Full Paper

Electrochemical Sensing of Aptamer-Protein Interactions Using a Magnetic Particle Assay and Single-Use Sensor Technology

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Abstract
A magnetic particle assay has been designed herein that can report the interactions of DNA aptamers with their cognate protein targets lysozyme (LYS) and human thrombin (THR). Electrochemical sensing of the biomolecular recognition between each aptamer and its target was explored by using a disposable graphite electrode, PGE, in combination with differential pulse voltammetry (DPV). The magnitudes of the oxidation signals of LYS and THR were measured at +780 mV and +680 mV, respectively, after interaction with the cognate aptamers attached to the surface of magnetic particles. The detection limits estimated for signal to noise ratios above 3.0 correspond to the concentrations of 10.77 μg/mL LYS (769 nM) and 2.00 μg/mL THR (54.5 nM). Our aptamer based approach that combines magnetic particles with a disposable graphite electrode performs well compared to other aptamer-based sensor-formats for quantitative protein detection with respect to sensitivity, selectivity, detection limit, and reproducibility.

Keywords: Aptamers, Proteins, Aptamer–protein interactions, Magnetic particles, Lysozyme, Thrombin

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1. Introduction

Aptamers are short single stranded nucleic acids, that bind their target molecules with high affinity and specificity comparable and sometimes superior to antibodies [1, 2]. The increasing demand for aptamers encouraged the development of automated high-throughput aptamer generation protocols. Various target molecules have been used for the identification of aptamers, including amino acids, drugs, RNA molecules, proteins and even living cells, that can be screened through systematic evolution of ligands by exponential enrichment (SELEX) process [3–7].

Aptamers represent nucleic acid based receptor molecules which can be considered as a highly versatile sensing element for the development of various types of biosensors or aptamer-based sensor arrays [8–13]. Among these, the specific and sensitive detection of aptamer-protein interactions and the development of different assay formats in combination with electrochemical sensor technology are of growing interest [14–23].

We have previously developed a prototype love-wave sensor for the quantitative measurements of alpha-human thrombin and the HIV-1 Rev peptide with a detection limit of approximately 75 pg/cm^2 [8], and the RNA/DNA aptamer coated surface acoustic wave (SAW) sensor for investigating the binding of heparin and antithrombin III to immobilized thrombin [9]. The signaling of aptamer-protein interaction was monitored by Jiang et al. [10] who used the luminescence signal change of the complex [Ru(phen)$_2$(dppz)$^{2+}$] resulted from its intercalation into a DNA aptamer as a function of the interaction between the aptamer and its target protein. In addition, a highly sensitive and selective simultaneous bioelectronic detection of several protein targets, namely lysozyme and thrombin, was explored by coating of each aptamer on an inorganic nanocrystal [19]. This resulted in a multi-analyte electrochemical biosensor based on quantum-dots coated with aptamers with a detection limit in the attomolar range.

Numerous materials, such as magnetic particles, nanoparticles, nanotubes, nanowires or various metal oxide films etc. have all been demonstrated capable of label-free electronic detection of small biomolecules, nucleic acids, and proteins. Thus, their specific properties offer an excellent prospect for biological recognition surfaces to develop highly selective and sensitive electrochemical sensor technology [24–46].

The electrochemical detection of nucleic acids based on a magnetic assay [35–44] was performed by labeling DNA with an enzyme [42–44], or by using label free system [35–38, 46, 47], or combining this magnetic assay with metal nanoparticles [39, 44]. These studies have proven the magnetic assays as a promising approach for the sequence specific DNA detection, that combines exceedingly low

detection limits with the advantage of the efficient magnetic separation.

Here, a magnetic particle assay was described for the electrochemical detection of proteins. We employed two different DNA aptamers for electrochemically monitoring their interaction with their cognate target proteins, namely the 129 amino acids long globular protein lysozyme (LYS) and the proteolytic enzyme thrombin (THR). Lysozyme is abundant in a large variety of biological fluids, and has been shown to serve as a diagnostic marker of leukaemia when detected in human urine. LYS also exhibits antiviral activity, and has been discussed as a potential marker for rheumatoid arthritis [48]. Thrombin is a highly specific serine protease and it is considered as a useful tumor marker in the diagnosis of pulmonary metastasis [49].

