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Determination of endotoxin through an aptamer-based impedance biosensor

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ABSTRACT

Lipopolysaccharide (LPS) often referred to endotoxin is an undesirable impurity frequently entrained with various recombinant protein therapeutics and plasmid DNA (pDNA) vaccines of bacterial origin. The inherent toxicities (e.g. fever, hypotension, shock and death) of LPS render its early and sensitive detection essential for several biological assays and/or parenteral administrations of biotherapeutics. In this study, an electrochemical biosensor using an LPS specific single stranded DNA (ssDNA) aptamer as a probe was developed. Amine-terminated aptamer exhibiting high affinity (K_d = 11.9 nM) to LPS was immobilized on a gold electrode using 3-mercaptopropionic acid (MPA) as a linker. Each step of the modification process was characterized by cyclic voltammetry (CV) and electrochemical impendence spectroscopy (EIS). A good linear relationship of the changes in the charge-transfer resistance (ΔR_{et}) and the logarithmic value of LPS concentration was demonstrated in a broad dynamic detection range of 0.001–1 ng/ml. Furthermore, the aptasensor showed a high selectivity to LPS despite the presence of pDNA, RNA and bovine serum albumin (BSA) and could be regenerated in low pH condition, offering a promising option for detecting LPS often present in a complex milieu.

1. Introduction

Endotoxins, also known as lipopolysaccharides (LPS) are an integral component of the outer membrane of all Gram-negative bacteria and consist of three distinct regions: O-specific antigen, core polysaccharide and lipid A (Raetz, 1990; Gutsmann et al., 2007). Since LPS is responsible for pyrogenic reaction (Murai et al., 1987), septic shock (Ding et al., 2007), diarrhea, hypotension and vascular blood clotting (Kotani et al., 1985), the detection and/or clearance of an extremely small amount of LPS is essential for medical, pharmacological and food security.

The limulus amoebocyte lysate (LAL) assay based on the clotting of the LAL and endotoxin has commonly been used for LPS detection (Cooper et al., 1971) with a detection limit reported to be around 10^{-9} g/ml (Nachum and Berzofsky, 1985; Sakti et al., 2001). However, it requires a rather long and complicated assay procedure primarily due to its enzymatic reaction required for clotting (Ong et al., 2006), and the assay results are susceptible to the presence of protease and/or impurities when applied to actual cell lysates or complicated biological liquors.

This renders the development of biosensors harnessing LPS affinity molecules a promising alternative to pursue for a rapid and accurate detection of LPS (Haas et al., 1998; Inoue et al., 2010; Matsumoto et al., 2010). Electrochemical or optical biosensors based on a variety of LPS binding proteins or peptides were reported (Jones and Jiang, 2005; Limbut et al., 2007). A biosensor based on polymyxin B was found to detect LPS in the range of 0.2–0.8 ng/ml (Ding et al., 2007). Voss et al. (2007) developed a biosensor to detect LPS in micromolar range using fluorescently labeled analogues of two peptide variants derived from the LPS-binding protein CD14.

In addition to harnessing LPS affinity biomolecules of natural source, a few artificial molecules cognitive of LPS were designed or synthesized on purpose for LPS detection. The first LPS sensor based on a synthetic receptor (i.e. amino acid-functionalized polydiacetylene liposome) was able to detect LPS at a range of 100 μM by mediating colorimetric changes upon its interaction with LPS (Rangin and Basu, 2004). Pyrene derivatives were designed and applied as a fluorescent probe to fabricate biosensors capable of sensing bacterial endotoxin with a detection limit of 10–100 ng/ml (Zeng et al., 2010).

However, these protein or synthetic LPS recognizing molecule-based sensors generally suffered from narrow dynamic detection ranges and high limitation of detection. They were largely prone to cross-binding to or fouling by other species frequently co-present with LPS because the specificity or sensitivity of the probe molecules used for LPS capture was not as high as expected. Especially those LPS sensors relying on designer biomolecules or synthetic LPS probes are usually cost-prohibitive and require complicated preparation and detection process for applications (Rustici et al., 1993; Little et al., 1994).

