used in the present study to develop an SPR biosensor for IgE detection [20].

Immobilization procedures for both the IgE-specific aptamer (5’ biotin-GCG CGG GGC ACG TTT ATC CGT CCC TCC TAG TGG CGT GCC CCG CGC 3’) (FC-2) and the poly-T DNA control (FC-1) were successful, with signal shifts of 1106 and 1881 RU observed after immobilization, respectively.

Binding experiments using concentrations varying from 5 ppm to 50 ppm IgE were performed. Results obtained using 20 ppm IgE are shown in Figure 6, where one can see that binding occurs exclusively on FC-2 (aptamer), giving a maximum response of 340 RU.

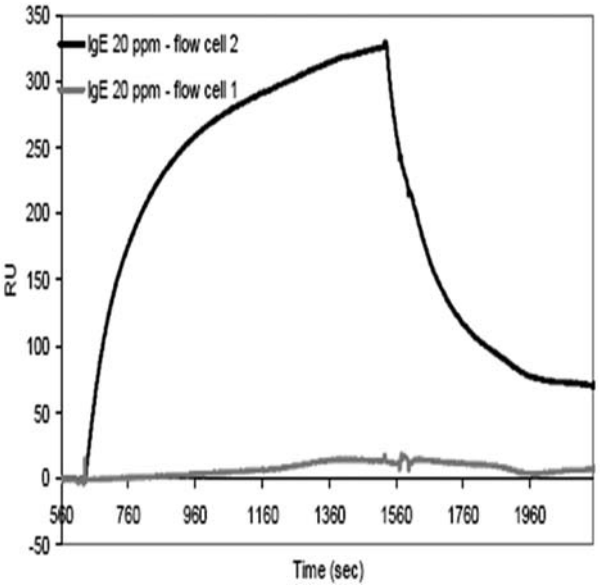

Figure 6. Sensorgrams recorded after the injection of IgE at 20 ppm (Biacore). Flow cell 1: non-specific DNA; Flow cell 2: IgE specific aptamer.

Similar responses were observed over the entire range of concentrations tested, with binding signals varying in accordance to the concentration tested, as shown in Figure 7. This therefore confirmed the absence of non-specific adsorption on the sensor surface (since no binding on FC-1 was observed). Furthermore, the specificity of the aptamer was

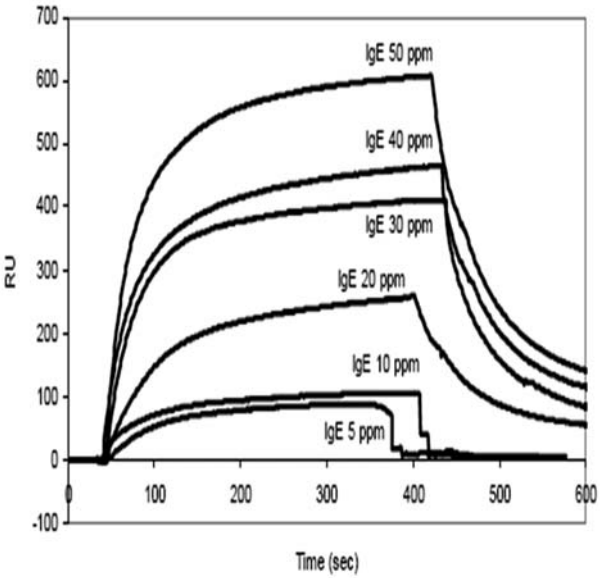

Figure 7. IgE binding responses at different concentrations (5-50 ppm) onto flow cell 2 (IgE specific aptamer).

evaluated by performing binding experiments using IgG as a negative control, with concentrations varying from 10 ppm to 50 ppm. In Figure 8, the response obtained using 50 ppm IgG is compared with that obtained using 10 ppm IgE, both on FC-2. One can clearly observe that the IgG signal is negligible compared to IgE and this was confirmed with all the tested concentrations of IgG.

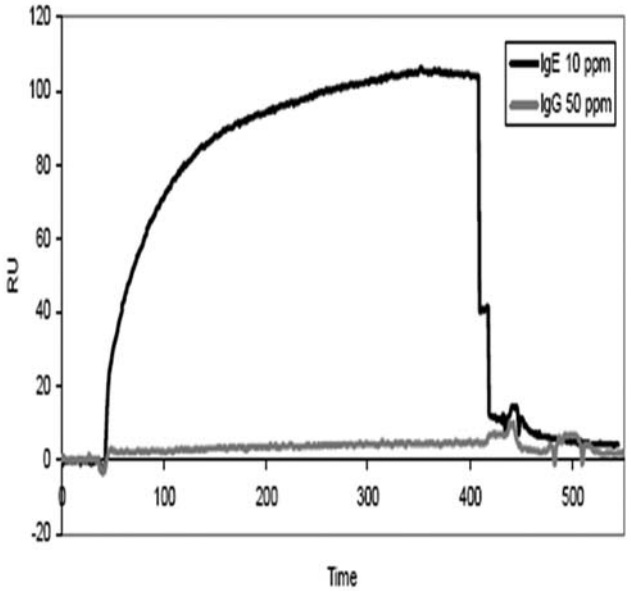

Figure 8. Sensorgrams recorded after the injection of IgE (10 ppm) and IgG (50 ppm) onto flow cell 2 carrying the IgE specific aptamer.

The regeneration procedure proved to be very successful, with one regeneration step being sufficient to regenerate the surface in most cases.

Also in this case the biosensor proved to be very selective and the aptamer proved to be able to bind to the target molecule also when immobilized onto a solid support. Anyway, especially due to the very rapid dissociation, the biosensor proved not to be sensitive enough for the detection of IgE in complex real samples.

CONCLUSIONS

This work demonstrated the potential role of aptamers as biorecognition elements in affinity sensing. These model systems were based on aptamers specific for thrombin, HIV-1 Tat protein and IgE. Different transducers were used for the development of the aptamer-based biosensors. The aptasensors developed proved to be specific, reproducible and reusable for the detection of the target proteins.

In addition to the very important aspect of having an unlimited source of identical affinity recognition molecules available due to the selection process, aptamers can offer advantages over antibodies that make them very promising for analytical applications. The use of aptamers as therapeutic tools is nowadays well established. On the contrary, the analytical application of aptamers in diagnostic devices or in systems for environmental and food analysis, is still under investigation and the scientific community still need further research to demonstrate the advancements brought by this new kind of ligands.

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