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A label-free and self-assembled electrochemical biosensor for highly sensitive detection of cyclic diguanylate monophosphate (c-di-GMP) based on RNA riboswitch

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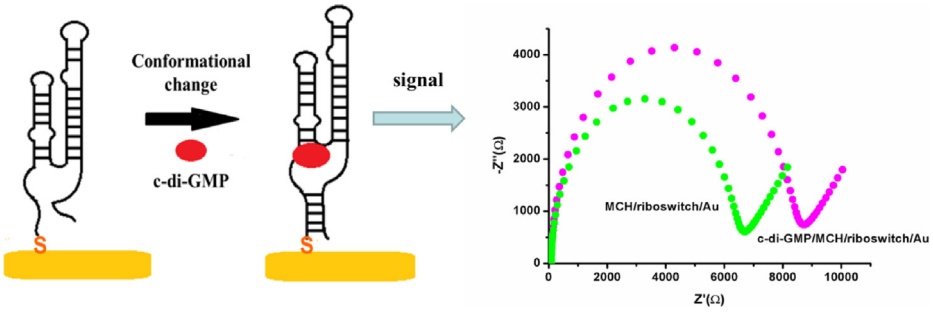
HIGHLIGHTS

- The RNA riboswitch based electrochemical biosensor could realize label-free c-di-GMP detection.
- The biosensor can detect c-di-GMP with highly sensitivity and specificity.
- The biosensor can be easily fabricated with low cost.
- The designed biosensor has an effective measuring range with low detection limit than previous reports.

GRAPHICAL ABSTRACT



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ABSTRACT

Cyclic diguanylate monophosphate (c-di-GMP) is an important second messenger that regulates a variety of complex physiological processes involved in motility, virulence, biofilm formation and cell cycle progression in several bacteria. Herein we report a simple label-free and self-assembled RNA riboswitch-based biosensor for sensitive and selective detection of c-di-GMP. The detectable concentration range of c-di-GMP is from 50 nM to 1 μM with a detection limit of 50 nM.

1. Introduction

Cyclic diguanylate monophosphate (c-di-GMP) was first described in 1987 as an allosteric regulator of cellulose synthesis in *Acetobacter Xylinum* [1]. Several decades later, it has become clear that c-di-GMP plays a central role in the regulation of a variety of complex physiological adaptations including motility, virulence, biofilm formation, and cell cycle progression [2,3]. For example, increased intracellular concentration of c-di-GMP promotes biofilm formation whereas decreased c-di-GMP concentration induces the biofilm to transform to planktonic lifestyle (a loose state for producing virulence factors) in many bacteria [4,5]. C-di-GMP is also involved in broader signaling processes, as it interacts with both the quorum sensing [6] and cAMP [7] signaling pathways, underscoring the importance and widespread effects

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of this second messenger. Therefore, to fully understand the wide range of bacterial physiological processes that c-di-GMP is involved in, methods of c-di-GMP detection need to be established.

To detect c-di-GMP, several approaches, such as tandem HPLC-MS [8], encoded fluorescence resonance energy transfer (FRET)-based biosensor [9], allosteric ribozyme-based biosensor [10] or fluorescent intercalator-based approach [11,12] have been developed. Some of these approaches require expensive and specialized instrumentations, complicated sample preparation or experimental operation, and more importantly, none of the approaches are able to obtain excellent detection range and are limited in a rapid measurement. For example, the fluorescent intercalator-based strategy which exhibited the strongest detection ability among the approaches above has a detection limit of 320 nM [13,14].

The electrochemical method is simple, sensitive, stable and low-cost, thus is widely used for small molecule detections in biological materials (i.e., metal ion, BPA, biological signal molecules) [15]. Riboswitches are a class of structured RNAs that regulate transcription, translation or alternative splicing through specific recognition of target molecules such as small molecule metabolites and second messengers [16–18]. Riboswitch (ligand-binding nucleic acids) could serve as the molecular recognition components of new array-based biosensor devices and are attractive alternatives to antibodies because of their structural stability and ease of manipulation [19]. Two structurally distinct classes of c-di-GMP riboswitches have been described [20,21], of which, the class-I riboswitches (c-di-GMP-I) are more widespread [22]. Herein we developed a biosensor for c-di-GMP detection combining the class-I riboswitch which is 8 nm in size with electrochemical method. The riboswitch was self-assembled as a monolayer on the surface of gold electrode through Au—S bond.