We explored herein the electrochemical sensing of the molecular recognition between each aptamer and its target protein LYS or THR, respectively, based on the oxidation by using a disposable graphite electrode, PGE, in combination with differential pulse voltammetry (DPV). Compared with other studies and detection systems, this novel biosensor format performs well with respect to detection limit, sensitivity, selectivity and reproducibility.

2. Experimental

2.1. Apparatus

The oxidation signals of lysozyme (LYS), thrombin (THR) and guanine were measured by using differential pulse voltammetry (DPV) with an AUTOLAB – PGSTAT 302 electrochemical analysis system and GPES 4.9 software package (Eco Chemie, The Netherlands). The raw data were treated using the Savitzky and Golay filter (level 2) of the GPES software, 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/KCl reference electrode and a platinum wire as the auxiliary electrode.

2.2. Chemicals

The biotinylated ssDNA aptamer and oligodeoxynucleotide were purchased from Metabion International AG (Germany).

Anti-lysozyme ss DNA aptamer (DNA-APT 1)

5′-biotin-ATC TAC GAA TTC ATC AGG GCT AAA GAG TGC AGA GTT ACT TAG

Anti-thrombin ssDNA aptamer (DNA-APT 2)

5′-biotin-GGT TGG TGT GGT TGG

ssDNA oligodeoxynucleotide (Random DNA-1)

5′-biotin-TTT TTT TTT CGA TCG AG-3′

DNA-APT1 and DNA-APT-2 were chosen according to related information given in the literature [9, 14]. Since the spacer in aptamer is required for optimal binding of thrombin [50], there have been used here a spacer length of twelve carbon atoms between the 5'-phosphate and the biotin moiety in aptamer.

The stock solutions of DNA aptamers and oligodeoxynucleotide were prepared with fresh ultrapure triple-distilled water and kept frozen. The diluted solutions of the aptamers and oligodeoxynucleotide were prepared with 5 mM Tris-HCl buffer supplemented with 20 mM NaCl (TBS, pH 7.00). Other chemicals were supplied from Sigma (USA) and Merck (Germany) in analytical reagent grade. Streptavidin coated magnetic particles in 0.94 μm diameter size were purchased from Estapor, Merck (France).

Lysozyme (LYS), thrombin (THR) and bovine serum albumin (BSA) were purchased as lyophilisates from Sigma. The stock solutions were prepared by dissolving the lyophilisates in fresh ultrapure triple-distilled water and they were stored at $-20\,^{\circ}C$. The diluted solutions of proteins were prepared in 50 mM phosphate buffer solution (PBS, pH 7.40)

2.3. Electrode Preparation

The disposable graphite electrode (PGE) was used in voltammetric measurements for the electrochemical detection of DNA hybridization. A Tombo 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 $+1.40$ V for 30 s in 0.5 M acetate buffer containing 20 mM NaCl (ABS, pH 4.80).

2.4. Preparation of ssDNA Aptamer-coated Magnetic Particles and Protein Accumulation onto the Surface of Particles

The preparation of aptamer coated magnetic particles was performed by using a magnetic separator MCB 1200 according to procedures found in the literature [14, 35, 36, 46]: 3 μL of streptavidin-coated magnetic particles were transferred into a 1.5 mL centrifuge tube. After these particles were washed with 50 μL TBS, they were resuspended in 10 μL of the same buffer solution. 15 μL of individual aptamers (in a required amount) were added and incubated for 15 min at room temperature by gentle mixing. The immobilized probe was separated and washed with 50 μL 10 mM phosphate buffer containing 0.1 M NaCl and 10 mM $MgCl_2$ (NaMgPBS, pH 7.40), and then resuspended in 50 μL PBS containing the desired amount of LYS or THR, respectively. The accumulation reaction was carried out for 15 min at room temperature. The hybridized magnetic particles were then washed with 50 μL of PBS and resuspended in 25 μL of 0.02 M NaOH solution for alkaline treatment and incubated for 5 min. After the alkaline treatment step in order to release the captured protein from the surface of particles, 25 μL of resulted sample was

transferred into the vial containing 85 μL 0.50 M ABS. Pretreated PGEs were then dipped into these vials and kept for adsorption step during 15 min. The electrodes were rinsed with ABS for 5 s. The PGEs were connected to three electrode system of electrochemical cell containing only ABS buffer for DPV measurement.