During the last two decades, aptamers have been screened against various classes of targets (Wilson and Szostak, 1999;

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Famulok et al., 2000). Aptamers with high specificity and affinity can, in principle, be selected in vitro for any given target, ranging from small to large molecules including proteins and cells, thus making them promising probes to be used for fabrication of a wide range of aptamer-based biosensors. Aptamers, once selected, can be synthesized with high reproducibility and purity. Also, in contrast to protein-based sensor probes such as antibodies or enzymes, DNA aptamers are usually highly stable. Therefore, the aptamer-based biosensors provide some advantages compared to biosensors relying on natural receptors such as antibodies, enzymes, and peptides (Drummond et al., 2003; Kim et al., 2008; Presnova et al., 2008; Song et al., 2008; Hianik and Wang, 2009; Lei et al., 2009; Sassolas et al., 2009; Xu et al., 2009). Nevertheless, few studies on LPS sensor based on an aptamer were found in the literatures.

In our previous study, an aptamer with a high affinity to LPS (K_d = 11.9 nM) was selected by a NECEEM (non-equilibrium capillary electrophoresis of equilibrium mixtures)-based non-SELEX (systematic evolution of ligands by exponential enrichment) method using capillary electrophoresis (CE) from a fluorescently labeled single stranded DNA (ssDNA) library (Wang, 2008). In the present study, the aptamer comprising 45 nucleotides was flanked by a forward primer and a reverse primer and used to develop a LPS biosensor by modifying its 5′ end to have an amine group. The amine-terminated aptamer was immobilized on the gold electrode surface covered in advance with a self-assembled monolayer (SAM) of 3-mercaptopropionic acid (MPA). The carboxyl groups of MPA reacted with the 5′-end amine groups of the aptamers (Lin et al., 2009) to produce peptide bonds which ensured tight anchoring of the aptamer molecules on the gold surface. The modification of the gold electrode was confirmed by cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS). While detecting LPS with the aptamer-based biosensor, EIS was used to directly probe and quantify the response of the aptamer–LPS interaction in $Fe(CN)_6^{3-/4-}$ solution. An equivalent circuit model of mixed kinetics and charge transfer control (Qiu et al., 2009) was adopted to interpret the obtained impedance spectra. The developed aptasensor exhibit high sensitivity, selectivity, and reproducibility and can be applied for quality control in the fields of food and pharmaceutical industries.

2. Experimental

2.1. Materials and apparatus

3-Mercaptopropionic acid (MPA), morpholinoethanesulfonic (MES), N-hydroxy-succinimide (NHS), N-(3-dimethyl aminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), $K_3Fe(CN)_6$, and $K_4Fe(CN)_6$ were purchased from Sigmar-aldrich and used without further purification. The aptamer, NH_2-5′-CTT CTG CCC GCC TCC TTC C- TAG CCG GAT CGC GCT GGC CAG ATG ATA TAA AGG GTC AGC CCC CCA -GGA GAC GAG ATA GGC GGA CAC T-3′, was purchased from Integrated DNA Technologies (IDT, Coralville, USA) and stored at −20 °C before use. LPS from *Escherichia coli 055*:B5 (L4524), Baker's yeast RNA (R6750) and BSA (A7906) were purchased from Sigma and stored at −20 °C before use. Baker's yeast RNA was stripped of LPS using LPS removal solution (Sigma, E4274). Purified pDNA (pcDNA3.1D) was prepared according to the protocol described elsewhere (Shin et al., 2011). All solutions were prepared with ultrapure water (resistivity: 18.2 MΩ/cm) through a Millipore MilliQ water purification system. All other chemicals were of analytical grade and used as received.

All electrochemical experiments were performed using VSP Potentiostat (Princeton applied research, USA) and the resulting curves were recorded with VSP EC-Lab software. CV and EIS were carried out in a conventional three-electrode cell with a gold disc electrode (CH Instruments Inc., USA; diameter: 2 mm), an Ag/AgCl and a platinum plate as the working, reference and counter electrodes, respectively. In the EIS measurement, a 10 mV amplitude sine wave was applied to a gold electrode in the frequency range of 0.1 Hz to 100 kHz and 2 mM $Fe(CN)_6^{3-/4-}$ in 10 mM pH 7.4 phosphate buffer saline (PBS) containing 137 mM NaCl and 2.7 mM KCl, was used as the electrolyte. The ZSimpWin EIS DATA analysis software (Perkin-Elmer, Version 2.00) was used to analyze the obtained EIS data and fit an equivalent circuit.

