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Voltammetric aptasensor combined with magnetic beads assay developed for detection of human activated protein C

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ABSTRACT

A sensitive and selective label free voltammetric aptasensor based on magnetic beads assay was performed for the first time in our study for monitoring of human activated protein C (APC), which is a serine protease (i.e., key enzyme of the protein C pathway). An amino modified DNA aptamer (DNA APT) was covalently immobilized onto the surface of carboxylated magnetic beads (MBs), and then, the specific interaction between DNA APT and its cognate protein, APC, was performed at the surface of MBs. Similarly a biotinylated DNA APT was immobilized onto the surface of streptavidin coated MBs. Before and after interaction process, the oxidation signal of guanine was measured at disposable pencil graphite electrode (PGE) surface in combination with differential pulse voltammetry (DPV) technique and accordingly, the decrease at the guanine signal was evaluated. The biomolecular recognition of APC was successfully achieved with a low detection limit found as 2.35 $\mu g\ mL^{-1}$ by using MB-COOH based assay. Moreover, the selectivity of this aptasensor assay was tested in the presence of numerous proteins and other biomolecules: protein C (PC), thrombin (THR), bovine serum albumin (BSA), factor Va (FVa) and chromogenic substrate (KS).

1. Introduction

Aptamers are nucleic acid ligands that can specifically recognize their targets such as amino acids, drugs, proteins or toxins. They are isolated from a synthetic nucleic acid pool by Systematic Evolution of Ligands by EXponential enrichment (SELEX) [1–7]. There is a growing interest for usage of aptamer in biorecognition area. Thus, fabrication of optic [8–10], piezoelectric [11,12] and electrochemical [13–23] aptasensors that were capable of analyzing of (bio)molecules—especially proteins has been reported in the literature.

Magnetic beads (MBs) are also called as magnetic particles that have extensive application areas related to sensitive recognition of numerous analyte molecules. Some requirements such as colloidal stability, homogenous size distribution, high and uniform magnetite content and having enough surface functional groups are essential for formation of analyte–MB complexes [24]. In addition, simplicity of washing and separation steps is a great advantage of MB-based assays. Also, unspecific adsorptions can be easily removed from MB surface by using effective magnetic separation [19]. Several electrochemical applications based on MB technology for monitoring of proteins [13,15], toxins [17], drugs [19] and nucleic acid hybridization [25,26] have been introduced in the literature. An enzyme-based electrochemical assay by using ochratoxin A (OTA) specific DNA aptamer functionalized paramagnetic microparticle beads (MB) was developed by Bonel et al. [16]. The functionalized MBs were immobilized onto the surface of disposable screen-printed carbon electrodes (SPEs) under a magnetic field, and the production of the enzymatic reaction between horseradish peroxidase and its substrate was detected by using a differential pulse voltammetry (DPV) technique.

A magnetic particle assay has been developed for voltammetric monitoring of interaction between DNA aptamers and their protein targets, thrombin (THR) and lysozyme (LYS) by Erdem et al. [15]. The oxidation signals of THR, LYS and guanine were measured by using pencil graphite electrode (PGE), and the detection limits (DLs) were reported as 10.77 $mg\ mL^{-1}$ (769 nM) and 2.0 $mg\ mL^{-1}$ (54.5 nM) respectively for LYS and THR.

An aptamer based inhibition assay for the electrochemical detection of tobramycin was generated by using a MB-based aptasensor [19]. First, tobramycin molecules were immobilized onto the surface of carboxylated MBs and then, the specific interaction between tobramycin and its biotinylated RNA aptamer was performed. After labeling the biotinylated tobramycin–aptamer complex with a streptavidin–alkaline phosphatase (SALP) conjugate, the enzymatic reaction was performed at the surface

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of SPEs under a magnetic field and the product, α-naphthol, was detected by using DPV. The selectivity of this approach was tested in the presence of kanamycin.

