Analytica Chimica Acta 897 (2015) 10–16

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

A regenerated electrochemical biosensor for label-free detection of glucose and urea based on conformational switch of i-motif oligonucleotide probe

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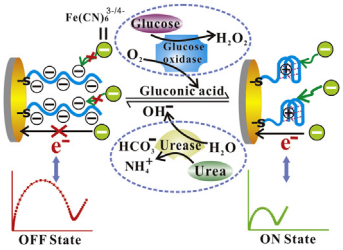
HIGHLIGHTS

- Conformational switch of i-motif is used for the detection of glucose and urea.
- The sensor can be regenerated.
- The proposed method is successfully applied in real sample assay.
- Our method is label-free and inexpensive.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 24 November 2014
Received in revised form
24 March 2015
Accepted 18 September 2015
Available online 1 October 2015

Keywords:
Regeneration
Electrochemical biosensor
Glucose
Urea
i-motif oligonucleotide

ABSTRACT

Improving the reproducibility of electrochemical signal remains a great challenge over the past decades. In this work, i-motif oligonucleotide probe-based electrochemical DNA (E-DNA) sensor is introduced for the first time as a regenerated sensing platform, which enhances the reproducibility of electrochemical signal, for label-free detection of glucose and urea. The addition of glucose or urea is able to activate glucose oxidase-catalyzed or urease-catalyzed reaction, inducing or destroying the formation of i-motif oligonucleotide probe. The conformational switch of oligonucleotide probe can be recorded by electrochemical impedance spectroscopy. Thus, the difference of electron transfer resistance is utilized for the quantitative determination of glucose and urea. We further demonstrate that the E-DNA sensor exhibits high selectivity, excellent stability, and remarkable regenerated ability. The human serum analysis indicates that this simple and regenerated strategy holds promising potential in future biosensing applications.

1. Introduction

DNA has now become a versatile building material in novel nanostructure fabrication and nanomachines design due to its conformational polymorphism and programmable sequence recognition [1,2]. A variety of nanodevices that use DNA conformational switch have recently been developed, which provide a new tool for biological detection and drug delivery application [3,4]. The electrochemical DNA (E-DNA) sensor, first developed by Fan et al., in 2003 [5,6], is one of the most promising sensors that can potentially be used in the detection of diverse analytes not only in pure buffer solutions but also in many complex matrixes [7–10].

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http://dx.doi.org/10.1016/j.aca.2015.09.045
0003-2670/

In recent years, E-DNA sensor has gained substantial popularity, owing to the various merits it possesses, which include easy to use, fast response, inexpensive and direct use in complicated samples [11,12]. Despite the obvious progress achieved in this area, the reproducibility of electrochemical signals from E-DNA sensor still remains further investigation. To circumvent this limitation, several strategies have been developed to construct regenerated sensor for small molecules [13], nucleic acids [11,14], and proteins detection [15,16]. In addition, our group recently has developed a regenerated DNA sensor for single-nucleotide polymorphisms detection [17]. In this way, improving the regenerability is a critical area for the development of E-DNA sensor moving forward.

Cytosine-rich (C-rich) nucleic acids, normally known as a kind of pH-sensitive materials, appear in the human genome frequently, especially the telomere of human chromosomes [18]. Under acidic conditions, a unique four-stranded nucleic acid secondary structure is formed, commonly known as i-motif [19,20]. This structure is formed from two parallel-stranded oligonucleotide duplexes in an antiparallel orientation containing hemi-protonated cytosine–cytosine ($C–C^+$) base pairs [21,22]. While in neutral or basic aqueous solution, i-motif is not stable and the strand adopts a random coil conformation [23,24]. Recently, i-motif oligonucleotide probe-based literature have focused on challenging task of identifying the physical mechanism [20,21], sensing pH [25,26], and constructing pH-induced nanoswitches [27,28]. However, few reports concentrate on the fabrication of i-motif oligonucleotide probe-based sensor for small molecular detection. An interesting design using conformational switch of i-motif DNA and non-crosslinking gold nanoparticles was developed by Qu and co-workers for visual detection of glucose [29]. Li and co-workers introduced conformational switch of i-motif DNA and unmodified gold nanoparticles for identification of pyruvic acid [30]. Although the above mentioned reports are well-conducted and inspiring, one potential limitation on currently reported sensor is inherently single-target detection protocols. Thus, the development of dual or multiplexed analysis biosensor is becoming one of the challenging topics in biosensor filed.