2.2. Preparation and modification of gold electrodes

The gold disc electrode was polished with 0.05 μm alumina slurry, and then ultra sonically cleaned in distilled water and absolute ethanol, respectively. After that, the gold electrode was immersed in Piranha solution [30% H_2O_2/98% H_2SO_4 (1/3, v/v)] and washed with distilled water. Subsequently, the gold electrode was electrochemically activated in 1 M $HClO_4$ aqueous solution by CV from 0 to 1.5 V at a scan rate of 100 mV s^{-1} for 20 cycles. Afterwards, the electropolished electrodes were dried and immediately immersed in the 20 mM MPA in water/ethanol (3/1, v/v) deposition solution for 3 h at room temperature, followed by rinsing the residual MPA molecules with ethanol. Then the MPA-modified electrode was soaked in 100 mM MES containing 20 mM EDC and 20 mM NHS to activate the carboxyl-terminated group of MPA for another 15 min (Geddes et al., 1995). Finally, the aptamer was immobilized on the activated MPA SAM electrode by dipping the gold electrode in 2 nM aptamer/PBS (10 mM, pH 7.4) solution for 1 h. Schematic diagram of the stepwise procedure of the aptasensor was shown in Scheme 1.

2.3. Characterization of aptasensor

The prepared aptasensor was incubated in 10 mM pH 7.4 PBS containing various concentration of LPS at room temperature for 15 min each. After washing with PBS to remove the non-chemisorbed LPS, EIS responses of the aptasensor were recorded in 10 mM pH 7.4 PBS containing 2 mM $Fe(CN)_6^{3-/4-}$. Since pDNA, RNA, and proteins are frequently co-existed with LPS, this study first investigated the affinity of the aptamer to those interferents (Li et al., 2005; Tan et al., 2007). To evaluate the selectivity of the aptasensor, 5×10^4 ng/ml BSA/PBS (10 mM, pH 7.4), 2.5×10^4 ng/ml RNA/PBS (10 mM, pH 7.4), and 100 ng/ml pDNA/PBS (10 mM, pH 7.4) were prepared and used as incubation solutions. The regeneration of the aptasensor was evaluated from the response to 1 ng/ml LPS in different pH PBS solutions.

3. Results and discussion

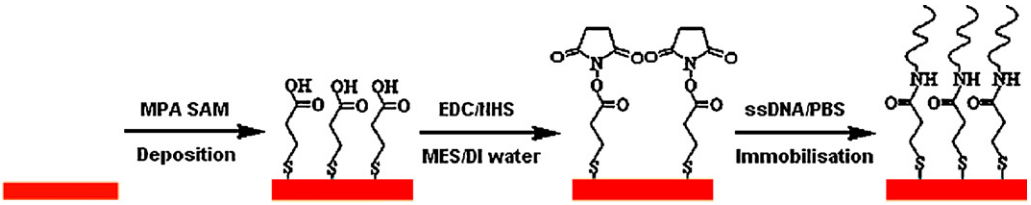
3.1. Electropolishing of gold electrode

Fig. 1 shows the repeated voltammetric cycling on gold electrodes when electrochemically polished in 1 M $HClO_4$ solution. The currents obtained upon successive cycles tended to reach a stable gold oxide formation and invariant reduction peaks, implying a clean and uniform electrode surface (Brett et al., 2003).

3.2. Fabrication of the aptasensor

The surface properties of the gold electrode at each modification step were characterized electrochemically. The CV results in 2 mM $K_3Fe(CN)_6$ aqueous solution containing 0.1 M KCl in the range of −0.3 to 0.6 V with the scan rate of 100 mV s^{-1} are shown in Fig. 2.

While the electrochemical activated bare gold electrode showed a couple of well-defined redox wave of $K_3Fe(CN)_6$ at 0.276 V and 0.201 V (ΔE_p = 75 mV, $I_{pc}/I_{pa} \approx 1$; Fig. 2a), the redox current of the



Scheme 1. The stepwise preparation of the aptasensor.

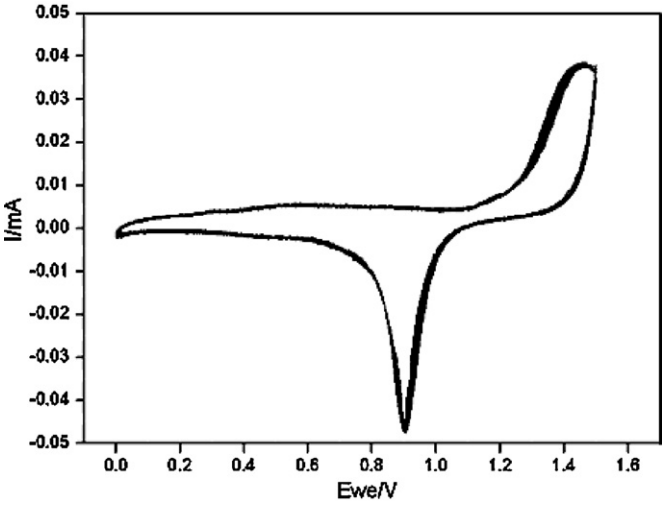


Fig. 1. Cyclic voltamograms of the bare gold electrode subjected to electropolishing between 0 V and 1.5 V in 1 M $HClO_4$ for 20 cycles. The scan rate is 100 mV/s.