Comparing to cyclic voltammetry (CV), differential pulse voltammogram (DPV) and square-wave voltammogram (SWV), etc., the electrochemical impedance spectroscopy (EIS) is a quantitative label-free characterization method with low destruction to biological samples [23,24], and it exhibits more significant response towards the changes of c-di-GMP concentration in our testing environment. So we designed the electrochemical biosensor for c-di-GMP detection based on EIS, and this proposed riboswitch-EIS-based biosensor has an effective linear detection range from 50 nM to 1000 nM as well as low detection limit of 50 nM with high sensitivity and selectivity.

2. Methods and materials

2.1. Apparatus and reagents

All impedance measurements (EIS) experiments were performed with a CHI660E electrochemical workstation (Chenhua Instruments Co., Ltd., Shanghai, China). The measurements were performed with a conventional three-electrode system consisting of a riboswitch modified gold electrode, a saturated calomel (SCE sat. KCl) reference electrode and a platinum plate auxiliary electrode. All potentials are referred to the reference electrode at room temperature. The ZSimpWin EIS DATA analysis software was used to analyze the obtained EIS data by fitting with a Randle equivalent circuit.

The 3 mm diameter gold electrodes and alumina powder (0.3 and 0.05 μM) were purchased from Aida Instrument Inc. (Tianjian, China). Cyclic diguanylate monophosphate (c-di-GMP), guanosine triphosphate (GTP), adenosine triphosphate (ATP) and adenosine monophosphate (AMP) were purchased from InvivoGen (USA), Thermo (USA), Amresco (USA) and Huamaike biotechnology Co., Ltd. (Beijing, China), respectively. 6-Mercapto-1-hexanol (MCH) was purchased from J&K Chemical (Beijing, China). Cyclic adenosine monophosphate and tris(2-carboxyethyl)phosphine hydrochloride (TCEP) were purchased from Sigma (USA). All chemicals mentioned above were of analytical grade and used without further purification. The Amicon 3 kDa centrifugal filter device (Millipore USA) was used to filter the *Escherichia coli* lysate.

Oligonucleotide tethered with a thiol linker was purchased from Takara (Dalian, China) and purified by HPLC. The sequence is 5′-UUGGUAGGUAGCGGGGUUACCGAUGGCAAUA-(CH2)-HS-3′ (HS-ssRNA-1). Its complementary sequence 5′-GGUGUCACGCA-CAGGGCAAACCAUUCGAAAGAGUGGGACGCAAAGCCUCCGGCCUA-AACCAA-3′ (ssRNA-2) was obtained through *in vitro* transcription by TranscriptAid T7High Yield Transcription Kit (Thermo #K0441), and purified by denature polyacrylamide gel [25]. ssRNA-1 and ssRNA-2 formed the free RNA riboswitch after being heated at 65 °C for 5 min and then gradually cooled down to room temperature.

2.2. Preparation of modified electrode

The Au electrode (3 mm in diameter) was polished sequentially with slurries of 0.3 and 0.05 μM alumina to mirror, followed by immersing in 30%/98% H_2SO_4 (1/3, v/v) solution for 15 min, then by