It has been demonstrated that glucose (Glu) can be oxidized to gluconic acid to decrease the pH by glucose oxidase (GOD)-catalyzed reaction [31,32], and urease can hydrolyze urea into NH_3 to increase the solution pH [33, 34]. Recently, Wang and co-workers proposed a sensing platform, based on chitosan-reduced graphene oxide/concanavalin A layer, for the detection of glucose and urea in one sample [35]. Although impressive results have been achieved, the sensor is not regenerated and the fabrication procedure suffers from cumbersome and time-consuming. Here we propose a regenerated E-DNA sensor for dual target, glucose and urea, detection based on conformational switch of i-motif oligonucleotide probe for the first time. The switchable property of the regenerated E-DNA sensor is controlled by *in situ* biochemical reactions. Specifically, the random coil conformation of C-rich DNA, which could fold into i-motif structure under acid conditions, was first modified on the gold electrode. Then, the modified sensor was immersed in the solution containing GOD or urease. When the target, Glu, appears in the assay, the *in situ* generation of gluconic acid provides H^+ by the GOD-catalyzed reaction and decreases the pH value of the solution, leading to the formation of i-motif structure and ON state of the sensing system. Similarly, the urease-catalyzed reaction can be triggered by the target (urea) and increases the pH value. In this case, the DNA recovered to random coil conformation, leading to the OFF state of interfacial electron transfer process. The Glu and urea detection processes are feasible by the target-controlled conformational transition of C-rich DNA. The conformational transition of C-rich DNA can be read out by EIS method. Compared to the traditional E-DNA sensor, this biosensor is unique in some characteristics, which are as follows: (i) i-motif structure as a molecular recognition module is first introduced into the dual detection of Glu and urea. (ii) A redox pair $Fe(CN)_6^{3-/4-}$ is utilized as a redox indicator for recording the conformational switch of C-rich DNA at the electrode interface. Our method is label-free and inexpensive. (iii) The regenerated E-DNA sensor, based on pH-programmed electrocatalytic reaction, can overcome the fatal weakness of the irreproducibility in previous reported assays. This sensing system is simple in design and can be carried out easily. It may also provide a potential application for the design of Glu/urea-triggered i-motif-based nanodevices and logic gates.

2. Experimental section

2.1. Materials

6-Mercaptohexanol (MCH), Tris-(2-carbozyethyl) phosphine hydrochloride (TCEP), HPLC-purified DNA oligonucleotides, glucose oxidase, and urease were purchased from Sangon Inc., Shanghai, China. Other chemicals employed were obtained from Aladdin Reagent Co., Ltd. All chemicals are of analytical reagent grade and were used without further purification. All DNA sequences are listed in Table S1 in Supporting Information. All buffer solutions were prepared by ultrapure water with a resistivity of 18.2 MΩ cm. The oligonucleotides probe was dissolved in 10 mM TCEP for 2 h in the dark to reduce disulfide bound. The volume of working solution was defined as 20 mL. The electrolyte solution used in this work was 5.0 mM $Fe(CN)_6^{3-/4-}$ containing 0.2 M NaCl, and HCl or NaOH was employed to adjust the pH of the electrolyte.

2.2. Electrochemical measurements

Electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV) measurements were carried out with a conventional three-electrode cell on a CHI660d electrochemical station (Chenhua Instruments Co., Shanghai, China). The three-electrode cell contains a gold electrode as the working electrode (2.0 mm diameter, CHI Co., Ltd., Shanghai, China), an Ag/AgCl reference electrode, and a platinum wire as the counter electrode. The cell was enclosed in a grounded Faraday cage. EIS was recorded at a frequency ranging from 10 kHz to 100 mHz by 5 mV amplitude. CV was recorded from −0.3 to +0.7 V at 0.1 V s^{-1}. All measurements were repeated for three times to obtain statistically meaningful results and conducted at room temperature.

2.3. Electrode cleaning and sensor preparation

The gold electrode was firstly polished for 3 min carefully with alumina powder (0.3 μm) and followed by polishing for 10 min using 0.05 μm alumina powder. The polished electrode was rinsed thoroughly by ultrapure water, and then sonicated in ultrapure water, ethanol, and ultrapure water for 3 min, respectively. Subsequently, the electrode was electrochemically cleaned by using cyclic voltammetry in 0.5 M H_2SO_4 for 25 cycles at the scan rate of 0.1 V s^{-1} in the range of −0.3–1.6 V and then dried with nitrogen.

The E-DNA sensors were fabricated according to our established procedure [36]. The thiolated DNA was prepared by 10.0 mM Na_2HPO_4–citrate acid buffer (1 M NaCl, pH 3.4). The cleaned gold electrode was modified with the relevant DNA probe by immersion in a 0.2 μM DNA solution for 30 min at room temperature. Then, the gold surface was rinsed with deionized water, and immersed in 2.0 mM MCH solution for 2 h. The DNA area percentage on the gold electrode surface was 97.1% calculated by our reported method [37]. The DNA-modified electrodes were stored at 4 °C in 10.0 mM Tris–HCl buffer (0.1 M NaCl, 5.0 mM $MgCl_2$, pH 7.5) before use. For

practical applications, human serum was obtained from healthy volunteers and diluted 500-fold for the detection.