electrode modified with MPA SAM was decreased dramatically (Fig. 2b), implying the formation of a compact SAM on the gold surface. As is well known, a MPA SAM has a blocking property that could prevent the redox reaction of $K_3Fe(CN)_6$ at the interface (Su et al., 2010). After the immobilization of aptamer, and the redox peak currents decreased further (Fig. 2c), which indicates the aptamer was successfully attached on the electrode surface. The immobilized aptamer acts as an insulator blocking the charge exchange of the $Fe(CN)_6^{3-}$ and the gold electrode.

The Nyquist plots in $Fe(CN)_6^{3-/4-}$/PBS (10 mM, pH 7.4), including a semicircle portion at higher frequencies corresponding to the electron-transfer-limited process and a linear part at lower frequency range representing the diffusion-limited process, are shown in Fig. 3. The bare gold corresponds to an almost straight line in the Nyquist plot of impedance spectroscopy (Fig. 3a), which is characteristic of a diffusion-controlled process over the whole frequency range. After the modification of MPA SAM, an obvious semicircle up to the mid-frequencies region reveals a charge transfer-controlled process (Fig. 3b). However, over low frequencies, the effect of mass transfer still persists. The EIS data indicates the successful formation of MPA SAM hindering the electron transfer of the electrochemical probe and confirms the CV results (Fig. 2b) which show an S-shaped voltammogram corresponding to a complete irreversible charge transfer process. When aptamer was immobilized on the MPA SAM, the semicircle of the EIS curve further increased (Fig. 3c) due to the electronegative phosphate skeleton of the aptamer that perhaps prevent $Fe(CN)_6^{3-/4-}$ ions from reaching the electrode surface for electron transfer during redox reaction (Patel et al., 2010). The aptamer layer on the electrode acts to block charge exchange and mass transfer, further insulating the conductive support and significantly hindering the access of redox probe towards the electrode surface (Sur et al., 2003). The impedance results were quite consistent with the conclusion from the CV experiments that the aptamer was immobilized firmly on the surface of the MPA SAM.

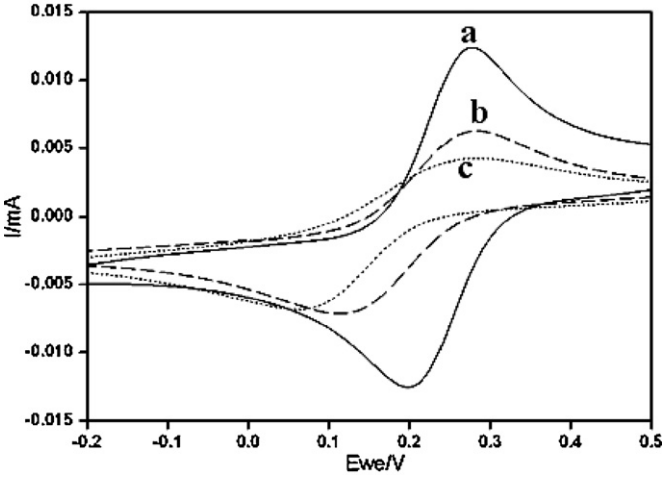


Fig. 2. Cyclic voltamograms of the bare gold (a), MPA SAM electrode (b), and aptamer immobilized electrode (c) between −0.3 and 0.6 V in 2 mM $K_3Fe(CN)_6$ aqueous solution containing 0.1 M KCl. The scan rate is 100 mV/s.

