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Label-free impedimetric aptasensor for lysozyme detection based on carbon nanotube-modified screen-printed electrodes

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ABSTRACT

We report on the direct electrochemical detection of aptamer–protein interactions, namely between a DNA aptamer and lysozyme (LYS) based on electrochemical impedance spectroscopy (EIS) technique. First, the affinity of the aptamer to LYS and control proteins was presented by using filter retention assay. An amino-modified version of the DNA aptamer-recognizing lysozyme was covalently immobilized on the surface of multiwalled carbon nanotube-modified screen-printed electrodes (MWCNT–SPEs), which were employed for measurements and have improved properties compared with bare SPEs. This carbon nanotube setup enabled the reliable monitoring of the interaction of lysozyme with its cognate aptamer by EIS transduction of the resistance to charge transfer (R_{ct}) in the presence of 2.5 mM $[Fe(CN)_6]^{3-/4-}$. This assay system provides a means for the label-free, concentration-dependent, and selective detection of lysozyme with an observed detection limit of 12.09 μg/ml (equal to 862 nM).

Aptamers are of increasing interest in being used as recognition module within sensors for proteins [1–3], DNAs [4,5], and small molecules [6–9]. As nucleic acid structures that are identified by in vitro selection approaches [10,11], aptamers bear several advantages such as high affinity and specificity with respect to target recognition. Moreover, they have a longer shelf life than antibodies, are resistant to repetitive cycles of denaturation/renaturation, and are less prone to degradation. Furthermore, they can be readily engineered by chemical synthesis and, thus, adopted to various biosensor formats. Consequently, several nucleic acid aptamer-based biosensors for protein detection have been developed [12–22]. The signal transduction approaches were mainly based on the quartz crystal microbalance (QCM)[1] [12,13], surface plasmon resonance (SPR) [14], or fluorescence approach [15,16] or on other optical methods [17,18]. However, only a few studies have yet reported electrochemistry methods for the transduction of the protein recognition events on electrode surfaces [19].

An electrochemical DNA aptamer biosensor was introduced recently by Erdem and coworkers [20]. Two cognate proteins, lysozyme (LYS) and human thrombin (THR), were selected as analytes. The interaction of the cognate aptamers with the analytes was maintained on magnetic particle surfaces, and electrochemical detection was facilitated by using disposable graphite electrodes with differential pulse voltammetry (DPV) techniques. The detection limits were determined to be 10.77 μg/ml for LYS and 2.00 μg/ml for THR.

Li and coworkers investigated an electrochemical LYS aptasensor developed by using gold nanoparticles [21]. The gold nanoparticles were applied to increase the immobilization of DNA probes due to the surface effect of nanomaterials; thus, it offers a significant amplification for the detection of lysozyme by using square wave voltammetry (SWV).

The modification of transducers with carbon nanotubes has recently attracted considerable attention in the field of electroanalytical chemistry. Carbon nanotube-based electrodes play an important role in DNA sensing for reasons similar to their role in enzyme biosensors: high surface area, fast heterogeneous electron transfer, and long-range electron transfer [23–31].

Here we describe a direct impedimetric aptasensor designed for direct lysozyme detection with aptamer-derivatized multiwalled carbon nanotube-modified screen-printed electrodes (MWCNT–SPEs) by using electrochemical impedance spectroscopy (EIS). The filter retention assay was first applied for proving the affinity of the aptamer to LYS and also control proteins, extracellular signal-regulated kinase 2 (ERK2), and Cytohesin1Sec7 domain (CYT1SEC7). The immobilization of amino-linked DNA aptamer onto

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[1] *Abbreviations used:* QCM, quartz crystal microbalance; SPR, surface plasmon resonance; LYS, lysozyme; THR, thrombin; DPV, differential pulse voltammetry; SWV, square wave voltammetry; MWCNT, multiwalled carbon nanotube-modified screen-printed electrode; EIS, electrochemical impedance spectroscopy; ERK2, extracellular signal-regulated kinase 2; CYT1SEC7, Cytohesin1Sec7 domain; APT, aptamer; TBS, Tris-buffered saline; BSA, bovine serum albumin; PBS, phosphate buffer solution.