The analysis of proteins has a key importance over the last decade due to their relation with biological pathways such as protein–protein interactions, protein–nucleic acid interactions. They have important roles for the regulation of intercellular and intracellular processes. The importance of human activated protein C (APC), which is a serine protease and the key enzyme of the protein C (PC) pathway, was earlier reported in the literatures [27,28]. APC plays cytoprotective, anti-inflammatory and antiapoptotic roles for protection of endothelial barrier function [27]. APC has three surface loops named as exosites (active sites), the 37-loop [27,29], the 60-loop, and the 70–80 loop, that play an important role for the inactivation of factors Va and VIIIa [29]. Disregulation of PC pathway caused APC resistance which is a lifelong effected process. Microvascular thrombosis in septicemia is contributed by acquired PC deficiency [29]. In addition, the recombinant APC has been used as prospective therapeutic intervention for the purpose of treatment of sepsis [30,31]. According to these information emphasized the regulation of APC, the importance for the development of sensitive and selective detection protocols based on the specific recognition and monitoring of APC has significantly increased day-by-day. Thus, there are several studies for detection of APC in the literature by using conventional methods, such as ELISA [32–34] and fluorometry [35].

No report has been presented yet in the literature for the label free voltammetric detection of human activated protein C (APC) by using single-use aptasensors in combination with magnetic beads assays. For this purpose, two types of magnetic beads (MB), carboxylated and streptavidin coated magnetic beads (respectively, MB-COOH and MB-STR), were used. The APC specific amino linked/biotinylated DNA aptamers (DNA APTs) were immobilized onto the surface of MBs, and then, the interaction between DNA APT and APC was performed. The oxidation signal of guanine was measured by using DPV before and after a surface-confined interaction process. The experimental conditions, such as the concentration of DNA APT and APC, and the interaction time between APC and DNA APT were optimized based on the changes at the guanine signal. In addition, the selectivity of aptasensor was investigated in the presence of several proteins and other biomolecules; protein C (PC), thrombin (THR), bovine serum albumin (BSA), factor Va (FVa) and chromogenic substrate (KS).

2. Experimental

2.1. Apparatus

All experimental measurements were carried by using a AUTOLAB–PGSTAT 302 electrochemical analysis system supplied with GPES 4.9 software package (Eco Chemie, The Netherlands). Differential pulse voltammetry (DPV) was used for electrochemical measurements. All measurements were performed in the Faraday cage (Eco Chemie, The Netherlands). The Savitzky and Golay filter (level 2) of the GPES software was used for treatment of raw data, followed by the moving average baseline correction with a "peak width" of 0.01. The three-electrode system consisted of the pencil graphite electrode (PGE), an Ag/AgCl/3 M KCl reference electrode (BAS, Model RE-5B, W. Lafayette, USA) and a platinum wire as the auxiliary electrode.

2.2. Chemicals

The buffer solutions that were used for experimental procedures are given as follow:

TBS-1: 5 mM Tris–HCl buffer solution containing 20 mM NaCl, pH 7.0.
TBS-2: 10 mM Tris–HCl buffer solution containing 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mg mL^{-1} BSA, pH 7.4.
TBS-3: 20 mM Tris buffer solution containing 150 mM NaCl and 2 mM $CaCl_2$, pH 7.5.
TBS-4: 20 mM Tris–HCl buffer solution containing 20 mM NaCl, pH 7.0.
PBS: 50 mM phosphate buffer solution containing 20 mM NaCl, pH 7.4.
PBSMg: 10 mM phosphate buffer containing 0.1 M NaCl and 10 mM $MgCl_2$, pH 7.4.
ABS: 0.5 M acetate buffer containing 20 mM NaCl, pH 4.8.

The single stranded APC specific DNA aptamer (DNA APT) was purchased from Ella Biotech (Germany). The DNA APT was chosen according to related information given in the literature [36,37].

5′-GCC TCC TAA CTG AGC TGT ACT CGA CTT ATC CCG GAT GGG GCT CTT AGG AGG C-C_6-NH_2-3′ DNA APT was capped 3′ or 5′ end with C_6–NH_2, or 3′ end with C_6-biotin for the experiments performed by using MB-COOH, or MB-STR.

The DNA APT stock solutions (500 μg mL^{-1}) were prepared in ultrapure water and kept frozen. More diluted amino linked DNA APT solutions were prepared in TBS-1 according to the related information given in the literature [15]. More diluted biotin linked DNA APT solutions were prepared in TBS-2 [36].

Human activated protein C (APC) was purchased from Haemtech. Protein C (PC), factor Va (FVa), chromogenic substrate (KS), bovine serum albumin (BSA), thrombin (THR), biotin, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich. The stock solutions of all proteins were prepared using ultrapure water, and kept frozen. More diluted solutions of protein were prepared in TBS-3. Biotin solution was prepared in PBS. The covalent attachment solution was prepared using 10 mM EDC and 10 mM NHS in PBS.

