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Short communication

Aptamers based electrochemical biosensor for protein detection using carbon nanotubes platforms

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ABSTRACT

A label-free bioelectronic detection of aptamer–thrombin interaction based on electrochemical impedance spectroscopy (EIS) technique is reported. Multiwalled carbon nanotubes (MWCNTs) were used as modifiers of screen-printed carbon electrotransducers (SPCEs), showing improved characteristics compared to the bare SPCEs. 5′amino linked aptamer sequence was immobilized onto the modified SPCEs and then the binding of thrombin to aptamer sequence was monitored by EIS transduction of the resistance to charge transfer (Rct) in the presence of 5 mM $[Fe(CN)_6]^{3-/4-}$, obtaining a detection limit of 105 pM. This study represents an alternative electrochemical biosensor for the detection of proteins with interest for future applications.

1. Introduction

Aptamers hold great promise for rapid and sensitive protein detections and for developing protein arrays (Mukhopadhyay, 2005). These synthetic nucleic acid sequences act as antibodies in binding proteins owing to their relative ease of isolation and modification, holding a high affinity and high stability (Jayesna, 1999; Hansen et al., 2006).

In the last years, there has been a great interest for developing aptasensors. Different transduction techniques such as optical (McCauley et al., 2003; Zhang et al., 2009; Pavlov et al., 2005), atomic force microscopic (Basnar et al., 2006), electrochemical (Radi et al., 2005; Zayats et al., 2006; Suprun et al., 2008) and piezoelectrical (Pavlov et al., 2004) have been reported. Aptamer based biosensors have a great promise in protein biosensing due to their high sensitivity, selectivity, simple instrumentation, portability and cost effectiveness (Minunni et al., 2005; Baker et al., 2006).

It is well-known that the sequence-specific single-stranded DNA oligonucleotide 5′-GGTTGGTGTGGTTGG-3′ (thrombin aptamer) acts as thrombin inhibitor. This thrombin aptamer binds to the anion-binding exosite and inhibits thrombin's function by competing with exosite binding substrates fibrinogen and the platelet thrombin receptor (Paborsky et al., 1993). This highly specific aptamer/thrombin binding interaction has been extensively approached to develop different biosensors for thrombin, as summarized in Table S1 at the supplementary material. Furthermore, the selectivity of thrombin aptasensors has been demonstrated to be excellent against possible interfering substances such as human serum albumin (HSA) or lysozymes (Hu et al., 2009; Ding et al., 2010) present in human serum.

In recent studies, electrochemical impedance spectroscopic transduction of aptamer based protein analysis has shown a great prospect for label-free detections (Bogomolava et al., 2009). Electrochemical impedance spectroscopy (EIS) has been proven as one of the most powerful analytical tools for diagnostic analysis based on interfacial investigation capability. EIS measures the response of an electrochemical system to an applied oscillating potential as a function of the frequency. Impedimetric techniques have been developed to characterize the fabricated biosensors and to monitor the catalytic reactions of biomolecules such as enzymes, proteins, nucleic acids, whole cells and antibodies (Steichen et al., 2007; Steichen and Herman, 2005).

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Herein we describe a label-free aptasensor design for direct protein analysis at multi walled carbon nanotube (MWCNT) enhanced disposable screen-printed carbon electrodes (SPCEs) surfaces. The offered detection limit shows that the designed thrombin aptasensor is sensitive enough, capable of performing label-free detection using a low-cost, fast and reliable electrochemical technique. In addition by using different aptamer sequences, different proteins could be detected using a similar detection system.

2. Experimental

2.1. Apparatus and electrodes

Electrochemical impedance spectroscopy (EIS) measurements were performed using AUTOLAB PGSTAT-30-FRA (Eco Chemie, The Netherlands).

The electrochemical transducers used were homemade screen-printed carbon electrodes, consisting of three electrodes: working electrode, reference electrode and counter electrode in a single strip fabricated with a semi-automatic screen-printing machine DEK248 (DEK International, Switzerland). The reagents used for this process were: Autostat HT5 polyester sheet (McDermid Autotype, UK), Electrodag 423SS carbon ink, Electrodag 6037SS silver/silver chloride ink and Minico 7000 Blue insulating ink (Acheson Industries, The Netherlands). (See the detailed procedure of the SPCEs fabrication as well as images of the sensors obtained, Fig. S1 in the supplementary material.)

Scanning electron microscopic images (SEM) of both SPCEs and MWCNT modified SPCEs were obtained using a Hitachi S-570 microscope (Hitachi Ltd., Japan).