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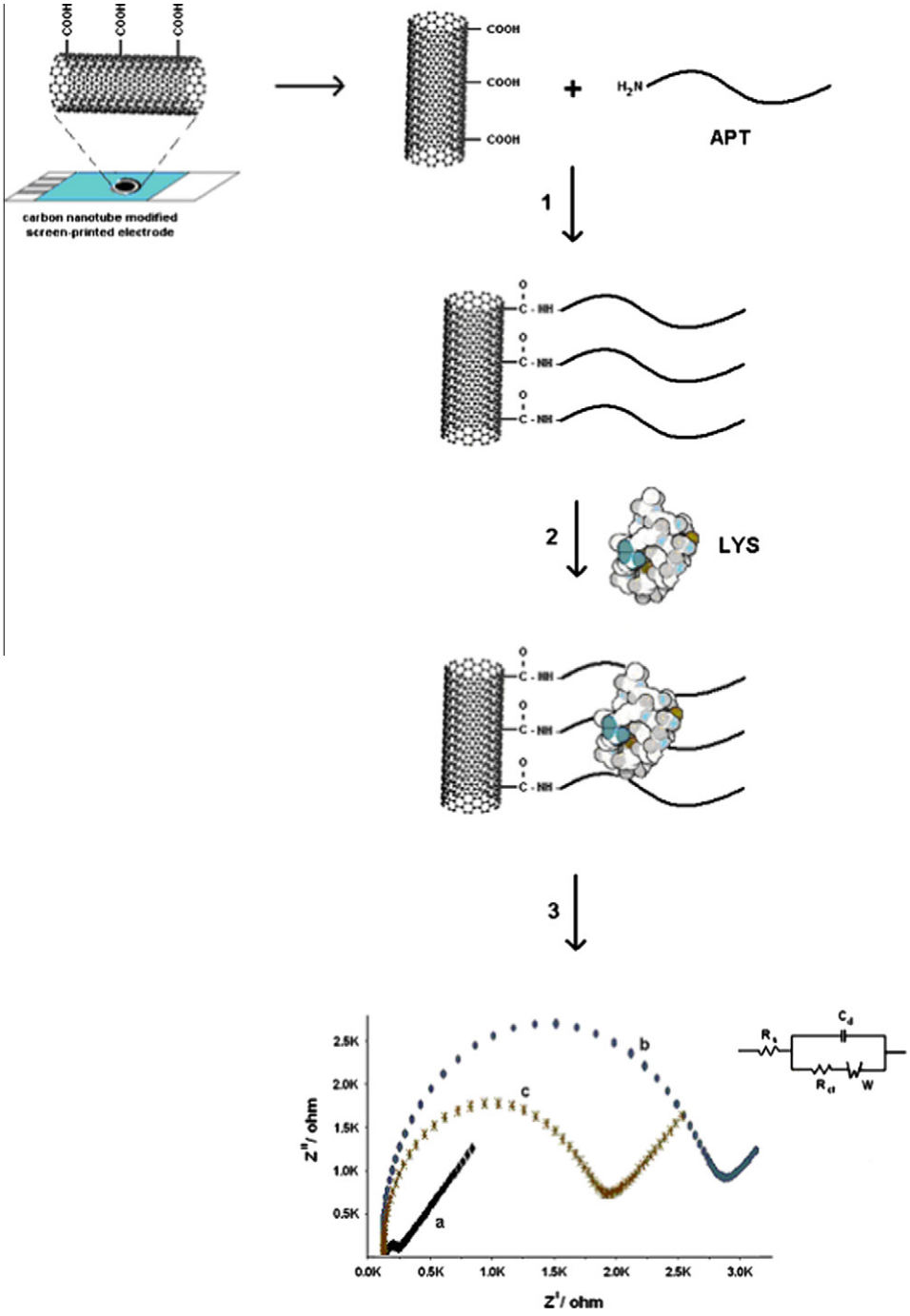