Other chemicals were of analytical reagent grade and they were supplied from Sigma and Merck. Ultrapure and deionized water were used in all solutions.

2.3. Electrode preparation

The disposable pencil graphite electrode (PGE) was used for voltammetric detection of interaction between DNA APT and its cognate protein, APC. A Tombow pencil was used as a holder for each new graphite lead. Electrical contact with the lead was obtained by soldering a metallic wire to the metallic part. The pencil was hold vertically with 14 mm of the lead extruded outside (10 mm of which was immersed in the solution). PGE was pretreated by applying +1.4 V for 30 s in ABS.

2.4. The preparation of DNA APT immobilized MBs and the interaction between DNA APT and APC at the surface of MBs

The preparation of DNA APT immobilized MBs was performed by using a magnetic separator MCB 1200 according to procedures found in the literature [15,38–40].

2.4.1. Preparation of amino linked DNA APT immobilized MB-COOH and its interaction with APC

3 μL of MB-COOH was transferred into a 1.5 mL centrifuge tube. After these particles were washed with 90 μL TBS-1, MBs were incubated in 50 μL of covalent attachment solution for 1 h in order to yield the stable amide linkages. After magnetic separation and washing step with 90 μL of PBSMg, a required amount of

3′ NH_2-DNA APT or 5′ NH_2-DNA APT prepared in 30 μL of TBS-1 was transferred into the vial containing the MB-COOH solution. The incubation of DNA APT was done during 15 min and then it was separated by following the washing step that was performed in 90 μL of PBSMg. DNA APT immobilized MB-COOH was resuspended in a required amount of APC prepared in 30 μL of TBS-3 in order to perform the interaction of aptamer with its target protein, APC, for a required interaction time. After washing step, the alkaline treatment was performed in 25 μL of 0.02 M NaOH solution for 5 min in order to release the APC–DNA APT complex from the surface of MB-COOHs [15].

25 μL of each resulted sample prepared was transferred into the vial containing 85 μL of ABS. 110 μL of each sample was mixed homogenously for 1 min, and then divided into 40 μL. The pretreated PGEs were then dipped into these samples and kept for passive adsorption step for 15 min. The electrodes were rinsed with ABS for 5 s. The PGEs were connected to a three electrode system of electrochemical cell and DPV measurements were accordingly performed.

2.4.2. Preparation of biotinylated DNA APT immobilized MB-STR and its interaction with APC

3 μL of MB-STR was transferred into a 1.5 mL centrifuge tube. After these particles were washed with 90 μL of TBS-4, a required amount of 3′ end biotinylated DNA APT prepared in TBS-2 was transferred into the vials containing MB-STR solution for 15 min. After a magnetic separation, the washing step with 90 μL of PBSMg was followed. Then, the MB-STR was separated and washed with TBS-2. The biotinylated DNA APT immobilized MB-STR was resuspended in 20 μg mL^{-1} biotin for 15 min to block unmodified parts of MB-STR using biotin. Then they were washed in TBS-2 and a required amount of APC prepared in 30 μL of TBS-3 was added into the vials in order to perform the interaction of DNA APT with its target protein, APC, for a required interaction time. After washing step, the alkaline treatment was performed in 25 μL of 0.05 M NaOH solution for 5 min in order to release the APC–DNA APT complex from the surface of MB-STRs [15].

25 μL of each resulting sample prepared was transferred into the vial containing 85 μL of ABS. 110 μL of each sample was mixed homogenously for 1 min, and then divided into 40 μL. The pretreated PGEs were then dipped into these samples and kept for passive adsorption step for 15 min. The electrodes were rinsed with ABS for 5 s. The PGEs were connected to the three electrode system of electrochemical cell and DPV measurements were accordingly performed.

A batch of control experiments were also performed in order to check any signal whether comes from magnetic particles. The MB-COOHs or MB-STRs were prepared following to the procedure itself, and the control measurement was performed voltammetrically in ABS by using PGE.

2.5. Voltammetric transduction

The oxidation signal of guanine was measured by using DPV in ABS scanning from +0.2 V to +1.4 V at the pulse amplitude as 50 mV with the scan rate as 50 mV/s.