2.2. Reagents and solutions

A 5′ amino modified DNA aptamer that is selective to human alpha thrombin was purchased from Alpha DNA (Canada). The aptamer sequence has the following base composition: 5′ NH_2-GGTTGGTGTGGTTGG-3′

Multiwalled carbon nanotube (MWCNT) powders (95% purity), human alpha thrombin (α-thrombin), human serum from human male AB plasma (HS), N-hydroxysulphosuccinimide (NHS), [N-(3-dimethylamino)propyl)]–N-ethylcarbodiimide (EDC), tri-Natrium citrate and tyrosinase from mushroom (lyophilized powder, >1000 unit/mg solid) were purchased from Sigma–Aldrich (Spain). Other chemicals were of analytical reagent grade and supplied Merck and Sigma (Spain). All chemicals were used as received, all aqueous solutions were prepared in double-distilled water and all experiments were performed at room temperature. Working solutions of α-thrombin were prepared in human serum.

2.3. Methods

2.3.1. Modification of screen-printed carbon electrodes with multi-walled carbon nanotubes

The working surface area of a bare SPCE was modified by depositing a 7 μl drop of MWCNT suspension (1 mg MWCNT/1 ml THF), followed by a drying process at room temperature for 24 h. The characterization process of the working electrode surface was performed by SEM to check if an homogeneous distribution of MWCNT over the surface was achieved. In order to have a conductive surface for SEM studies the electrodes were mounted on adhesive carbon films and then sputtered with gold following the standard sample preparation procedure.

2.3.2. Electrochemical activation

Both bare SPCEs and MWCNT modified SPCEs were electrochemically pretreated by applying an oxidative potential of +1.40 V during 2 min.

2.3.3. Aptamer-modified biosensing surface preparation

SPCEs surfaces were chemically modified by exposure to 50 mM phosphate buffer containing 5 mM EDC and 8 mM NHS used for free amino group coupling. After that, the thrombin sensitive aptamer was immobilized onto the modified SPCEs surfaces by immersing these in a 54.5 nM aptamer solution, in acetate buffer pH 4.8, for 1 h. After that, the modified electrodes were immersed in a 1% bovine serum albumine (BSA) solution during 1 h to prevent non-specific adsorptions.

2.3.4. Aptamer/thrombin complex formation

α-thrombin solution at a concentration of 1.95 nM is accumulated onto the aptamer-modified SPCEs by immersing during 1 h the sensors into a α-thrombin solution prepared in human serum. Blank assays were performed by following the same protocol but using tyrosinase instead of α-thrombin.

2.3.5. Electrochemical impedance spectroscopy based detection

50 μL of a 5 mM $[Fe(CN)_6]^{3-/4-}$ solution prepared in PBS 50 mM pH 7.4 were dropped on the working area of the SPCEs and a potential of +0.24 V was applied. A frequency range from 10 kHz to 50 mHz and an AC amplitude of 10 mV were fixed. The impedance data was fitted to an equivalent circuit, and the value of the resistance to charge transfer (Rct) was chosen as the analytical signal (see the detailed equivalent circuit to fit the frequency scans at Fig. S2 in the supplementary material). All the measurements were done in quintuplicate.

3. Results and discussion

3.1. Effect of the MWCNTs on the impedimetric signal

A label-free impedimetric aptasensor system has been developed for the direct detection of human α-thrombin as model protein, using MWCNT modified SPCE as electrochemical transducers. 5′ amino linked aptamer sequence is immobilized onto the MWCNT modified SPCE surface via the carbodiimide chemistry and finally the thrombin detection was accomplished by EIS transduction of aptamer–protein interaction. A general scheme of the experimental procedure is shown in Fig. 1A.

MWCNTs are used as modifiers of the SPCE electrotransducer surface because of their notable charge-transfer capability between heterogeneous phases. Furthermore, they modify the electrode surface providing higher rugosity with an increased active surface to further aptamer immobilization. In addition to this, aptamers can self assemble in carbon nanotubes by π–π stacking interactions between the nucleic acid bases and the carbon nanotubes walls (Zelada-Guillén et al., 2009) or they can be anchored to the carboxylic groups of oxidized nanotubes by chemical reactions. The changes in the morphology of the working electrode surface of the SPCE after the MWCNTs and aptamers immobilizations are evidenced in the SEM images shown in Fig. 1B, C. Furthermore in order to oxidize the MWCNTs and generate carboxylic groups an electrochemical pretreatment is applied. These are necessary for the further aptamer immobilization through the carbodiimide interaction and represent another advantage of MWCNTs. In Fig. S3 (see supplementary material) are summarized the analytical signals obtained for bare SPCEs electrodes after the sequential biomodifications (without aptamer, with aptamer and with aptamer + thrombin) (a) and also the effect of the previous SPCEs modification with MWCNTs electrochemically pretreated (b) and non pretreated (c). As it was expected, the impedimetric signal increases when increasing the SPCEs modification and this effect is enhanced when electrochemically pretreated MWCNTs are previously immobilized onto the SPCE surface.