Scheme 1. Before (1) and after (2) immobilization of amino-linked DNA aptamer onto the surface of MWCNT–SPEs and (3) the interaction of LYS with APT and by using EIS measurement. Representative Nyquist diagrams show the EIS measurements for (a) bare (unmodified) electrode, (b) APT-modified electrode, and (c) APT+LYS-modified MWCNT–SPEs.

the MWCNT–SPE surface containing carboxylic groups and interaction with LYS is illustrated in Scheme 1. Compared with previous reports presenting detection systems for aptamer–protein detection, this aptasensor performs well with respect to sensitivity, selectivity, and reproducibility.

Materials and methods

Chemicals

The amino-linked single-stranded DNA (ssDNA) aptamer and oligodeoxynucleotide were purchased from Ella Biotech (Germany):

anti-lysozyme DNA aptamer (APT): 5′-NH_2-ATC TAC GAA TTC ATC AGG GCT AAA GAG TGC AGAGTTACT TAG-3′
ssDNA oligodeoxynucleotide (random DNA): 5′-NH_2-TTT TTT TTT CGA TCG AG-3′.

The stock solution of the DNA aptamer and the random DNA molecule were prepared with fresh ultrapure triple-distilled water and kept frozen. The diluted solutions of the aptamers and oligodeoxynucleotide were prepared with Tris-buffered saline (TBS: 5 mM Tris–HCl buffer supplemented with 20 mM NaCl, pH 7.0). Other chemicals were supplied from Sigma (USA) and Merck (Germany) in analytical reagent grade.

LYS 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 were stored at −20 °C. The diluted solutions of proteins were prepared in 50 mM phosphate buffer solution (PBS, pH 7.4).

Filter retention assay

APT (10 pmol) and random DNA were radioactive 5′-phosphorylated by 20 U of T4 Polynucleotide Kinase (New England Biolabs, USA) in 70 mM Tris–HCl buffer (pH 7.6) containing 10 mM $MgCl_2$, 5 mM dithiothreitol, and 300 μM [γ-^{32}P]ATP. [γ-^{32}P]ATP was purchased from PerkinElmer (USA).

LYS, ERK2, and CYT1SEC7 proteins were incubated with 100 nM ^{32}P-labeled DNA for 30 min at 37 °C in PBS containing 1 mg/ml BSA and 3 mM $MgCl_2$. After incubation, the reactions were passed through 0.45-μm nitrocellulose membranes (Whatman, USA) and washed three times with 200 μl of PBS containing 3 mM $MgCl_2$. Bound DNA was quantified with the Fujifilm FLA 3000 PhosphorImager. CYT1SEC7 and ERK2 were expressed in *Escherichia coli* and purified by nickel–nitrilotriacetic acid agarose beads and fast protein liquid chromatography. The concentration was measured by OD_{280} .

Electrochemical assay

Electrochemical measurements for MWCNT–SPEs were performed by using an AUTOLAB PGSTAT 302 electrochemical analysis system supplied with an FRA 2.0 module for impedance measurements and GPES 4.9 software package (Eco Chemie, The Netherlands). For electrochemical measurements, EIS was used. The EIS measurements were performed in a Faraday cage (Eco Chemie).

Electrode preparation

The planar screen-printed electrode (3.4 × 1.0 × 0.05 cm, length × width × height) consists of three main parts: a graphite working electrode modified with MWCNTs, a graphite counter electrode, and a silver pseudo reference electrode. The graphite working SPE surface is 4 mm in diameter, and these electrodes are modified with MWCNTs containing approximately a 5% ratio of carboxylic groups. The diameter of the carbon nanotubes is approximately 10 nm, and the average length is 1–2 mm. These disposable SPEs were purchased from DropSens (Spain); further details about disposable MWCNT–SPEs can be obtained from the supplier's website at http://www.dropsens.com.