2.5. Voltametric Transduction

The oxidation signals of protein and guanine were measured by using DPV in ABS by scanning from −0.10 to +1.40 V at the pulse amplitude as 50 mV and the scan rate as 30 mV/s.

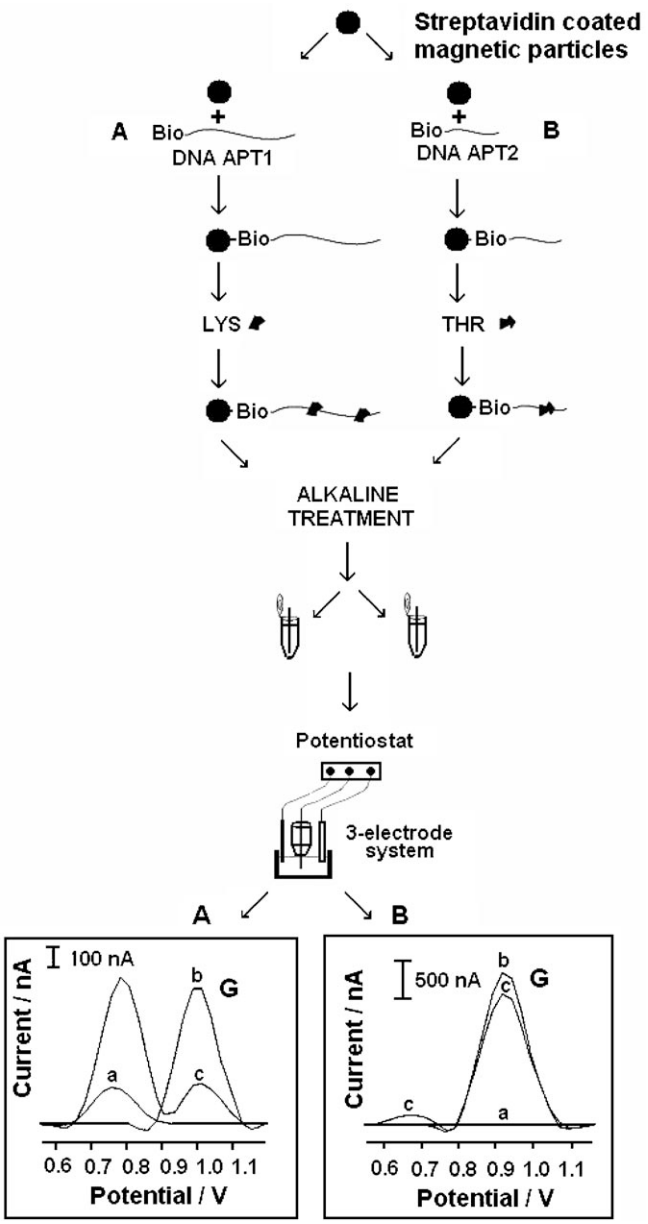
3. Results and Discussion

After the magnetic assay (Scheme 1) was performed the electrochemical behavior of the DNA aptamers and their specific target proteins, LYS and THR, were explored by using PGEs and DPV technique.

3.1. Electrochemical Detection of Lysozyme

The electrochemical detection of the biotinylated DNA aptamer-1 (DNA APT-1) immobilized on magnetic particles was carried out by measuring the guanine oxidation signal at +1.03 V in the absence of LYS. As shown in scheme 1Ab to c, the decreased guanine signal (as about 78%) was detected in the presence of LYS. The decrease at the guanine oxidation signal may be attributed to the interaction of lysozyme with the guanine nucleobases of the DNA aptamer. Similarly to our results, Rodriguez et al. [51] also reported a decrease at the magnitude of guanine signal. It was explained by this group that the structural changes occurred in the aptamer after the interaction with lysozyme pockets could be responsible for this decrease, since the aptamer preferentially interacts with the pockets or clefts on proteins and it can produce important structural changes in the aptamer as well as in the protein.