3. Results and discussion

3.1. Principle of glucose and urea assay

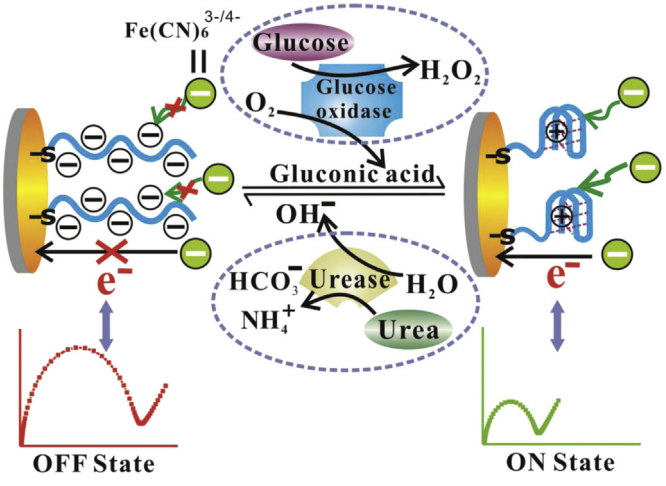
The E-DNA sensor architecture and signaling mechanism is shown in Scheme 1. The C-rich DNA modified electrochemical sensor is able to switch interfacial electron transfer reactions ON and OFF upon the addition of Glu and urea. This pH-switchable DNA interface allows transduction of pH changes, generated upon GOD or urease-catalyzed reaction, into electronic signals, which can be read out by EIS. Specifically, the *in situ* generation of gluconic acid provides proton to the solution by the GOD-catalyzed reaction and decrease the pH value (eq (1)), leading to the formation of i-motif structure. In this situation, the hemi-protonated DNA skeleton is favorable for the interfacial electron transfer, and the system presents ON state with a lower electron transfer resistance value. On the other hand, the solution pH is converted to alkaline by the urease-catalyzed reaction due to the generated ammonia (eq (2)). Thus, random coil conformation of DNA is recovered, switching OFF the interfacial electron transfer processes. A high electron transfer resistance can be observed due to the inaccessibility of the DNA for $Fe(CN)_6^{3-/4-}$ probe. Based on these enzyme-catalyzed reactions, the concentrations of Glu and urea can be quantitatively determined by EIS.

$$\text{glucose} + O_2 + H_2O \xrightarrow{\text{GOD}} \text{gluconic acid} + H_2O_2 \quad (1)$$

$$NH_2CONH_2 + 3H_2O \xrightarrow{\text{urease}} 2NH_4^+ + HCO_3^- + OH^- \quad (2)$$

3.2. pH-switchable behavior of the sensor toward standard buffer

The modification process was first characterized by CV. As shown in Fig. 1A, the bare gold electrode exhibits a couple of typical redox peaks of $Fe(CN)_6^{3-/4-}$ (curve a). With the successive modification of DNA (curve b) and MCH (curve c), the difference of peak position was increased gradually, whereas the peak current was decreased. The conformational switch progress of C-rich DNA at various pH values was then characterized by EIS (Fig. 1B). In week acidic Britton–Robinson buffer (B-R buffer) solution, intramolecular noncanonical base pair interactions between a protonated and an unprotonated cytosine residue interdigitated to form i-motif conformation. The negative charged DNA was partially neutralized by the proton-bridged i-motif structure, switching ON the interfacial electron transfer processes between the electrode surface and the redox label. Thus, lower interfacial electron transfer resistances were obtained. The Faradaic impedance spectra were presented as Nyquist plots. The obtained results were fitted to the Randles circuit: $R_s(Q(R_{ct}W))$ (inset, Fig. 1B), where R_s is the solution resistance, Q is the constant phase element, R_{ct} is the charge transfer resistance, and W is the Warburg impedance. Under the neutral or alkaline conditions, the C-rich DNA maintained an extended single strand configuration. The electron transfer was hindered between gold surface and electrolyte solution due to the negatively charged oligonucleotides skeleton, presenting higher interfacial electron transfer resistance and turning OFF the electron transfer reactions. The ΔR_{ct} value was linear to pH value in the range from 4.5 to 7, and it increased slightly with increasing pH value. The calibration equation is $\Delta R_{ct} = 1.299\ \text{pH} - 5.661$, with a correlation coefficient $R^2 = 0.9852$ (Fig. S1). The EIS responses of ON–OFF behavior between pH 7.0 and 6.0 in B-R buffer are shown in Fig. 1C, indicating that the proposed E-DNA sensor has good stability and reversibility. In addition, the ON-OFF behavior was further confirmed by CV. The CV diagrams of the sensor at different stages are presented in curves c and d in Fig. 1A. The observed CV behavior was in accordance with that of EIS test. A control experiment was done with a random DNA sequence, which cannot form i-motif structure. Slight change of impedance spectrum was observed between pH 6.0 and 7.0 (Fig. S2). The incubation time was also optimized. It can be seen from Fig. S3, it takes 10 min to form i-motif structure and 15 min is needed to recover to random single strand DNA. Based on the above results, we assume that the design of regenerated strategy driven by Glu and urea biocatalysis is enabled.