A specific DropSens connector (DSC) allows the connection of the MWCNT–SPEs to the potentiostat.

Impedance measurements

The EIS measurements were performed in the presence of a 2.5 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1) mixture as a redox probe prepared in 0.1 M KCl. The impedance was measured in a frequency range from 100 mHz to 100 KHz at a potential of +0.23 V versus Ag/AgCl with a sinusoidal signal of 10 mV. The frequency interval divided into 98 logarithmically equidistant measure points. The respective semicircle diameter corresponds to the charge transfer resistance (R_{ct}), the values of which are calculated using the fitting program AUTOLAB 302 (FRA, version 4.9, Eco Chemie).

EIS measurements on MWCNT–SPEs were performed by placing a 40 μl drop of the corresponding solution to the working area at the SPE surface. Before APT immobilization onto the surface of MWCNT–SPEs, a 40 μl droplet of redox probe was added and the EIS measurements were performed. After APT immobilization onto the electrode surface, each disposable strip was first covered by a 40 μl droplet of 200 μg/ml APT for 30 min by the formation of covalent coupling between the carboxylic groups of the nanotubes and the amino groups of aptamers without using any chemical agents for covalent binding such as *N*-(3-dimethylamino)-propyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS). Each electrode was then rinsed with PBS for 5 s to remove unbound aptamer from the electrode surface. The EIS measurements were performed by dropping a 40 μl redox probe onto the APT-modified electrode.

A 40 μl droplet of a required concentration of LYS (or BSA) was added onto the surface of APT-modified electrode, and it was kept for the interaction process for 15 min. Then, each electrode was then rinsed with TBS for 5 s. After the 40 μl redox probe was dropped onto the surface of electrode, the EIS measurement was performed.

Results and discussion

The binding of free APT and control DNA to LYS and two control proteins is measured by filter retention assay. For this well-established assay, the DNA is labeled with [^{32}P] phosphate and the affinity of the aptamer to the protein is measured by immobilizing the protein on a nitrocellulose membrane and measuring the radioactivity after washing off all unbound sequences [32].

The results in Fig. 1 demonstrated the binding of APT to LYS beginning at a concentration of 250 nM protein. APT and the control DNA showed no affinity to the control proteins ERK2 and CYT1SEC7. The control DNA exhibited 55% lower binding compared with APT at a concentration of 1000 nM LYS.

The impedimetric detection of the interaction between a DNA aptamer and its target protein, namely LYS, on the surface of MWCNT–SPEs was explored before and after immobilization of the DNA aptamer on the surface of MWCNT–SPEs (Scheme 1). The interaction of LYS with its aptamer was investigated by using EIS technique.

First, the selectivity of the aptamer at a concentration of 200 μg/ml and in the presence of 50 μg/ml target protein (LYS) or the control protein (BSA) was analyzed and compared with signals derived from experiments employing the random control DNA molecule. The obtained R_{ct} values after EIS measurement are shown in Fig. 2.

After modification of the MWCNT–SPE surface with APT, the average R_{ct} value (Fig. 2, column b) was calculated as 980 Ω, which is 3 times higher than the one obtained by bare MWCNT–SPE. This indicates a strong verification that APT has been immobilized onto the MWCNT–SPE surface. The higher R_{ct} value can best be explained by reduced efficiency of the redox couple, $[Fe(CN)_6]^{3-/4-}$, to reach the electrode surface in the presence of DNA, presumably due to charge repulsion between the negatively charged phosphate backbone of oligonucleotide and $[Fe(CN)_6]^{3-/4-}$. The net positive charges of the interaction between APT and LYS in turn led to a 35.2% decrease of the R_{ct} value (Fig. 2, column c). Due to the interaction of APT with LYS that may have a higher negative charge density, the lower R_{ct} value was observed at the sensor surface. This observation is in accordance with the results shown in the studies of Peng and coworkers [33] and Liao and Cui [34].

