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Ultrasensitive electrochemical detection of cancer-associated circulating microRNA in serum samples based on DNA concatamers

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ABSTRACT

MicroRNAs (miRNAs), a kind of endogenous, noncoding RNAs (19–24 nucleotides), play vital roles in regulating gene expression and cellular processes. In recent years, it has been found that circulating miRNAs are differentially expressed in patients and healthy controls. This leads to the suggestion that circulating miRNAs are promising biomarkers for cancer classification and prognosis. However, it is still difficult to detect circulating miRNAs directly from real samples such as human serum without prior extraction and purification. In this work, we developed an ultrasensitive electrochemical biosensor for detection of cancer-associated circulating miRNAs based on DNA concatamers amplification. The proposed biosensor showed a high sensitivity for target miRNA-21 in a concentration range from 100 aM to 100 pM with a detection limit of 100 aM. Furthermore, the biosensor was successfully employed for direct detection of circulating miRNAs in human serum. Due to the high sensitivity, good selectivity and stability, the proposed electrochemical biosensor might have potential clinical application for circulating miRNAs in relation to diagnosis and prognosis.

1. Introduction

MicroRNAs (miRNAs) are endogenous non-coding RNAs, which consist of 19–24 nucleotides in length. They play an important role in regulating gene expression by blocking the translation or triggering the degradation of the target mRNAs and preventing protein synthesis (Bartel, 2004; Farh et al., 2005). It is also shown that at least three-fifths of all human genes are subject to miRNA regulation (Friedman et al., 2009). Calin's group (Calin et al., 2002) first discovered the connection between miRNAs and human cancer, and altered expression of miRNAs has been found in some tumor types (Calin and Croce, 2006; Tricoli and Jacobson, 2007). Some act as tumor suppressors, while others can act as oncogenes, either directly or indirectly (Esquela-Kerscher and Slack, 2006; Ma et al., 2007). Moreover, it has also been demonstrated that the circulating miRNAs are resistant to RNase digestion, extreme pH and temperature, and they can be reliably extracted and assayed in either serum or plasma (Chen et al., 2008; Mitchell et al., 2008). These findings highlight the significance of using serum circulating miRNAs as biomarkers for cancer diagnosis, prognosis and prediction. Therefore, fast and inexpensive miRNA detection methods become more attractive in biological research and clinical diagnosis.

Recently, many methods have been developed for analyzing miRNA expression, including northern blotting, microarray-based detection, and quantitative polymerase chain reaction (qPCR; Válóczi et al., 2004; Thomson et al., 2004; Chen et al., 2005). Northern blotting is considered as a standard method, while it is time-consuming and requires a large amount of total RNA sample. Microarray-based method is excellent for high-throughput screening and profiling. Nevertheless, its sensitivity and specificity for detecting miRNA are not satisfactory. Another method widely used to detect miRNA expression is qPCR, due to its advantage that small amounts of miRNA in samples can be significantly amplified. However, the main obstacle lies in the requirements for special laboratory skills in miRNA extraction and purification. Besides, the short length of miRNA sophisticates the design of PCR primers and fluorescence-labeled DNA probes, which increases the experimental cost and complexity. Furthermore, false-positives may happen during the amplification step.

Compared with the aforementioned methods, electrochemical method is particularly attractive due to its low-cost, good specificity and simplicity of instrumentation (Wei et al., 2005; Xiao et al., 2007; Lusi et al., 2009). In recent years, various new strategies have been developed to improve the detection sensitivity, such as nanoparticle-amplified assay (Gao and Yang, 2006; Yang et al., 2009; Zhang, et al., 2009; Dong et al., 2012) and enzyme-based assay (Fan et al., 2007;

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Pöhlmann and Sprinzl, 2010; Gao et al., 2013; Cai et al., 2013). Although these methods show low detection limit at about fM level, they are laborious and always require expensive equipment. More importantly, prior extraction and purification are necessary when detecting miRNAs in biological samples.

Herein, we develop a simple and sensitive electrochemical miRNA biosensor by introducing two auxiliary probes (auxiliary probe 1, AP1, and auxiliary probe 2, AP2). AP1 and AP2 can self-assemble to form one-dimensional DNA concatamers, which serve as carriers for signal amplification. In our previous work, we have demonstrated that the length of the DNA concatamers can achieve ~μm level under optimized conditions and can be used for amplified detection of DNA (Chen et al., 2011, 2012). However, it has not been applied to real biological samples. In this work, we attempt to use the DNA concatamers for direct detection of circulating miRNAs in human serum. As a model case, we choose miRNA-21, which has high expression in breast, ovarian, diffuse large B-cell lymphoma and myocardial diseases. (Wang et al., 2010; Corcoran et al., 2011; Yan et al., 2008; Resnick et al., 2009; Lawrie et al., 2008; Thum et al., 2008). Fig. 1 shows a schematic representation of amplification detection of miRNA-21. Hexaammineruthenium(III) chloride ($[Ru(NH_3)_6]^{3+}$, RuHex) was selected as a signal reporter, which can bind to negatively charged DNA strands (Steel et al., 1998). Screen-printed gold electrodes (SPGEs) were used as substrate for surface immobilization of capture probes. The capture probe comprises a loop component, which includes a sequence complementary to the target. In the absence of target miRNA-21, the capture probe exits predominantly in the hairpin form. The DNA concatamers cannot bind to the capture probe through the complementary base pairing. In this state, only small electrochemical signals were observed. However, in the presence of target miRNA-21, stem-loop structure of capture probe is unfolded. Then DNA concatamers can hybridize with terminus of capture probe. Consequently, numerous redox indicators $[Ru(NH_3)_6]^{3+}$ can be gathered to the working electrode via DNA concatamers and eventually produce an amplified electrochemical signal. This biosensor can detect as low as 100 aM target miRNA-21, with sensitivity similar to that of PCR. The main advantage of our work is that miRNA-21 can be directly detected in human serum without enrichment, which can decrease the operation complexity and increase the detection accuracy.