Scheme 1. Schematic representation of glucose and urea detection processes.

3.3. Feasibility of glucose and urea assay

In order to demonstrate the feasibility of Glu and urea assay, we investigated the enzyme-catalyzed reactions activated by Glu and urea, respectively. We first applied GOD-catalyzed reactions to change the pH value. The experiments started under the neutral state of the bioelectrocatalytic system (OFF state, 5.0 U mL^{-1} GOD, 5.0 mM $Fe(CN)_6^{3-/4-}$, 0.2 M NaCl, pH 7.0). In this situation, the C-rich DNA-modified electrode was redox inactive and not able to mediate the electron transport, presenting a high electron transfer resistance (Fig. 2A, curve a). The system was saturated with air and included O_2 as a natural electron acceptor for GOD. To activate the redox process and switch ON the system, 5.0 mM Glu was added to the solution. This resulted in the production of gluconic acid biocatalyzed by GOD, thus leading to the formation of i-motif structure and activation of the interfacial electron transfer reactions. The impedance spectrum recorded at this state of the system exhibits a low electron transfer resistance (Fig. 2A, curve b). In addition, H_2O_2 was the by-product of GOD-catalyzed reaction. According to the previous report [30], H_2O_2 alone cannot result in the efficient oxidative cleavage of DNA. So the cleavage effect of H_2O_2 to DNA can be ignored. Then, the investigation of urease-catalyzed reaction was included (Fig. 2B). The initial state of the experiment is weak acid (ON state, 1.0 U mL^{-1} urease, 5.0 mM $Fe(CN)_6^{3-/4-}$, 0.2 M NaCl, pH 6.0). First, the C-rich DNA modified sensor was incubated in this weak acid solution for 10 min to form i-motif structure. Then, 0.1 mM urea was added to the solution to switch OFF the bioelectrocatalytic process. This resulted in the formation of NH_4^+ and OH^-, thus increasing the pH value of the solution. As a result, i-motif structure was destroyed and DNA probe recovered to random

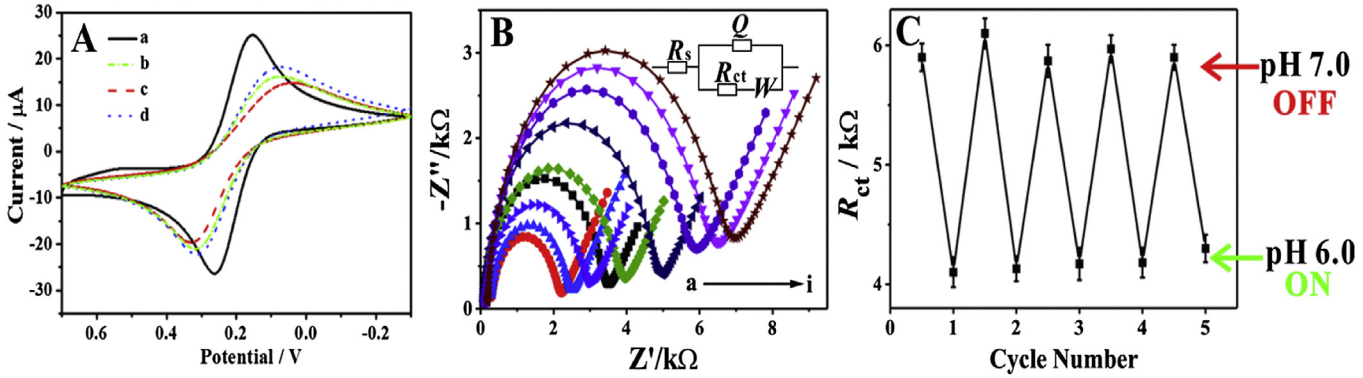


Fig. 1. (A) CV diagrams of the DNA sensor at different stages: (a) bare gold electrode (5.0 mM $Fe(CN)_6^{3-/4-}$, 0.2 M NaCl, pH 7.0); (b) assembly of the C-rich DNA probe (5.0 mM $Fe(CN)_6^{3-/4-}$, 0.2 M NaCl, pH 7.0); (c) assembly of the C-rich DNA probe and MCH (5.0 mM $Fe(CN)_6^{3-/4-}$, 0.2 M NaCl, pH 7.0); (d) assembly of the C-rich DNA probe and MCH (5.0 mM $Fe(CN)_6^{3-/4-}$, 0.2 M NaCl, pH 6.0) (B) EIS plots of E-DNA sensor at different pH stages (a–i): 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0. The EIS measurements were carried out in different pH B-R buffers containing 5 mM $Fe(CN)_6^{3-/4-}$ and 0.2 M NaCl (C) Reversibility of E-DNA sensor in pH 6.0 and 7.0 B-R buffer solutions containing 5 mM $Fe(CN)_6^{3-/4-}$ and 0.2 M NaCl. Error bars represent the standard deviation of three replicates.