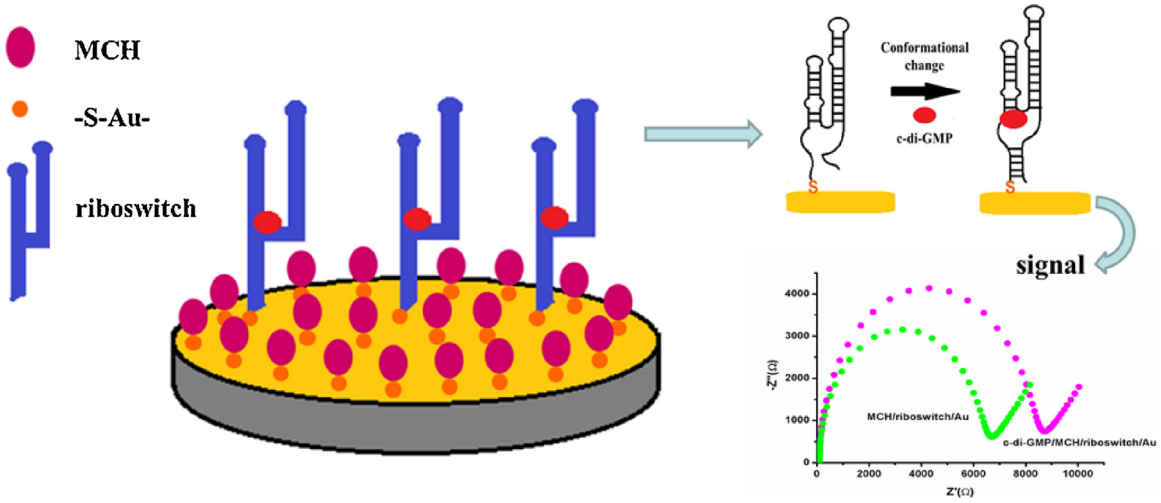

Fig. 1. Schematic representation of the electrochemical riboswitch-based biosensor for c-di-GMP.

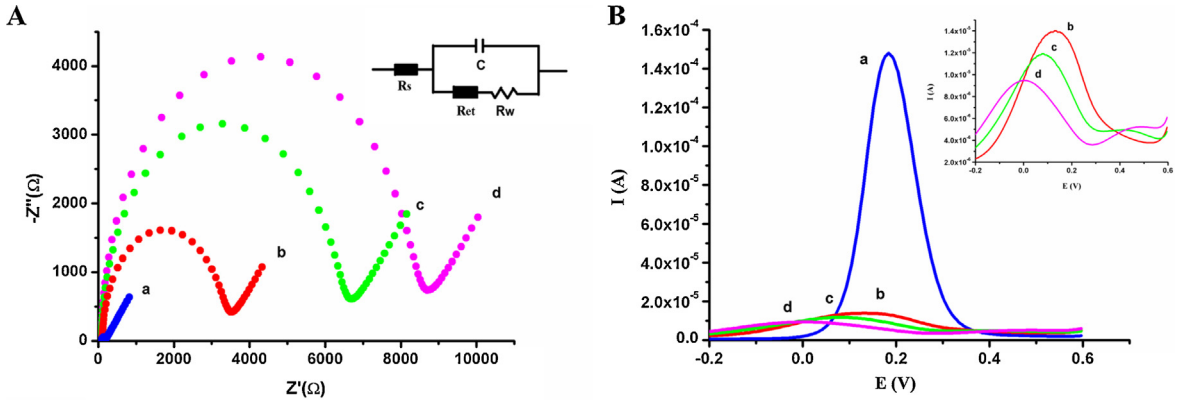

Fig. 2. (A) Nyquist plots of different electrodes in 10 mM PBS buffer solution containing 5 mM $[Fe(CN)_6]^{3-/4-}$ and 100 mM KCl (pH 7.4) (a) Au electrode, (b) riboswitch/Au electrode, (c) MCH/riboswitch/Au electrode, (d) c-di-GMP/MCH/riboswitch/Au electrode (the concentration of c-di-GMP is 100 nM). Insert is the modified Randles equivalent circuit. (B) Typical DPVs of different electrodes in 10 mM PBS buffer solution containing 5 mM $[Fe(CN)_6]^{3-/4-}$ and 100 mM KCl (pH 7.4). The color and letters are consistent with A. Insert is the typical DPVs of the modified electrodes.

ultrasonic washing with ethanol and ultrapure water for 5 min each. Electrochemically cleaning was carried out in 0.5 M H_2SO_4 by a potential scanning between 0 and 1.6 V until a characteristic cyclic voltammogram of a clean Au electrode was obtained. Finally, the electrode was washed with ultrapure water and dried. To fabricate the biosensor, 10 μl solution (10 mM Tris–HCl and 100 mM NaCl, pH 7.4) containing 2 μM free riboswitch (8 nM in size) and 10 mM TCEP (which was used to reduce the disulfide bonds) was dropped onto the electrode surface with incubation for 12 h at 4 °C to allow the riboswitch to assemble on the electrode surface as a monolayer through Au—S bond [13]. The electrode was then soaked in 100 μl of 1 mM 6-mercaptohexanol (MCH) for 30 min to get rid of non-specifically absorbed RNAs and increase the order of the riboswitch monolayer on the electrode surface. Finally, a thoroughly rinse was taken with washing-buffer (10 mM Tris–HCl and 100 mM NaCl, pH 7.4), and the MCH/riboswitch/Au electrode was finally assembled. The schematic representation of this riboswitch-based biosensor for c-di-GMP was shown in Fig. 1 [25].