The selectivity of the impedimetric aptasensor was further analyzed by measurements in the presence of BSA instead of LYS. R_{ct} values that reflect a 79.8% increase were measured in the presence of BSA (Fig. 2, column d), indicating that there was no specific interaction between APT and BSA. These data are in accordance with results reported in previous studies [20,35]. In addition, compared with the data of filter retention assay (shown in Fig. 1), this ensures the specific interaction of LYS with the APT-modified

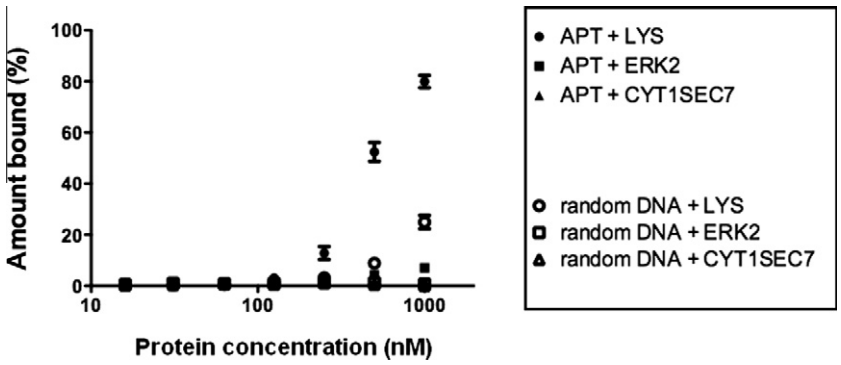

Fig.1. Filter retention assay analyzing affinity and selectivity of APT and random DNA to different protein concentrations. APT (100 nmol/L) and random DNA were incubated with 16 to 1000 nmol/L LYS, ERK2, and CYT1SEC7, and the relative photostimulated luminescence (PSL) was measured. Beginning at a concentration of 250 nmol/L, APT exhibits an increased binding to LYS compared with the random DNA. No binding to the control proteins ERK2 and CYT1SEC7 was observed for APT and the random DNA.

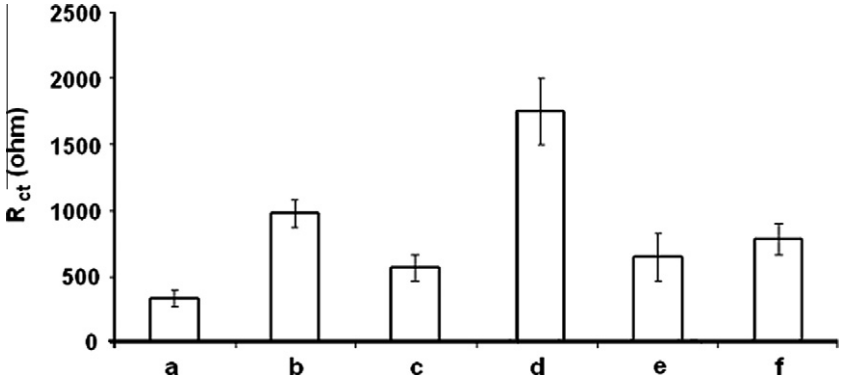

Fig.2. Histograms representing the average R_{ct} values ($n = 3$) measured at MWCNT–SPE surface before (a) and after (b) modification with 200 μg/ml APT, interaction of 50 μg/ml LYS (c), interaction of 50 μg/ml BSA (d), MWCNT–SPE surface after modification with 200 μg/ml random DNA (e), and interaction of 50 μg/ml LYS (f).

MWCNT–SPEs by excluding an unspecific surface adsorption effect as a main mode of action of the impedimetric sensor.

Second, the selectivity of the aptasensor was analyzed by measuring the interaction between LYS and a control DNA molecule. The average R_{ct} value (Fig. 2, column e) was first measured as 580 ± 142 Ω ($n = 3$) when the control DNA was immobilized on the MWCNT–SPE surface, which is 1.8 times higher than the one measured for unmodified MWCNT–SPEs. A 21.4% increase of the average R_{ct} value was obtained (Fig. 2, column f) after the addition of LYS. This result indicates that almost no interaction between the control DNA and LYS is detectable and that the signal measured for the specific anti-LYS aptamer is specific (Fig. 2, column c).