3. Results and discussion

3.1. Electrochemical detection of interaction between DNA APT and APC using magnetic particle assay developed by MB-COOH

The concentration dependent behavior of 3′-NH_2-DNA APT at the surface of MB-COOH upon the response was first investigated. The representative voltammograms and histograms are respectively shown in Fig. 1A and B. It was shown that the guanine signal sharply increased while 3′-NH_2-DNA APT concentration increased, and then it levelled off at concentration level of 100 μg mL^{-1}. According to the three repetitive measurements by using 100 μg mL^{-1} DNA APT, the average guanine signal was found as 1554.5 ± 125.2 nA with a relative standard deviation % (R.S.D. %) as 8.1%. Thus, 100 μg mL^{-1} 3′-NH_2-DNA APT was chosen as the optimum concentration for further studies.

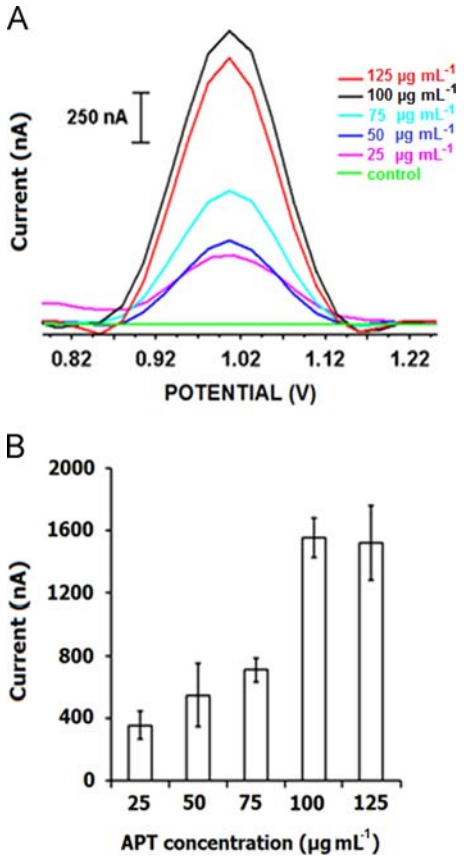

Fig. 1. The effect of DNA APT concentration upon response: (A) voltammograms and (B) histograms representing the guanine oxidation signal after immobilization of 3′ NH_2-DNA APT in different concentrations ranging from 25 to 125 μg mL^{-1} (n=3). Control experiment done in ABS.

The effect of the linkage side at DNA APT immobilization onto the surface of MB-COOH was also investigated by using 100 μg mL^{-1} 5′ or 3′ end amino linked DNA APT. The results were shown in Online Resource 1. When DNA APT was capped with amino group at the 3′ end, the highest guanine signal was recorded. It was concluded that 3′ end amino linked DNA APT could successfully bind onto the surface of MB-COOH. On the other hand, 5′ end amino linked DNA APT could not bind effectively onto the surface of MBs due to steric hindering of APT structure [36]. Because of this reason, the guanine signal was measured very lower (Online Resource 1-I and II-a) compared to the one obtained by using 3′-NH_2-DNA APT.

Next, the DNA APT interaction with APC in the protein concentration ranging from 5 to 25 μg mL^{-1} was performed by using different interaction durations: 5 and 15 min. Fig. 2 shows the changes at the guanine signals measured before and after aptamer interaction with APC for 5 min (Fig. 2A) and 15 min (Fig. 2B). According to these results, the DNA APT interaction with APC definitely is depended on APC concentration and also, the interaction time. In the case of interaction for 5 min, the changes at the guanine signal were not found as remarkable compared to the ones obtained by using 15 min interaction time. The guanine signal