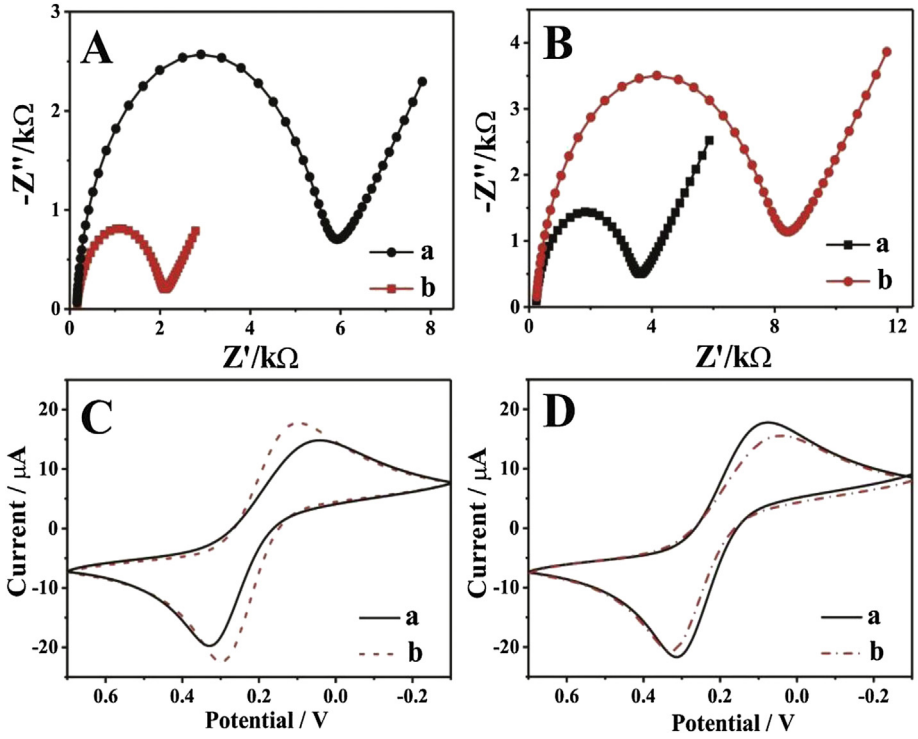


Fig. 2. EIS (A) and CV (C) responses of the E-DNA sensor in Glu assay (a) 5.0 mM $Fe(CN)_6^{3-/4-}$ solution (0.2 M NaCl, 5.0 U mL^{-1} GOD, pH 7.0), and (b) 5.0 mM $Fe(CN)_6^{3-/4-}$ solution (0.2 M NaCl, 5.0 U mL^{-1} GOD, pH 7.0) + 5.0 mM Glu; EIS (B) and CV (D) responses of the E-DNA sensor in urea assay (a) 5.0 mM $Fe(CN)_6^{3-/4-}$ solution (0.2 M NaCl, 1.0 U mL^{-1} urease, pH 6.0), and (b) 5.0 mM $Fe(CN)_6^{3-/4-}$ solution (0.2 M NaCl, 1.0 U mL^{-1} urease, pH 6.0) + 0.1 mM urea.

coil conformation, which inhibited the electron transfer process. Fig. 2B, curve a, shows the impedance spectrum at the initial ON state of the E-DNA sensor with the low electron transfer resistance. Addition of the urea resulted in the inactive interfacial electron transfer reactions and the increase of the R_{ct} to a high value (curve b). To further confirm the conformational switch, we adopted CV to study the "ON-OFF" behavior. It can be seen that the redox current of the solution containing 5.0 mM Glu was higher than the neutral state (Fig. 2C). And a lower redox current was obtained after the urease-catalyzed reaction occurred (Fig. 2D). The above results reveal that our proposed strategy might offer a potential for sensitive and selective detection of Glu and urea.