2.3. Electrochemical detection

All electrochemical experiments were performed in an electrochemical cell containing 10 ml 5 mM $[Fe(CN)_6]^{3-/4-}$] redox probe solution with conventional three-electrode system. The MCH/riboswitch/Au electrode was incubated with a 10 μl droplet containing various concentrations of c-di-GMP in binding buffer (50 mM Tris–HCl, 100 mM NaCl, 100 mM KCl, 10 Mm $MgCl_2$, pH 7.4) for 30 min at room temperature. The electrode was then washed with washing-buffer and transferred into the electrolyte for electrochemical measurement. To determine selectivity, the procedures were the same as before, except that c-di-GMP was replaced by analogues (cAMP, GTP, ATP and AMP). To test the capability of the biosensor to detect c-di-GMP in real sample, a series of *E. coli* lysate samples with different concentrations of c-di-GMP were prepared: firstly, filtered the lysate with the 3 kDa centrifugal filter; then boiled them for 10 min at 95 °C to inactive the residual enzymes to eliminate the background effect of c-di-GMP that possibly existed in samples; finally, added different concentrations of c-di-GMP into the processed samples. All measurements were carried out at room temperature.

3. Results and discussion

3.1. Characterization of the riboswitch modified electrode

In order to monitor immobilization and binding steps of the c-di-GMP/MCH/riboswitch/Au electrode assembly, the corresponding EIS was performed in 10 mM PBS buffer solution containing 5 mM $[Fe(CN)_6]^{3-/4-}$ and 100 mM KCl (pH 7.4) in a frequency range from 0.1 Hz to 100 kHz. The EIS fitting spectra for

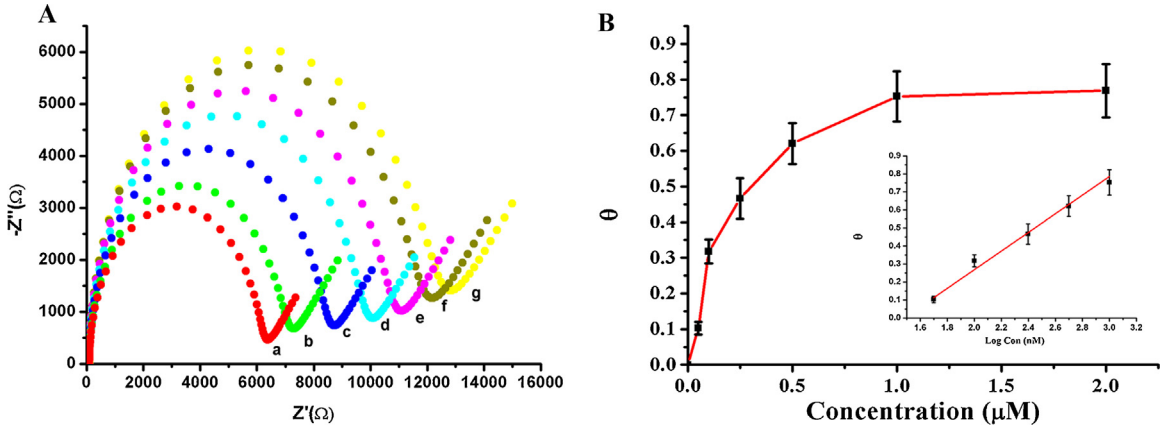

Fig. 3. (A) Nyquist plots of the MCH/riboswitch/Au electrode incubated with the reaction mixture containing different concentrations of c-di-GMP: (a) 0 nM, (b) 50 nM, (c) 100 nM, (d) 250 nM, (e) 500 nM, (f) 1 μM, (g) 2 μM. (B) Relationship between impedance increase rate θ and c-di-GMP concentrations. Insert shows a proportional linear relationship of increasing θ values upon increasing c-di-GMP concentrations. The error bars represent the SEMs (standard error of the mean) of four independent experiments.

Table 1
Values of the modified Randles equivalent circuit parameters with different concentrations of c-di-GMP.