To further analyze the selectivity of the aptasensor, we used 200 μg/ml APT and either the target protein (LYS) or a control protein (BSA) at a higher concentration such as 400 μg/ml (Fig. 3).

After the addition of LYS (Fig. 3, column b) and BSA (Fig. 3, column c) to the MWCNT–SPE surface and in the absence of aptamers, the average R_{ct} values were calculated as 120 and 740 Ω, which are 2.5 and 9.0 times higher, respectively, than the one obtained by unmodified MWCNT–SPEs. The average R_{ct} value (Fig. 3, column d) was calculated as 2372 ± 102 Ω ($n = 3$) after modification with APT, which is 30.0 times higher than the one measured by MWCNT–SPEs (Fig. 3, column a). The net positive charges between APT and LYS interaction led to a 43.4% decrease of the R_{ct} value (Fig. 3, column e), similar to the results shown in Fig. 2 (column c) and reports published elsewhere [33,34]. This decrease of R_{ct} value can be attributed to the lower rate of resistance faced by the electrons due to specific interaction between APT and its target protein (LYS) having a higher negative charge density.

In addition, the selectivity of the aptasensor was further analyzed using BSA instead of LYS, whereas a 49.4% increase of the

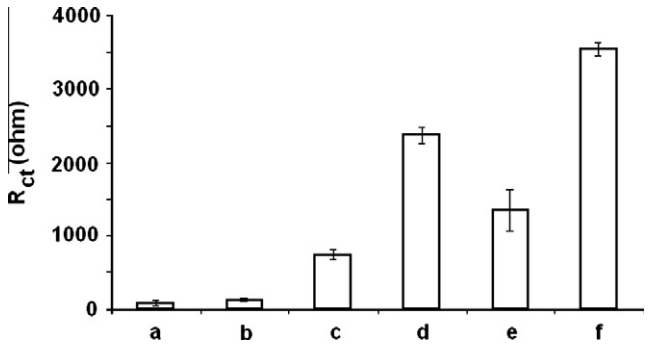

Fig.3. Histograms representing the average R_{ct} values ($n = 3$) measured at MWCNT–SPE surface (a) and 400 μg/ml proteins LYS (b) and BSA (c) after 200 μg/ml aptamer immobilization onto electrode surface (d) and the interaction of APT with 400 μg/ml proteins LYS (e) and BSA (f).

average R_{ct} value was observed (Fig. 3, column f). This result shows that there was no specific interaction between APT and BSA, as suggested in previous studies employing different assay systems [20,35].

The complex impedance is presented as the sum of the real and imaginary components (Z' and Z''). The value of the charge transfer resistance (R_{ct}) depends on the dielectric and insulating features at the electrode/electrolyte interface. To obtain detailed information on the impedance spectroscopy, a simple equivalent circuit model (Fig. 4A, inset) was used to fit the results. In the Nyquist plot of impedance spectra, the semicircle section at higher frequencies characterizes the electron transfer limited process and the linear section seen at lower frequencies may be attributed to diffusion.