3.3. Selectivity of the aptasensor

Since the main components of Gram-negative bacteria are proteins and nucleic acids, BSA, RNA, and pDNA were chosen as possible interfering substances in order to evaluate the selectivity of the developed aptasensor. The aptasensor was incubated

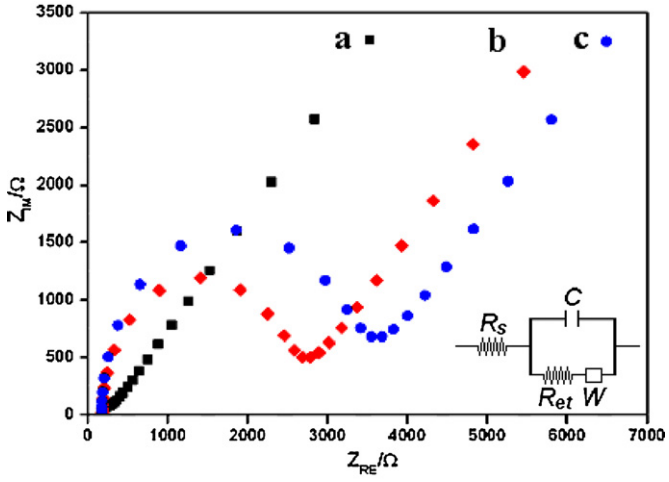


Fig. 3. Nyquist plots of the different electrode in PBS (10 mM, pH 7.4) solution containing 2 mM $Fe(CN)_6^{3-/4-}$: (a) bare gold, (b) MPA SAM electrode, (c) immobilized with aptamer. The frequency range was from 0.1 to 100 Hz with perturbation amplitude of 10 mV. The inset is the equivalent circuit used to model impedance data in the form of R_s, W, R_{et} and C representing the solution resistance, the Warburg diffusion resistance, the electro-transfer resistance and the double layer capacitance, respectively.

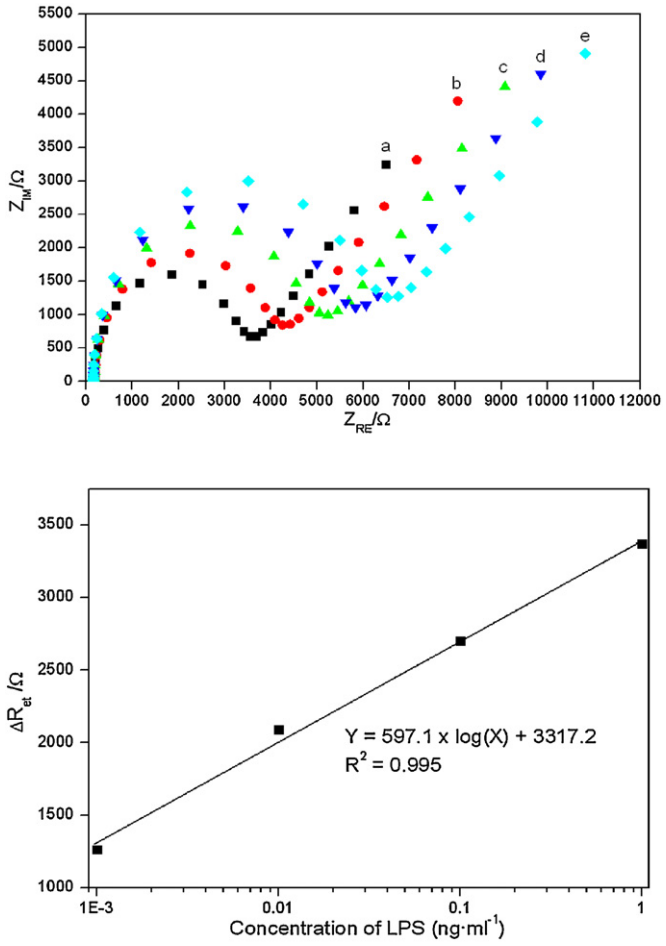


Fig. 4. Nyquist plots of the aptasensor after incubating in LPS/PBS solution (a: aptamer immobilized electrode; b–e: 0.001, 0.01, 0.1, and 1 ng/ml) and the linear relationship between the ΔR_{et} and logarithmic value of LPS concentration.

in 5×10^4 ng/ml BSA/PBS, 2.5×10^4 ng/ml RNA/PBS, and 100 ng/ml pDNA/PBS, respectively. After the aptasensor was washed with PBS solution, the EIS response was recorded as shown in SFig. 1 (the data are shown in the supplementary file). The EIS response of the aptasensor did not change before (SFig. 1a) and after each incubation (SFig. 1b–d) indicating the aptamer has no affinities for those materials. One of the potential advantages of using aptamer as recognition elements in biosensors is the selectivity of the biological molecules for the analytes.