The changes of the LYS oxidation signal were monitored electrochemically in the presence and absence of the LYS-specific DNA aptamer. The voltammetric signals of LYS after the interaction between LYS and DNA APT-1, are shown in Scheme 1A. The oxidation signals of LYS before and after interaction with aptamer were measured at ca. +0.78 V (Scheme 1Aa and c). Additionally, another small and not reproducible oxidation signal of LYS was observed at ca. +1.28 V (not shown), that is overlapping the peak potential of DNA base, adenine observed at +1.2 V. For that reason, we focused our analysis to monitor the changes at the LYS signal only observed at +0.78 V. Due to the strong binding of LYS to the DNA aptamer, an increase in the magnitude of the LYS oxidation signal by ca. 3.5 times was observed (Scheme 1Aa to c) in the presence of the DNA APT-1.



Scheme 1. Electrochemical sensing of biomolecular recognition between each aptamer and its target protein: A) lysozyme (LYS) and B) thrombin (THR) following steps: the immobilization of biotinylated DNA aptamers (DNA APT-1 and DNA APT-2) onto streptavidin-coated magnetic particles; the interaction of LYS or THR with the their aptamers at the surface of magnetic particles; alkaline treatment step; wet-adsorption procedure and measurement for both oxidation signals of proteins and guanine bases (G) of DNA aptamers by using differential pulse voltammetry (DPV): The oxidation signals of each protein and guanine measured in the presence of a: protein alone; b: DNA aptamer alone; c: after interaction of DNA aptamer with its target protein.

The selectivity of LYS binding to DNA aptamer-1 was also analyzed in the presence of a biotinylated random DNA sequence (17-mer bases) that does not bind lysozyme and subsequently, no LYS signal was obtained ca. +0.78 V (Fig. 1Aa). In the absence of LYS, the signals were analyzed coming from DNA APT-1 and also, a buffer solution-ABS in

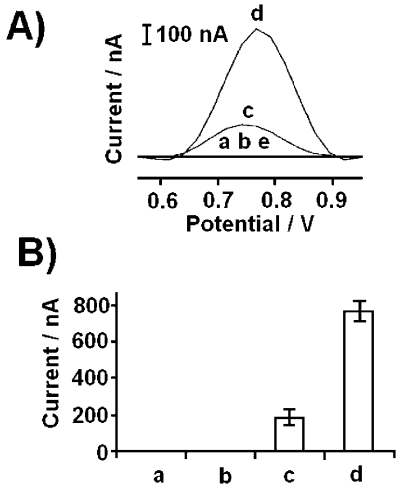


Fig. 1. A) Differential pulse voltammograms and B) histograms for the relative oxidation signals of lysozyme (LYS) obtained in the presence of a) biotinylated random DNA (no aptamer) and LYS; b) DNA aptamer-1 in the absence of LYS; c) LYS in the absence of DNA aptamer-1; d) DNA aptamer-1 and LYS, and e) blank signal (shown only in the voltammogram).

a control study, and the results were correspondingly shown in Figure 1A-b and Figure 1Ae.

The histograms presenting the relative oxidations signals of LYS were also shown in Figure 1B. After LYS interaction with its DNA APT-1, a higher magnitude increase (ca. 3.5 times) was observed at LYS oxidation signal due to the strong binding of the protein to its DNA aptamer in good reproducibility.

A series of three repetitive DPV signals obtained with 500 μg/mL and 200 μg/mL respectively of LYS and DNA aptamer-1 at PGE surface resulted in reproducible results namely a mean response of 766 nA for LYS with a relative standard deviation of 8.6% ($n=3$).