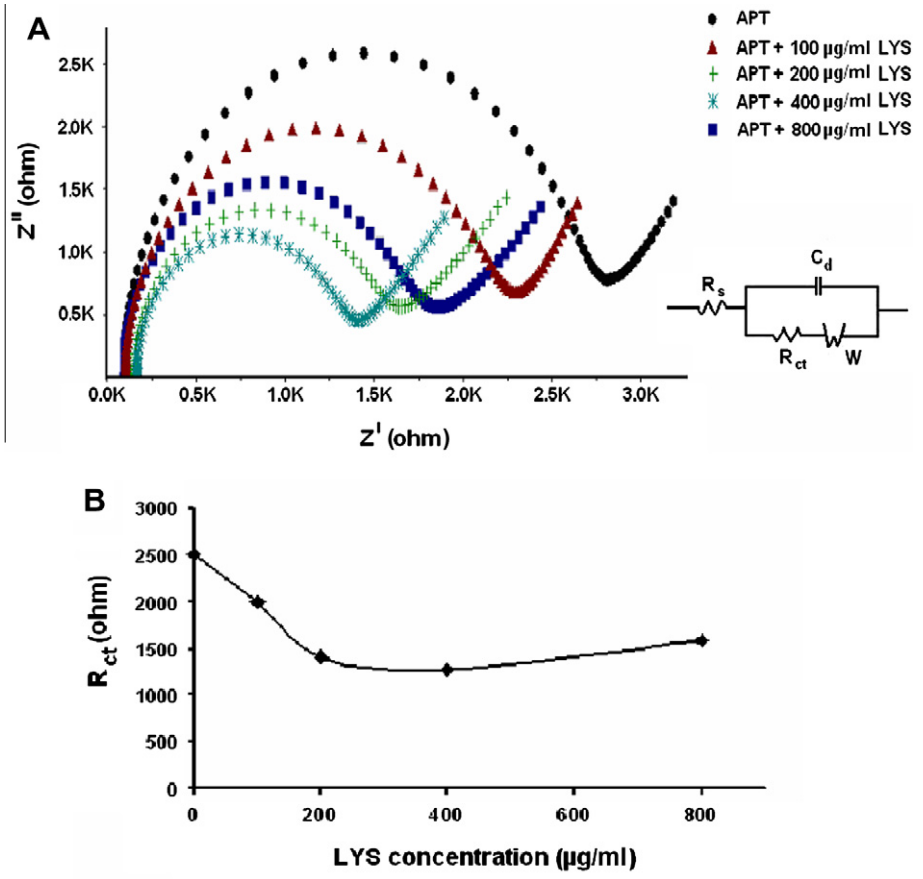

Fig.4. (A) Nyquist diagrams recorded with supporting electrolyte solution 2.5 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1) containing 0.1 M KCl by using MWCNT–SPEs with immobilized 200 µg/ml aptamer for different LYS concentrations such as 0, 100, 200, 400, and 800 µg/ml. The inset shows the equivalent circuit model used to fit the impedance data, the parameters of which are listed in the text; R_1 is the solution resistance. The constant phase element C_d is then related to the space charge capacitance at the DNA/electrolyte interface. R_2 is related to the charge transfer resistance (R_{ct}) at the DNA/electrolyte interface. The constant phase element W is the Warburg impedance due to mass transfer to the electrode surface. (B) Line graph for relation between LYS concentration and R_{ct} values.

The effect of LYS concentration on the response of the impedimetric aptasensor was also studied in the presence of an interaction between aptamer and LYS at various concentrations (Fig. 4A and B). Fig. 4A shows the impedance changes of the MWCNT–SPEs associated with the stepwise interaction process. The R_{ct} value decreased linearly from 0 to 400 µg/ml LYS concentration and then leveled off. The curve shows that there is a good linear relationship ($y = -5.515x + 2527.8$, with a correlation coefficient of 0.9984) in the LYS concentration range of 0 to 400 µg/ml. The limit of detection was calculated as 12.09 µg/ml (equal to 862 nM).

Conclusion

An impedimetric aptasensor was developed for the label-free detection of lysozyme using MWCNT–SPEs. The selectivity of the aptasensor was investigated by using BSA. The lysozyme detection limit was calculated as 12.09 µg/ml (equal to 862 nM). MWCNT–SPEs have many advantages, such as good reproducibility and sensitivity and being cost-effective as well as portable, which are crucial properties of devices for chip technology, since MWCNT–SPEs provide a high surface coverage for enhanced adsorption of the nucleic acids, aptamers, and other molecules [27,36,37].

In conclusion, the aptasensor developed by MWCNT–SPEs could lead to the development of advanced aptasensor platforms for the expansion of fast and cost-effective detection systems with respect to protein chip technologies with further improvements.

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