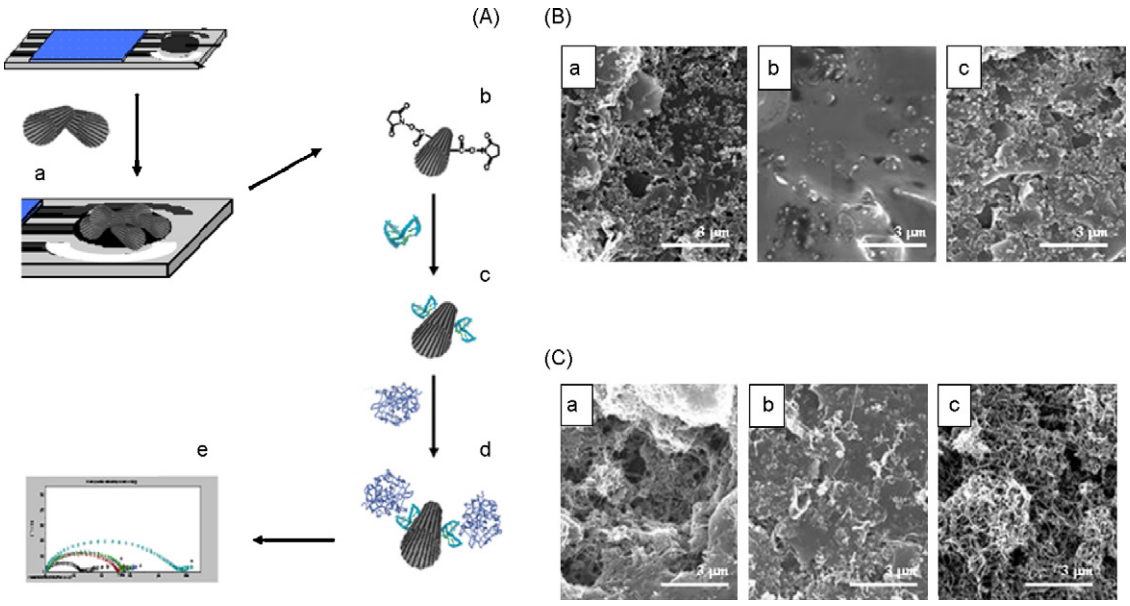

Fig. 1. (A) Schematic representation of the experimental procedure followed for the obtaining of the analytical signal: (a) MWCNTs modification of the SPCEs; (b) surface modification with covalent agents; (c) aptamer binding; (d) α-thrombin interaction; (e) EIS detection. (B) SEM images of the working surface area of bare SPCES after electrochemical pretreatment (a), after aptamer immobilization (b) and after its interaction with thrombin (c). (C) SEM images of the working surface area of MWCNT modified SPCEs after the same modifications detailed in (B). SEM resolution: 3 μm.

3.2. Selectivity of the impedimetric aptasensor

The selectivity of the sensor was tested doing different reference assays, using tyrosinase as negative control. The Rct values obtained for each assay are summarized in Fig. 2. First of all, the effect of the direct adsorption of both thrombin and tyrosinase on the MWCNT modified SPCE was evaluated. A significant increase in the Rct values was observed from both proteins due to bounding at the sensing surface via carbodiimide chemistry, being this increase of the same magnitude in both cases (b and c columns). This assay demonstrates that the direct adsorption of both proteins on the MWCNTs surface takes place in a similar magnitude, so further differences in the aptamer-based signals will be due to

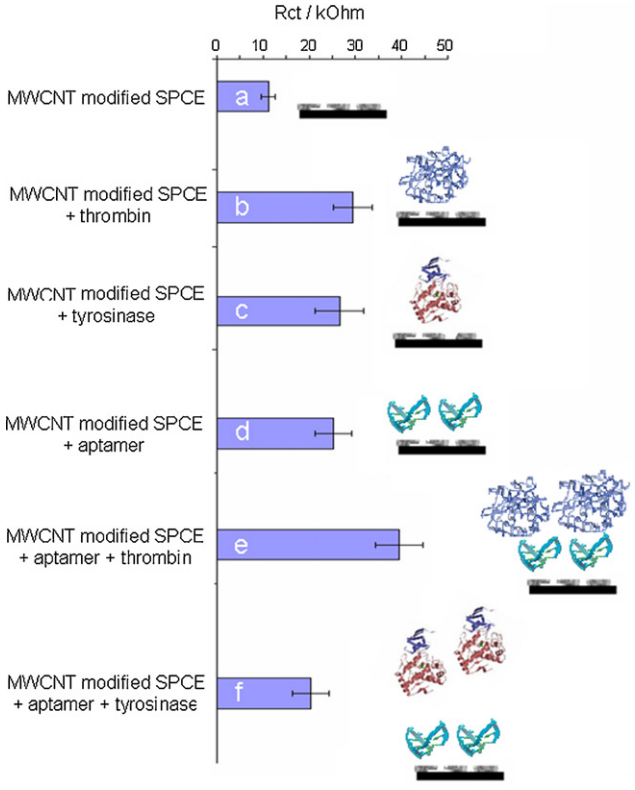

Fig. 2. Rct values obtained in 5 mM $[Fe(CN)_6]^{3-/4-}$ following the experimental procedure detailed in Section 2.3 for pretreated MWCNT modified SPCEs after performing different specificity assays.