3.4. Electrochemistry of the aptasensor

The prepared aptasensor was used to detect the LPS concentration in a series of samples containing pDNA (100 ng/ml), RNA (2.5×10^4 ng/ml), BSA (5×10^4 ng/ml) and different concentration LPS (0.01 pg/ml to 100 ng/ml) in PBS (10 mM, pH 7.4). As shown in Fig. 4, the increase in electron transfer resistance (R_{et}) was proportional to the logarithmic value of LPS concentration over the range of 0.001–1 ng/ml and the linear regression equation is $\Delta R_{et} = 597.1 \times \log([LPS]) + 3317.2$ with $R^2 = 0.995$. The low limit of detection (1 pg/ml) and good linear relationship suggest that the developed aptasensor could be used to detect endotoxin in actual use. The EIS data of the aptasensor performed in a concentration range lower than 0.001 ng/ml and more than 1 ng/ml was shown in SFig. 2 (the data are shown in the supplementary file).

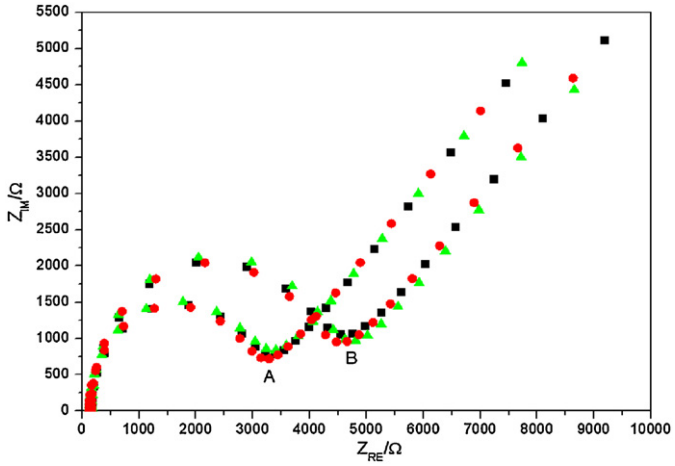


Fig. 5. Nyquist plots of the aptasensor after the regeneration in pH 4 PBS (first sensing, ■; first regeneration, •; second regeneration, ▲): (A) aptamer immobilized electrode and (B) LPS captured electrode.

3.5. Regeneration of the aptasensor

The regeneration of an aptamer-based sensor is important for its practical application. Generally, highly acidic or alkaline surroundings would damage the immobilized aptamer or affect the interaction between aptamer and its target (Qiu et al., 2009). To develop a feasible regeneration process, the proposed aptasensor was immersed in 1 ng/ml LPS of 10 mM PBS with pH value from 4 to 8, respectively (the data are shown in the supplementary file, SFig. 3). For pH 4, the EIS response was similar to that of the original aptamer-immobilized electrode (SFig. 3a and b), indicating that LPS was not captured by the aptasensor. For pH 5–8, the EIS data was response to the aptasensor capturing LPS. The result suggested that the aptasensor was suitable for the environment of pH 5–8 and could be recovered in the pH 4 PBS.

The regeneration of the aptasensor was also evaluated based on its response to 1 ng/ml LPS/PBS (10 mM, pH 7.4) sample. The EIS response was recorded in 2 mM $Fe(CN)_6^{3-/4-}$/PBS (10 mM, pH 7.4) solution before and after the incubation in 1 ng/ml LPS/PBS (Fig. 5A and B black). And the aptasensor was incubated in pH 4 PBS for 10 min and washed with pH 7.4 PBS for several times in order to accomplish aptasensor recovery and LPS detachment. Continuously, the regenerated aptasensor was subjected to 1 ng/ml LPS/PBS solution again and recorded by EIS before and after the incubation. The results indicated that the two R_{et} values and ΔR_{et} values were almost the same as those obtained before the regeneration. Successive experiments showed that the LPS aptasensor could be regenerated twice without losing its sensitivity (Fig. 5A and B, green and red). After a series of three measurements, the regenerated aptasensor yielded in a relative standard deviation (RSD) of 5%, indicating a successful regeneration process.

4. Conclusions

In this study, the impedance biosensor based on aptamer was developed and used to detect LPS at a wide concentration range (1 pg/ml to 1 ng/ml) in the present of pDNA, RNA and BSA. The aptasensor was regenerated under low pH conditions. The deviation of the detection was less than 5% after regeneration. The developed aptasensor has a high affinity for LPS, a low detection limit (1 pg/ml), a good linear relationship with LPS concentration, simple operation and low cost of production. These features demonstrate that the LPS aptasensors may replace or complement the rather expensive antibody/antigen approach which is the major

research method nowadays. Using aptamer for selective detection of LPS has a tremendous prospect in further practical application.

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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.2011.11.009.

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