Con (nM)	R_s (Ω cm^2)	C (F/cm^2)	R_{et} (Ω cm^2)	Z_w (S s$^{0.5}$/cm^2)
0	95.94	3.755×10^{-7}	6005	7.074×10^{-4}
50	94.16	3.636×10^{-7}	6784	4.528×10^{-4}
100	93.90	3.737×10^{-7}	8184	4.536×10^{-4}
250	88.20	3.922×10^{-7}	9457	4.423×10^{-4}
500	92.63	3.713×10^{-7}	10380	3.801×10^{-4}
1000	94.60	4.429×10^{-7}	11340	3.297×10^{-4}
2000	99.36	4.420×10^{-7}	11900	2.954×10^{-4}

bare gold, riboswitch/Au and MCH/riboswitch/Au electrode were presented in Nyquist plots (Fig. 2A), where a semicircle in the high frequency region represents an electron transfer limited process and a straight line in the low frequency region is associated with a diffusion controlled process [26,27]. The bare gold electrode conveyed a very small semicircle at high frequency region with a calculated electron transfer resistance (R_{et}) value of 92.57 Ω indicating a fast electron transfer process (Fig. 2A, curve a). In contrast, the R_{et} value of the electrode had a significant increase after the self-assembly of free riboswitch onto the gold electrode (Fig. 2A, curve b). This could be attributed to the fact that the riboswitch monolayer generated an interfacial barrier on the bare gold electrode surface, restricting the access of the electrolyte (i.e., $[Fe(CN)_6]^{3-/4-}$) [28]. After treatment with MCH, the semicircle diameter further increased, and R_{et} increased to 6240 Ω (Fig. 2A, curve c). MCH molecules worked as blockers which could minimize exposed bare gold surface and block the $[Fe(CN)_6]^{3-/4-}$ from the electrode. A more distinct growth with a R_{et} value of 8723 Ω was observed after binding of c-di-GMP with the immobilized-riboswitch (Fig. 2A, curve d), probably due to the conformational changes of the nucleic acid sequence that is closed to the electrode surface: more base pairs have been introduced by the compact conformation after binding (Fig. 1). This result is consistent with previous report that the electrocatalytic effect of nucleic acids can be attenuated after the formation of base pairs [29]. The EIS results demonstrate that the riboswitch-based biosensor was successfully assembled.

Differential pulse voltammetry (DPV) was also conducted to confirm the assembly of monolayer on the gold surface. The electrochemical behavior of these electrodes measured by DPV in the same conditions was presented in Fig. 2B. The distinct DPV peak current (I_{max}) value of bare gold was 148 μA (Fig. 2B, curve a). After the free riboswitch was immobilized on the gold electrode, I_{max} value obviously decreased to 14.1 μA (Fig. 2B, curve b), which was agreed with the result that the self-assembled riboswitch monolayer on the electrode surface hindered electron transfer. After treatment with MCH, the I_{max} value decreased to 11.9 μA (Fig. 2B, curve c), and then further decreased to 9.5 μA after incubation with c-di-GMP (Fig. 2B, curve d). In addition, negative shifts in the peak potential were observed during the self-assembly process of the biosensor, which suggested that immobilization of nucleic acids and MCH through Au—S bond and binding of c-di-GMP to nucleic acids changed the electrostatic interactions environment so that the availability of electrons on the electrode surface is reduced, thus the reduction reaction of $[Fe(CN)_6]^{3-/4}$ was hindered. This finding is also consistent with previous report [30]. The results of DPV were in agreement with EIS data, and demonstrated that the c-di-GMP riboswitch was successfully immobilized on the gold electrode surface through Au—S bond.

3.2. Performance of the riboswitch-based electrochemical biosensor on c-di-GMP detection

Next, the electrochemical response of MCH/riboswitch/Au electrode upon binding of different concentrations of c-di-GMP was studied using EIS in 10 mM PBS buffer solution containing 5 mM $[Fe(CN)_6]^{3-/4-}$ and 100 mM KCl (pH 7.4). As shown in Fig. 3A, the EIS signals increased significantly with the increasing concentrations of c-di-GMP. The values of the modified Randles equivalent circuit parameters were detailed in Table 1, where the R_{et} values presented a larger change compared to the changes of double layer capacitance (C) and Warburg diffusion (Zw). The relative impedance increase rate θ was calculated by the following equation: $\theta = (R_i - R_0)/R_0$ (R_0 and R_i were the R_{et} value before and after incubation with c-di-GMP, respectively). Relationship between θ and concentrations of c-di-GMP from 0 nM to 2 μM was shown in Fig. 3B. A linear relationship of the logarithmic concentration of c-di-GMP in a range of 50–1000 nM with a detection limit of 50 nM was presented (Fig. 3B, insert. Unit of concentration is nM). The regression equation of the impedance increase rate is $\theta = 0.517\log(\text{Con}) - 0.766$ and the regression coefficient is 0.979. With further increase in the c-di-GMP concentration to 2 μM, the EIS signals began to level off, which is presumably due to the saturation of the binding sites in the riboswitches immobilized on the electrode surface.