The selectivity of DNA APT-1 checked in the presence of its target protein, LYS or as a negative control protein, BSA. While a well-defined oxidation signal of LYS is measured at ca. +0.78 V in the presence of the DNA APT-1 and LYS (shown in Fig. 2b), a negligible small response is observed with BSA measured at ca. +0.68 V (shown in Fig. 2c). To investigate the specificity of DNA APT-1, we performed further experiments. In the presence of a mixture sample containing LYS:BSA in a molar ratio of 1:1 (Fig. 2d), we found that the binding of LYS to DNA APT-1 can be performed successfully even in the presence of BSA.

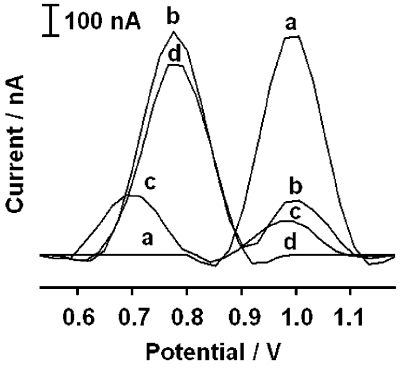


Fig. 2. Differential pulse voltammograms for the relative oxidation signals of protein and guanine after the interaction of DNA aptamer-1: in the absence of a) only lysozyme (LYS) and in the presence of b) LYS, 500 μg/mL, c) BSA, 500 μg/mL, d) a mixture sample containing 500 μg/mL of LYS and BSA.

The changes at the magnitude of guanine signals were monitored before/after interaction and shown in Figure 2a to d. A decrease of the guanine signal was obtained after any interaction of aptamer with LYS or BSA (shown respectively in Fig. 2b and 2c) and as a result, the decrease ratio was found respectively as 78% and 82% in contrast to the guanine signal from the DNA aptamer in the absence of protein (Fig. 2a). When using mixture samples containing LYS and BSA, the guanine signal completely disappeared (Fig. 2d) due to specific and strong binding of LYS to DNA APT-1. These results demonstrate that the magnetic assay combined with single-use graphite sensor architecture developed for aptamer-protein interaction is highly selective.

The concentration dependence of the magnitude of the LYS signal after its interaction with the DNA APT-1 was also monitored, and shown in Figure 3. Well defined LYS signals were observed in the range of 100 μg/mL to 1000 μg/mL employing a 15 min interaction time with DNA APT-1. The LYS signal increased linearly from 100 μg/mL to 1000 μg/mL and leveled off after further increasing the concentration of lysozyme (data not shown).

The limit of detection (DL) of LYS was calculated with the aid of the section of the calibration plot close to the origin, which is linear, utilizing both the regression equation $y = 6.27x + 67.5$, $R^2 = 0.97$, as outlined in reference [52]. The detection limit (DL) of LYS was found to be 10.77 μg/mL (equal to 769 nM).

Earlier studies reported the development of aptasensors by using different transducers, e.g., indium tin oxide electrode [18], or labeling of the aptamers with quantum dots [19]. The approach explored in our study for the detection of the interaction between LYS and aptamer represents a simple, cheap, and fast alternative method with reliable and selective results by the advantage of efficient magnetic separation. The detection limit of LYS

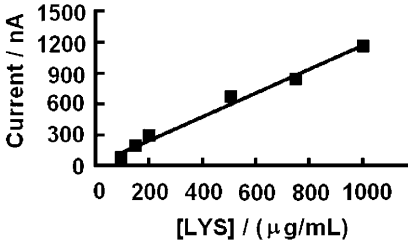


Fig. 3. Calibration plot showing the lysozyme (LYS) oxidation signal obtained by using DNA aptamer-1 immobilized onto the surface of particles in various concentrations of LYS from 100 to 1000 μg/mL. The conditions as in Fig. 1.

is in the same range as observed in other approaches [14, 51].

3.2. Electrochemical Detection of Thrombin

The electrochemical detection of a biotinylated anti-THR DNA aptamer-2 (DNA APT-2) immobilized on the surface of magnetic particles was carried out by first measuring the magnitude of the guanine oxidation signal in the absence of THR. The guanine signal was slightly decreased after specific binding of THR to DNA APT-2 (Scheme 1Bb to c).