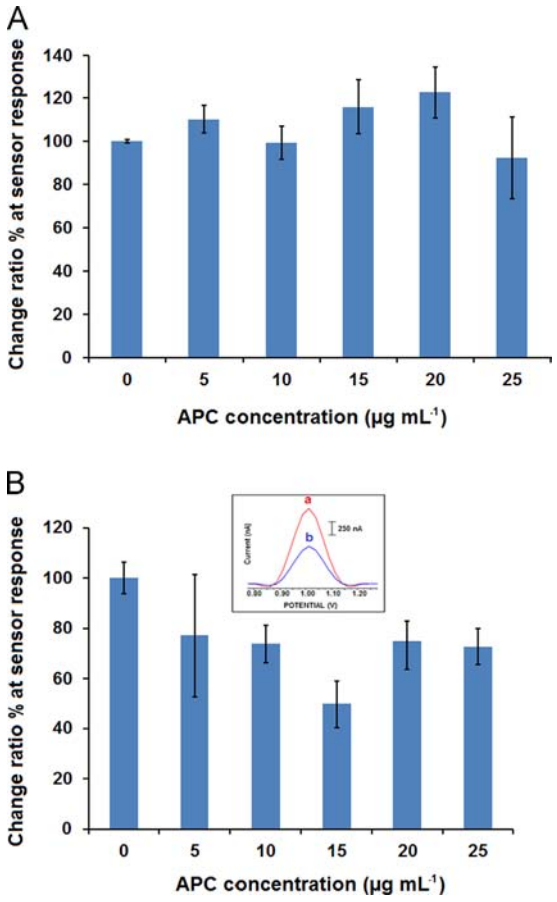

Fig. 2. Histograms representing the response related to the change ratio % at the guanine signal obtained before and after 100 μg/mL^{-1} DNA APT interaction with APC in different concentrations from 5 to 25 μg mL^{-1} by using (A) 5 min and (B) 15 min interaction time (n=3). Inset of (B): voltammograms representing the guanine signals measured before (a) and after (b) DNA APT interaction with 15 μg mL^{-1} APC.

decreased as 50.3% after DNA APT interaction with 15 μg mL^{-1} APC during 15 min (Fig. 2B) with an average response as 728.3 ± 137.7 nA (R.S.D. %=18.9%, n=3). The calibration plot was shown in Online Resource 2 based on guanine signal before and after interaction of APC from 0 to 25 μg mL^{-1}. The detection limit (DL) was calculated according to the method of Miller and Miller [41] with a regression equation $y=-46.22x+1587.9$ and coefficient of determination (R^2)=0.9855, and it was found as 2.35 μg mL^{-1}.

The selectivity of APC aptasensor based assay is then tested in the presence of numerous proteins and other biomolecules, such as, protein C (PC), thrombin (THR), bovine serum albumin (BSA), factor Va (FVa) and chromogenic substrate (KS) and the changes % at guanine signal [23] are presented in Fig. 3. The average guanine signal was found as 612.3 ± 113.1 nA (% R.S.D.=18.5%, n=3) after interaction between DNA APT and 15 μg mL^{-1} APC. In the case of DNA APT interaction with 15 μg mL^{-1} of selected proteins and biomolecules APC, PC, BSA, THR, FVa or KS, % decrease was recorded respectively 54.2%, 37.2%, 38.4%, 40.8%, 9.6% and 35.4%. Concerning the results, the voltammetric aptasensor combined with magnetic beads assay was found not only very sensitive, but also a selective recognition for human activated protein C.

3.2. *Electrochemical detection of interaction between DNA APT and APC using magnetic particle assay developed by MB-STR*

The optimization of experimental parameters of the MB-STR based assay was performed first followed by the same experimental steps of the MB-COOH based assay. The concentration dependent

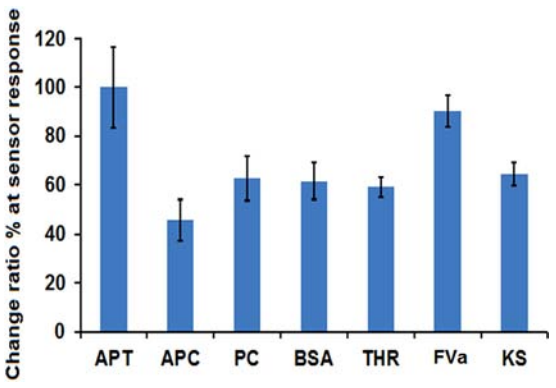

Fig. 3. Histograms representing the response related to the change ratio % at the guanine signal measured before and after 100 μg mL^{-1} DNA APT interaction with 15 μg/mL of APC, or numerous proteins and other biomolecules (n=3).