3.3. Selectivity of the c-di-GMP riboswitch-based biosensor

Besides sensitivity, selectivity is also an important feature for a biosensor. In order to assess the sensor performance in the view of selective recognition of c-di-GMP, control experiments had been performed, and the results were shown in Fig. 4. Without riboswitch monolayer, the impedance value of the MCH-modified electrode showed almost no change before and after incubation

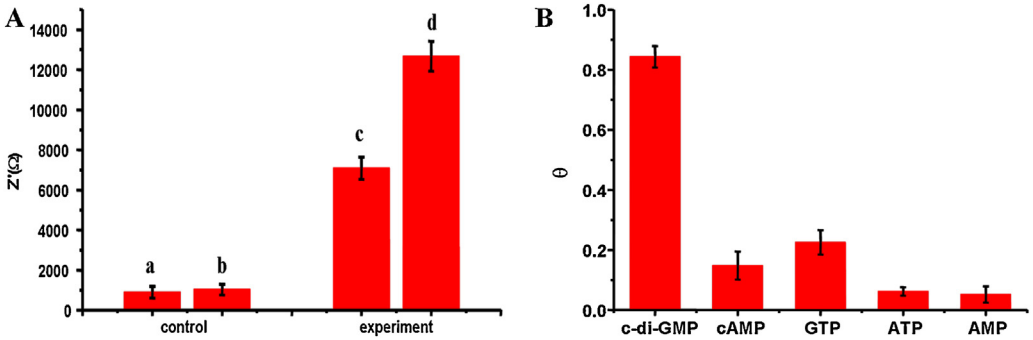

Fig. 4. (A) Impedance value (R_{et}) of the gold electrode at different states: (a) MCH/Au electrode, (b) MCH/Au electrode after incubation with 1 μM c-di-GMP, (c) MCH/riboswitch /Au electrode, (d) MCH/ riboswitch /Au electrode after incubation with 1 μM c-di-GMP. (B) Selectivity of the riboswitch-based biosensor for c-di-GMP compared to its analogues. (1 μM of c-di-GMP, cAMP, GTP, ATP and AMP, respectively). All measurements were performed in PBS buffer solution containing 5 mM $[Fe(CN)_6]^{3-/4-}$ and 100 mM KCl (pH 7.4). The error bars represent the SEMs (standard error of the mean) of three measurements.

with 1 μM c-di-GMP. Furthermore, 1 μM analogues with similar structure were used to replace c-di-GMP under the same experimental conditions as before. As summarized in Fig. 4B, for cAMP, GTP, ATP or AMP, only a small impedance change can be observed. However, the impedance increase rate θ increased significantly in the presence of c-di-GMP. These results demonstrated that this designed riboswitch-based biosensor can detect c-di-GMP selectively. It is noteworthy that despite of the lager molecular weight of riboswitch compared to other nucleic acid probes (i.e., aptamer), our results demonstrate that this nanoscale structured RNA still can realize the sensitive detection of target molecules.

The capability of the proposed biosensor to test c-di-GMP in real sample has also been investigated. We found that the EIS signal is greatly interfered in the unfiltered bacteria lysate, which is consistent with previous report that the proteins in the biological samples can easily affect the reaction surface due to the adsorption and blocking [31–33]. However, the interference can be weakened upon protein removal with a 3 kDa filter (Fig. S1). Notably, the proposed biosensor still has sensitivity and selectivity in the filtered *E. coli* lysate (Fig. S2). The regression equation of the impedance increase rate in the filtered *E. coli* lysate is $\theta = 0.393\log(\mathrm{Con}) - 0.519$ and the regression coefficient is 0.925. This result indicates that the proposed electrochemical biosensor is competent for c-di-GMP detection in biological samples as well.

4. Conclusions

In this study, we have developed a label-free electrochemical biosensor based on self-assembled riboswitch for sensitive and selective detection of c-di-GMP. The detection limit of this biosensor is 50 nM, which is much lower than previously reported value which is about 320 nM [14]. Furthermore, this biosensor has provided a possible promising approach for multichannel detection of small molecules using specific riboswitch based on its merits of high specificity, low cost and easy fabrication.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aca.2015.04.061.

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