3.4. Influence of different lengths of DNA

The oligonucleotides with 21-mer, 34-mer, and 47-mer, named C-21, C-34, and C-47, were investigated by EIS and CV measurements in this experiment. The measurements were conducted in 5.0 mM $Fe(CN)_6^{3-/4-}$ solution containing 0.2 M NaCl between pH 6.0 and 7.0 and results are shown in Fig. S4. It can be observed that signal changes of EIS markedly decreased with increasing DNA length (Fig. S4A). Fig. S4B shows the anodic peak current changes (ΔI_{pa}) after CV measurements in pH 7.0 and 6.0 solutions. We found that the anodic peak currents were all decreased under different lengths of DNA assembled conditions, which further confirm the

structure of C-rich DNA monolayer was changed on the electrode surface. It is worth noting that as the length of DNA probe increased from 21 to 47, the ΔI_{pa} value decreased from the best 3.01 to 1.47 μA. The reason might be that the linear DNA is inherently flexible, and the longer DNA probe has lower mechanical rigidity than shorter DNA probe, leading to the accessibility of redox probe to gold surface [38]. In addition, the E-DNA sensor modified with longer DNA probe has a rather complicated situation, including poor orderliness and strong interstrand entanglement [39], which might be another reason. Thus, 21-mer DNA probe was employed in our assay.

3.5. Sensitivity and selectivity of the DNA sensor

We investigated the sensitivity of the E-DNA sensor to verify its potential to being a simple protocol to detect Glu and urea. According to Fig. 3A, as the concentration of Glu increased, the interfacial electron transfer resistances decreased. The corresponding calibration plot (Fig. 3B) of Glu concentration *vs.* ΔR_{ct} (the difference of impedance before and after incubation with the Glu) exhibits a dynamic range from 0.4 to 0.8 mM with a R^2 of 0.9812. The regression equation is $\Delta R_{ct} = 5.88\ C_{Glu} - 1.38$. A detection limit of 41 μM is obtained based on 3σ/slope (where σ is the standard deviation). The sensitivity of the described strategy towards urea was also examined. Fig. 3C shows the corresponding EIS responses with increasing urea concentrations. The ΔR_{ct} value possesses a linear correlation to the concentration of urea in the range from 5 to 40 μM (Fig. 3D). After that, a plateau is reached and further increase of urea concentration does not cause an obvious change in interfacial electron transfer resistance. The calibration equation is $\Delta R_{ct} = 0.068\ C_{urea} + 0.017$, with a correlation coefficient $R^2 = 0.9857$. The detection limit for urea is determined to be 2.2 μM. The sensitivity of the proposed method is better to some previously reported sensors for Glu and urea detection [35,40]. The time-dependent pH changes in the presence of different amounts of Glu and urea were also recorded. We found that the pH changes produced *in situ* remained stable at a reaction time of 60 min in the presence of Glu (Fig. S5A). And 10 min incubation was required for urease-catalyzed reaction to allow the pH value to reach saturation (Fig. S5B).

Furthermore, we performed a series of contrast experiments using several common interferences, including fructose (Fru), lactose (Lac), saccharose (Sac), maltose (Mal), uric acid (UA), and dopamine (DA), to evaluate the selectivity of the proposed E-DNA sensor. As indicated in Fig. 4A, no obvious interfacial electron transfer resistance changes were obtained toward these interferences. However, a significant change of interfacial electron transfer resistance was observed in the presence of Glu. And, Fig. 4B shows that, UA, DA, Fru, Mal, and Glu caused no noticeable changes in interfacial electron transfer resistance, while the signal was greatly increased by the addition of urea. The experimental results indicate that the proposed method exhibited high specificity for Glu and urea detection.

3.6. Practical application

To evaluate the applicability of the proposed strategy to real samples, recovery experiments for human serum with different Glu or urea concentrations were carried out. The experimental results are summarized in Tables S2 and S3 in the Supporting Information, from which it can be seen that Glu concentration recoveries of 98.0–103.8% were achieved with the relative standard deviation