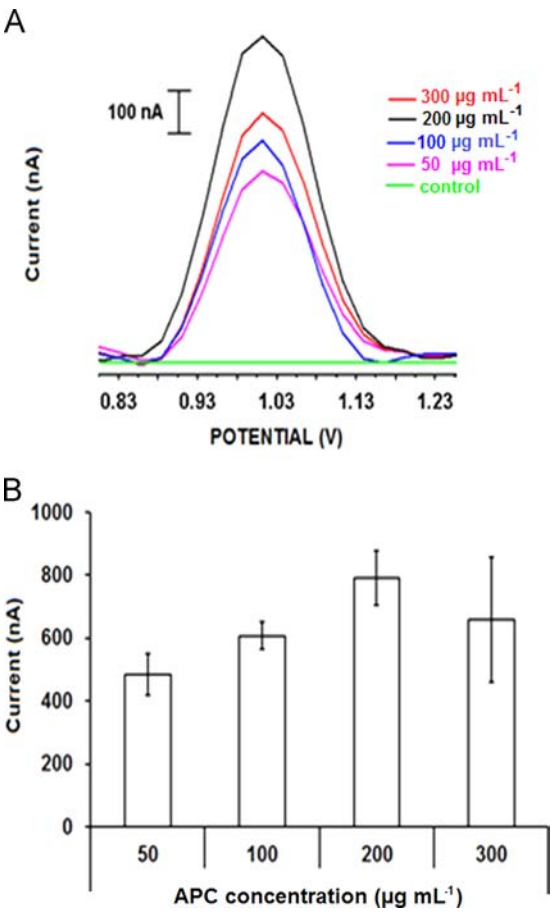

Fig. 4. The effect of DNA APT concentration upon response: (A) voltammograms and (B) histograms representing the guanine oxidation signal after immobilization of 3′ biotinylated DNA APT in different concentrations ranging from 50 to 300 μg mL^{-1} (n=3). Control experiment done in ABS.

behavior of 3′ biotinylated DNA APT immobilized onto the surface of MB-STR was investigated based on the guanine signal by using DPV (Fig. 4). The guanine signal gradually increased while aptamer concentration increased, and then it levelled off at 200 μg mL^{-1} DNA APT concentration. The average guanine signal was found as 790.5 ± 86.3 nA with R.S.D. %=10.9% (n=4) at 200 μg mL^{-1} DNA APT concentration, and thus it was chosen as the optimum one for further studies.

The interaction between 200 μg mL^{-1} DNA APT and APC in the different concentration levels of protein ranging from 25 to

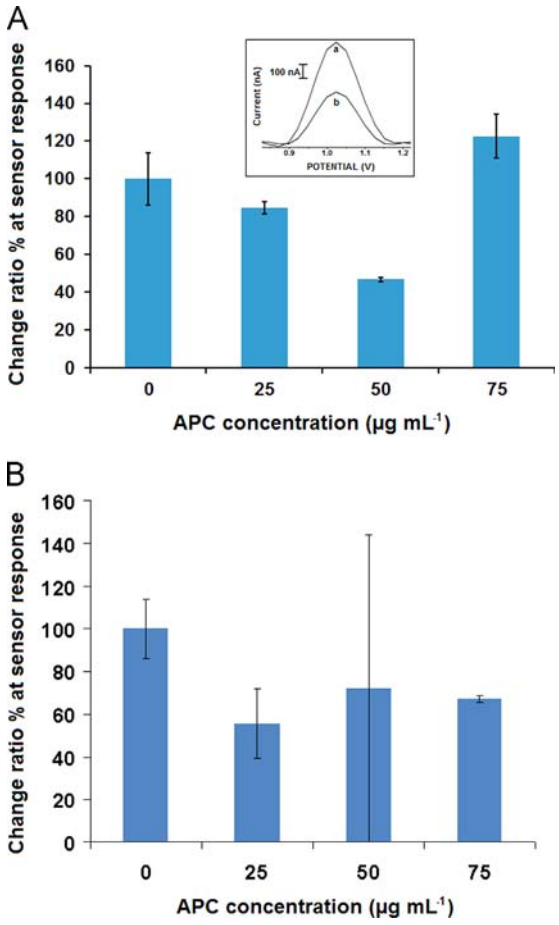

Fig. 5. Histograms representing the response related to the change ratio % at the guanine signal obtained before and after interaction between 200 μg mL^{-1} DNA APT and APC in different concentrations from 5 to 25 μg mL^{-1} by using (A) 5 min and (B) 15 min interaction time (n=3). Inset of (A): voltammograms representing the guanine signals measured before (a) and after (b) DNA APT interaction with 50 μg/mL APC.