The changes of the THR oxidation signal were also monitored electrochemically in the same voltammetric scale before/after interaction between THR and aptamer. After the interaction between THR and DNA APT-2, the oxidation signals of THR were shown in scheme 1Ba to c. Before and after interaction of THR with its DNA aptamer, the oxidation signals of THR were measured at ca. +0.7 V. Due to the strong binding of THR to DNA aptamer-2, a high magnitude increase (shown in scheme 1Bc) was observed at THR oxidation signal after its interaction with DNA aptamer-2 in comparison to the one obtained in the absence of aptamer (shown in scheme 1B-a). By means of the specific and strong binding of Thrombin to the T–T loop in the G-quadruplex conformation of DNA aptamer [21], an increase of the THR signal was measured in this system in contrast to a gradual decrease of the guanine signal using the double-surface technique based on magnetic particles.

The binding selectivity of THR was investigated in the presence of the biotinylated random DNA sequence-1 (17-mer bases) and no THR signal was obtained (data not shown).

The histograms of the relative oxidations signals of THR and guanine are shown in Figure 4. After THR interaction with DNA aptamer-2, an increase at THR signal was observed attributable to the strong binding of THR to its aptamer. For a control experiment, the signals derived from DNA APT-2 and ABS solution were also analyzed in the absence of THR (Figure 4A and B).

A series of three repetitive DPV signals obtained with 20 μg/mL and 200 μg/mL, respectively, of THR and DNA APT-2 at PGE surface resulted in reproducible results namely a mean response of 493 nA for THR with a relative standard deviation of 9.5% ($n=3$).

The selectivity of DNA APT-2 was also analyzed in the presence of its target protein THR or a negative control protein, e.g, BSA. While a well-defined THR signal is measured at ca. +0.68 V for the aptamer/THR-complex (Fig. 5a), almost no signal was observed with DNA APT-2 in the presence of BSA (Fig. 5b). When there is a large excess of BSA in the mixture sample containing THR: BSA in ratio 1:2 (Fig. 5c). The binding of THR to DNA APT-2 was still reliably detected even though there are present unwanted constituents; such as, BSA by the advantages of this magnetic assay.

According to the changes at the guanine signals observed at +1.03 V, the interaction was also monitored, and then the

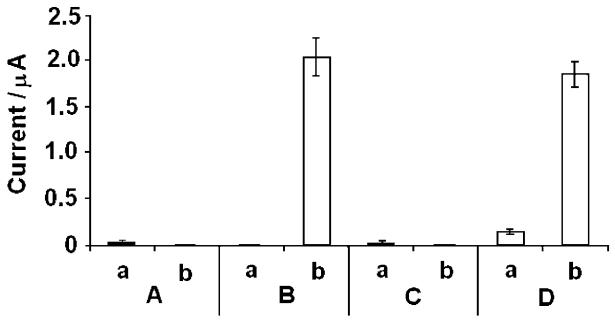


Fig. 4. After the interaction between DNA aptamer-2 and Thrombin (THR), the relative oxidation signals of THR (a) and guanine (b) are shown in the histograms. A) in the absence of aptamer and THR, B) in the presence of only aptamer, C) THR in the absence of aptamer, and D) in the presence of THR and aptamer.

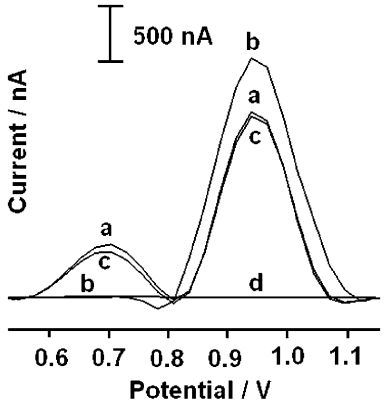


Fig. 5. The relative oxidation signals of THR and guanine presented in the voltammograms after the interaction between DNA aptamer-2 and protein: In the presence of a) THR, 50 μg/mL, b) BSA 50 μg/mL, c) a mixture sample containing both THR and BSA (respectively, 50 and 100 μg/mL), and d) blank signal in the absence of aptamer and THR.

results were presented in Figure 5a to d. When the interaction was performed in the mixture sample containing THR:BSA (1:2), guanine signal was slightly decreased after the specific binding of THR to DNA APT-2 (shown in Fig. 5c) even though a double amount of unwanted constituent, BSA is co-existing in the sample.