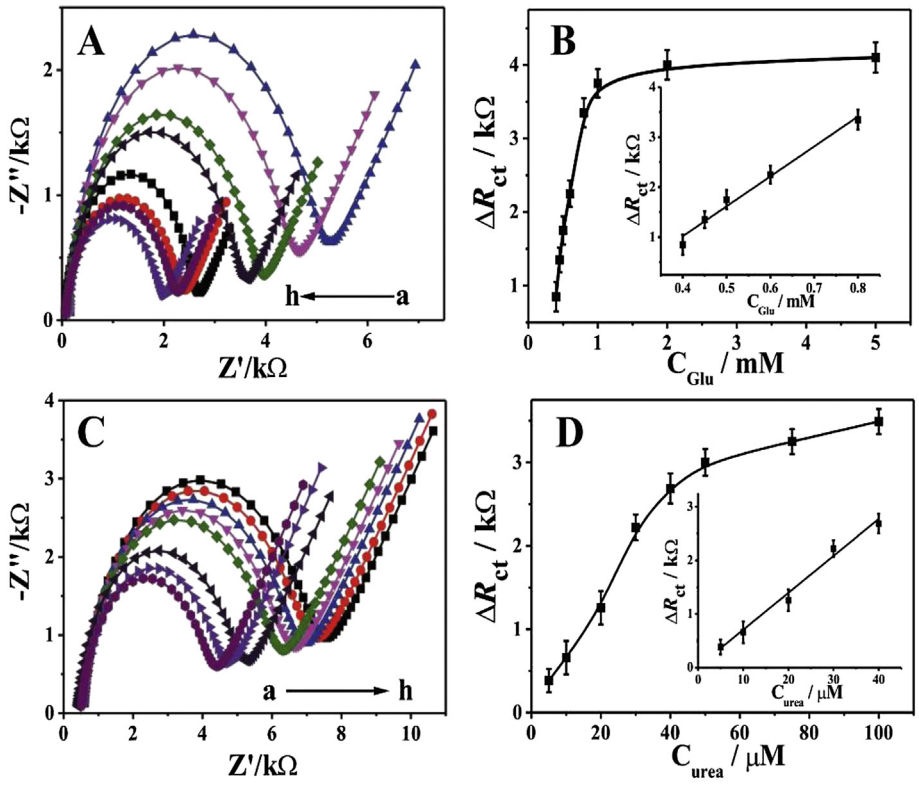


Fig. 3. (A) EIS plots of the E-DNA sensor in 5.0 mM $Fe(CN)_6^{3-/4-}$ solution (0.2 M NaCl, 5.0 U mL^{-1} GOD, pH 7.0) after injection of different concentrations of Glu (a–h: 0.4 mM, 0.45 mM, 0.5 mM, 0.6 mM, 0.8 mM, 1.0 mM, 2.0 mM and 5.0 mM) (B) The corresponding calibration curve (C) EIS plots of the E-DNA sensor in 5.0 mM $Fe(CN)_6^{3-/4-}$ solution (0.2 M NaCl, 1.0 U mL^{-1} urease, pH 6.0) after injection of different concentrations of urea (a–h: 5.0 μM, 10.0 μM, 20.0 μM, 30 μM, 40.0 μM, 50.0 μM, 80.0 μM, and 100.0 μM) (D) The corresponding calibration curve. Error bars represent the standard deviation of three replicates.

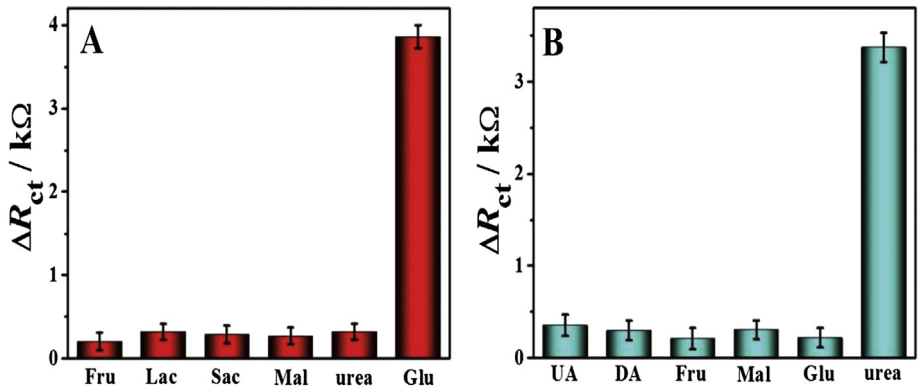


Fig. 4. Selectivity investigations of Glu sensing (A) and urea sensing (B). All interfering substances were tested at 5.0 mM. The concentration of Glu was 5.0 mM in Glu sensing and 0.1 mM urea was employed in urea sensing. Error bars represent the standard deviation of three replicates.

(RSD) of 4.72–5.15% and satisfactory recoveries of urea ranged from 99.3% to 106.0% were obtained with RSD of 3.55–4.02%. The results indicate the reliability of our proposed assay for Glu and urea determination in practical samples.

3.7. Regenerability and stability of the DNA sensor

The electrochemically reversible redox process of the E-DNA sensor was studied *in situ* by EIS upon the application of GOD-catalyzed and urease-catalyzed reactions, respectively. The GOD-catalyzed experiments were started at pH 7.0 when the system was in the OFF state, and the conformational changes of DNA at the sensor surface were monitored by EIS. A large electron transfer resistance was observed in the OFF states (Fig. S6A). The addition of glucose (5 mM) resulted in the ON state and R_{ct} was decreased to a much smaller value after biocatalytic reaction. After 10 min incubation in the solution of pH 7.0, OFF state was recovered. The E-DNA sensor exhibits high reproducibility over the 5 cycles of regeneration. Fig. S6B shows typical impedance spectra recorded when the E-DNA sensor was in the ON state at pH 6.0, and when it was in the OFF state after 0.1 mM urea added. The ON state demonstrates a low R_{ct} value corresponding to the proceeding of interfacial electron transfer process. The OFF state shows a high electron transfer resistance, reflecting the inhibition of the interfacial electron transfer process due to the "successful" urease-catalyzed reaction. It should be noted that the E-DNA sensor exhibits good renewability after 5 cycles replicated test as well. To further exploit the stability of our proposed E-DNA sensor, the C-rich DNA modified electrode was stored in Tris–HCl buffer (10 mM, 0.1 M NaCl, 5 mM $MgCl_2$, pH 7.5) in the refrigerator at 4 °C over 9 days. We found that even ~98% initial signal was remained, indicating a quite satisfactory stability (Fig. S7).