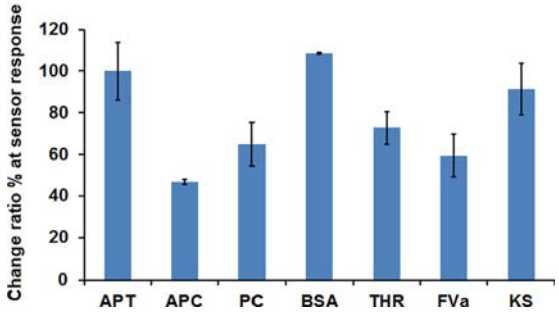

Fig. 6. Histograms representing the response related to the change ratio % at the guanine signal measured before and after 200 μg mL^{-1} DNA APT interaction with 50 μg mL^{-1} of APC, or numerous proteins and other biomolecules (n=3).

100 μg mL^{-1} was also analyzed by using 5 and 15 min interaction time (shown in Fig. 5). Concerning to the changes at the guanine signal, it was shown that the behavior of DNA APT–APC interaction is significantly related to two main experimental parameters: APC concentration and interaction time. After DNA APT interaction with APC in the different concentrations of protein by using 5 min interaction time, the changes at the guanine signal was found more remarkable with a better reproducubility compared to the ones obtained by using 15 min interaction time. A decrease % at the guanine signal was about 53.2% after DNA APT interaction with 50 μg mL^{-1} APC in 5 min (Fig. 5A) with an average guanine signal as 368.3 ± 9.3 nA (R.S.D. %=2.5%, n=3).

Further, the selectivity of our assay combined with MB-STR for APC detection is tested in the presence of protein C (PC), thrombin (THR), bovine serum albumin (BSA), factor Va (FVa) and chromogenic substrate (KS) and the changes % at guanine signal [23] are presented in Fig. 6. The average guanine signal was found as 368.3 ± 9.3 nA with a R.S.D. %=2.5%, (n=3) after interaction between 200 μg mL^{-1} DNA APT and 50 μg mL^{-1} APC. In the case of aptamer interaction with 50 μg mL^{-1} of APC, PC, THR, FVa or KS, % decrease ratio at guanine signal was found respectively as 53.2%, 35.1%, 27.2%, 40.6% and 8.6%, whereas a 8.7% increase ratio at response was obtained in the presence of aptamer interaction with BSA. These results claimed that our electrochemical assay combined with MB-STR was capable of sensitive and selective recognition of APC in the presence of APC selective DNA aptamer.

4. Conclusion

A sensitive and selective voltammetric single-use aptasensor technology based on magnetic beads assay was progressed for the first time in the present study for monitoring of human activated protein C (APC), which is a serine protease (i.e., key enzyme of the protein C pathway). Different types of magnetic beads (MB), carboxylated and streptavidin coated magnetic beads (respectively, MB-COOH and MB-STR), were used herein and consequently, an amino linked DNA APT and a biotinylated DNA APT were immobilized individually onto the surface of MB-COOH and MB-STR, respectively. According to our records obtained herein, more sensitive detection of APC-aptamer interaction was performed at the surface of MB-COOH and consequently, the voltammetric detection of APC was successfully achieved with a low detection limit as 2.35 μg mL^{-1}. Moreover, the selectivity of both aptasensor technologies combined with MB-COOH and MB-STR was tested. Under the optimum conditions, the selective aptasensor responses using these magnetic beads assays were recorded against numerous proteins, which were assessed in the prothrombotic and hyperinflammatory pathways [36,37]: protein C (PC), thrombin (THR), bovine serum albumin (BSA), factor Va (FVa) and chromogenic substrate (KS).

Selective biorecognition of proteins has been one of the important topics in biochemistry since many years. Researchers have fabricated various detection platforms to monitor the proteins and to recognize them by using different biorecognition elements, antibodies. Concerning to advantages of aptamers, such as having stabile structure, high affinity and specificity to their target molecules, they are useful candidates for the fabrication of aptasensing platforms, which are combination of MBs and single use graphite electrodes [15]. Although APC analysis was performed by using a magnetic bead based flourimetric assay in the literature [35], the single-use electrochemical aptasensor developed in this study has presented many advantages due to electrochemical techniques possess an accurate, reliable and fast analysis protocol, which are essential for the development of aptasensor technologies.

As a conclusion, these magnetic beads assay combined with single-use aptasensor technology has achieved selective and sensitive biorecognition of human activated protein C (APC). We expect that this label-free electrochemical aptasensor technology can be further progressed as a device after miniaturization in order to bed-side diagnosis of APC in the future.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.04.082.

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