Figure 6 shows the histograms representing the relative oxidation signals of proteins and guanine before/after the interaction between DNA aptamer and protein.

The magnitude of THR signals after its interaction with DNA APT-2 was monitored in various THR concentrations (Figure 7). Well defined THR signals were measured between the range of 10 to 100 μg/mL concentration range using 15 min interaction time with DNA APT-2. The THR signal linearly increased from 10 to 100 μg/mL and leveled off after 100 μg/mL THR concentration (not shown).

The detection limit (DL) of THR was calculated according to the procedure given by McCreery [52] utilizing the regression equation $y=1.16x+12.5$, $R^2=0.98$, and it was found as 2.00 μg/mL (equals to 54.5 nM).

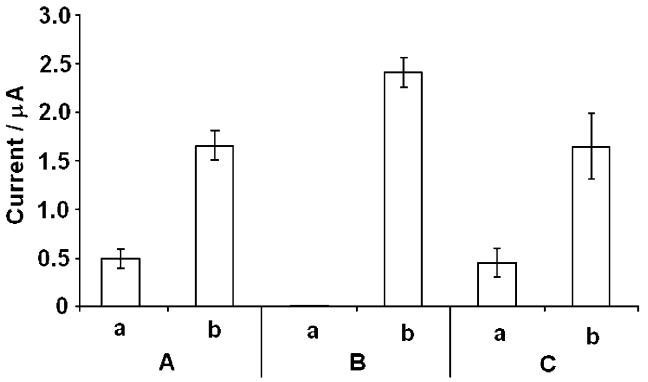


Fig. 6. Histograms representing the relative oxidation signals of a) protein and b) guanine after the interaction between DNA aptamer-2 and protein: In the presence of A) THR and aptamer, B) BSA and aptamer, and C) a mixture sample containing both THR and BSA.

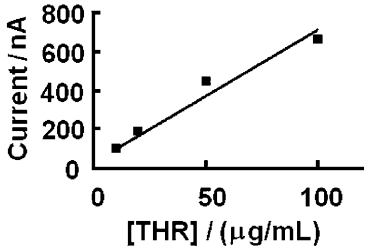


Fig. 7. Calibration plot showing the THR oxidation signal obtained by using DNA aptamer-2 immobilized onto the surface of particles in various concentrations of THR from 10 to 100 μg/mL. The conditions as in Fig. 4.

Similar to earlier methods [13, 21–23], the interaction between THR and its DNA aptamer was detected with high sensitivity and selectivity. Measuring the changes of the THR oxidation signal avoided the use of indicator tagged oligonucleotides, a necessity in other approaches that used, for example, methylene blue as an electroactive tag [21], or streptavidin labeled with peroxidase [22], or alkaline phosphatase [13]. Our electrochemical assay thus can be performed in a short time without using any advanced surface chemistry or external indicators [15].

4. Conclusions

Magnetic beads assays have previously been performed for aptamer–protein interactions [13, 14, 17]. In our study, aptamer-based magnetic bead assays have successfully been implemented in combination with single-use graphite electrodes (PGEs). The detection of the interaction between the anti-thrombin-aptamer and its target was explored for the first time in our study by measuring the oxidation signals of THR and an electroactive base, guanine in the same voltammetric scale. The magnetic assay worked also well in the second application, which we detected in the interaction of the anti-lysozyme DNA aptamer with LYS. Our method allowed for quantifying both interactions in a label-free fashion without using any indicator tagged oligonucleotide, or enzyme.

The combination of magnetic bead assays with disposable PGEs is advantageous to similar assays with respect to reproducibility, cost, and ease of implementation (single-use, portable detection system), which are important properties of devices applied in chip technology compared to other transducers such as gold-, or indium tin oxide electrodes.

We expect that a wide range of proteins and other small molecules could be monitored using the magnetic assay combined with aptasensor technology, and thus, it may possibly open a challenging direction for further applications in proteomics and diagnostics by the development of aptamer-based electronic devices based on nanotechnology.

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