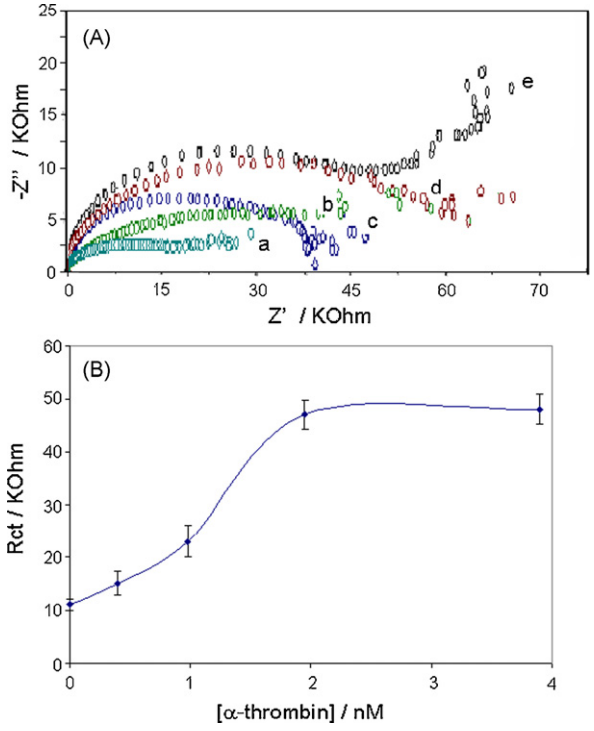

Fig. 3. (A) Impedance spectra obtained in 5 mM $[Fe(CN)_6]^{3-/4-}$ following the experimental procedure detailed in Section 2.3, using pretreated MWCNT modified SPCEs with immobilized aptamer for different α-thrombin concentrations: 0 nM (a), 0.39 nM (b), 0.98 nM (c), 1.95 nM (d) and 3.9 nM (e). (B) Corresponding relation between the α-thrombin concentrations and the Rct values (analytical signals).

the specific interaction and not to non-specific adsorptions. After that, the aptasensor was evaluated. When only the aptamer is immobilized onto the surface of the MWCNTs modified SPCE, an increase in the Rct values is also observed, considered as the reference value (d column). In addition, if the specific reaction with the α-thrombin is performed, a high increase (of about 15 kOhm) in the Rct value is registered (e column). However, when the assay is carried out with the tyrosinase, no changes are observed from the reference value, demonstrating the selectivity of the assay (f column).

3.3. α-thrombin quantification in human serum

Finally, the effect of the α-thrombin concentration on the analytical signal (Rct) for the thrombin sensitive aptamer-modified MWCNT SPCEs was evaluated. In Fig. 3A are shown the impedimetric spectra obtained for increased concentrations of α-thrombin from 0 to 3.9 nM. The curve presented in Fig. 3B shows that there is a good linear relationship (correlation coefficient of 0.99) between the α-thrombin concentration and the value of the analytical signal in the range of 0.39–1.95 nM, according to the equation:

$$\text{Rct (kOhm)} = 10.86\,[\alpha\text{-thrombin (nM)}] + 4.41\,(n = 5)$$

The limit of detection (calculated as the concentration of α-thrombin corresponding to 3 times the standard deviation of the estimate) was 105 pM. The reproducibility of the method shows a relative standard deviation (RSD) of 7.5%, obtained for a series of 5 repetitive assays performed for a 1.95 nM concentration of α-thrombin.

4. Conclusions

An impedimetric aptasensor for direct detection of human alpha thrombin at multiwalled carbon nanotube modified enhanced surfaces is developed. The selectivity of the aptasensor was evaluated by using the EIS response differences between aptamer–thrombin and aptamer–tyrosinase interaction. The thrombin detection limit of 105 pM shows that the designed aptasensor is capable to perform a label-free and sensitive detection. Considering also advantages related to low-cost, fast and reliable electrochemical detection mode applied in this methodology different other aptamer sequences for other proteins can be expected to be used in the future. The extension and application of the developed technique to other analytes and fields should be a matter of further investigations related also to a better understanding and improvements of the aptamer immobilization quality onto the carbon nanotube modified electrodes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2010.07.090.

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