3.8. Switchable behavior of the sensor toward glucose oxidase and urease coexistence solution

In order to prove that the designed E-DNA sensor could work in complicated biochemical systems, we applied glucose and urea to generate the pH changes in one sample without separation. The time-dependent pH changes measured *in situ* are shown in Fig. 5A. We designed the experiments started at pH 6, when i-motif structure formed on the electrode surface and the system presented ON state. Impedance spectrum recorded at this pH shows a low R_{ct} value (Fig. 5B). After 40.0 μM urea was added to the solution, the urease-catalyzed reaction was activated and increased the pH of solution to 7.0 in about 20 min, thus resulting in the OFF state and a high interfacial electron transfer resistance. Then, 0.6 mM Glu was added to reset the initial pH, switching ON the interfacial electron transfer process. The *in situ* produced gluconic acid decreased the solution pH to 6.0 in about 40 min. Meanwhile, the interfacial electron transfer resistance recovered to the similar results at the beginning of the experiment. After another 40.0 μM urea was added, the solution became neutral and interfacial electron transfer progress was changed to the OFF state again in about 30 min. The observed longer reaction time might attribute to the following factors: (1) the neutralization reaction between the residual

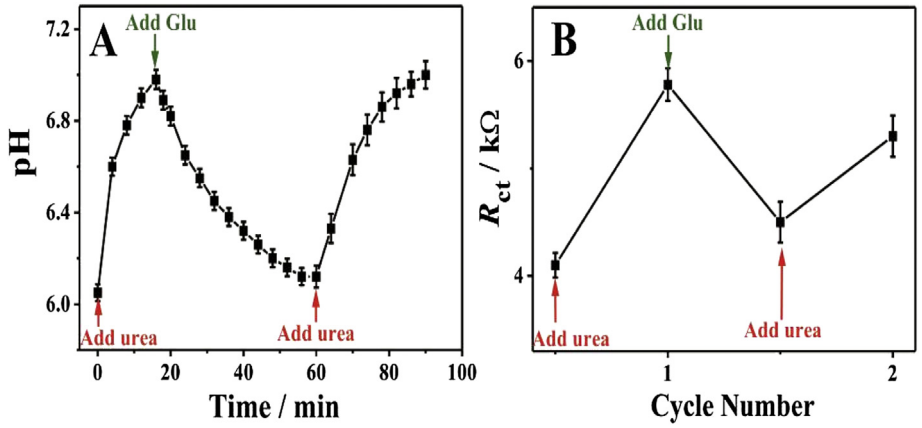


Fig. 5. (A) Time-dependent pH changes and (B) R_{ct} changes triggered by *in situ* enzyme-catalyzed reactions upon addition of urea (40 μM) and Glu (0.6 mM). The initial state of the working solution containing 5.0 mM $Fe(CN)_6^{3-/4-}$ solution (0.2 M NaCl, 5.0 U mL^{-1} GOD, 1.0 U mL^{-1} urease, pH 6.0). Error bars represent the standard deviation of three replicates.

gluconic acid and *in situ* generated ammonia; (2) the dilution effect, owing to the increase of solution volume with the alternating addition of urea and Glu.

4. Conclusions

We have developed a regenerated electrochemical method for label-free detection of dual targets, Glu and urea, based on conformational switch of i-motif oligonucleotide probe for the first time. The regenerated E-DNA sensor can be fabricated without any sophisticated equipment, so the preparation of the device is simple and inexpensive. Furthermore, the detection progress is reversible, and does not require a cumbersome thermal annealing step. Of course, barriers to successful *in vivo* detection and in−home point-of-care self-testing still exist. Nonetheless, regenerated oligonucleotide probe-based biosensor is likely to be a valuable technique for biological diagnosis. We hope this methodology could bring new perspective to construct i-motif structure-based "smart" nanomachines and logic gates, which use Glu and urea as triggers instead of H^+ and OH^- in the future.

Acknowledgment

This work was financially supported by the National Natural Science Foundation of China (Nos. 21273174, 20975083), the Municipal Science Foundation of Chongqing City (No. CSTC−2013jjB00002), and the Innovation Foundation of Chongqing City for Postgraduate (CYB14052).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.aca.2015.09.045.

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