2. Materials and methods

2.1. Materials

Hexaammineruthenium(III) chloride ($[Ru(NH_3)_6]^{3+}$, RuHex), 6-mercapto-1-hexanol (MCH), and tris(2-carboxyethyl) phosphine hydrochloride (TCEP) were purchased from Sigma-Aldrich. Other chemicals employed were all of analytical grade and purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). All solutions were prepared with ultrapure water obtained from a Milli-Q water purification system (Millipore Corp., Bedford, MA) with resistivity of 18.2 MΩ cm.

The buffers and solutions involved in this work were as follows: DNA immobilization buffer (I-buffer; 10 mM Tris–HCl containing 1 mM EDTA, 1000 mM NaCl and 10 mM TCEP, pH 7.4), DNA hybridization buffer (H-buffer; 10 mM Tris–HCl containing 1 mM EDTA, 300 mM NaCl and 1 mM $MgCl_2$, pH 7.4), voltammetry testing buffer (V-buffer; 10 mM Tris–HCl containing 5 μM $[Ru(NH_3)_6]^{3+}$, pH 7.4), electrochemical impedance spectroscopy testing buffer (E-buffer; 100 mM PBS containing 5 mM $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ and 0.1 M KCl, pH 7.4), and MCH solution (2 mM MCH in water).

All the oligonucleotides used in this study were synthesized and purified through HPLC by Shanghai Sangon Biotechnology Co. (Shanghai, China) and used without further purification. The RNA sequences are synthesized by TaKaRa Biotechnology Co. (Dalian, China) and purified by HPLC. The sequences of the used oligonucleotides are given below.

Capture probe (CP): 5′-GGCCG*TCAACATCAGTCTGATAAGCTA*AA-CATGATGACGGCC-3′
Target miRNA-21: 5′-UAGCUUAUCAGACUGAUGUUGA-3′
Auxiliary probe 1 (AP1): 5′-GCACCTGGGGGAGTAAGTGGCCGT-CATCAT-3′
Auxiliary probe 2 (AP2): 5′-TACTCCCCCAGGTGCATGATGACGGC-CACT-3′
Single-base mismatch target (1MT): 5′-UAGCUUAUCGGACU-GAUGUUGA-3′
Two-base mismatch target (2MT): 5′-UAGCUUAUCGGACUCAU-GUUGA-3′
Noncomplementary sequence (NC): 5′-AAAAAAUCUGCUGAG-GAUAAAA-3

The italic fragments of CP are complementary sequences to target miRNA-21. 1MT is a single-base-mismatch target, while 2MT is a two-base-mismatch target. The underlined bases are the mismatched bases. NC is a noncomplementary sequence.

2.2. Sensor preparation

The screen-printed three gold electrodes (SPCEs; *L*3.4 × *W*1.0 × *H*0.05, cm) were purchased from DropSens (Spain). The SPCE consists of a 4 mm diameter disk gold working electrode, gold counter electrode and a silver pseudo-reference electrode. Prior to experiments, the SPCE was washed thoroughly with a copious amount of ultrapure water and blowed dry with nitrogen.

2.3. DNA self-assembly on gold electrode

The electrochemical sensor was constructed via the self-assembly of the –SH group on the gold electrode. At first, 8 μL of I-buffer containing 1 μM thiolated capture probe DNA (CP) was dropped on the surface of the SPGE and incubated in a dark place for 180 min. The electrode was then thoroughly rinsed with ultrapure water and dried under a stream of nitrogen gas. The resulting electrode was incubated in 2 mM MCH solution for 90 min to remove nonspecific DNA adsorption on the gold surface. After rinsing thoroughly, 8 μL of H-buffer containing target miRNA was placed on the modified SPGE for 120 min. Then the electrode was rinsed and dried. Finally, 10 μL of a freshly prepared H-buffer containing 1 μM AP1 and 1 μM AP2 was dripped on the surface of the electrode, and incubated for 120 min to self-assemble to be

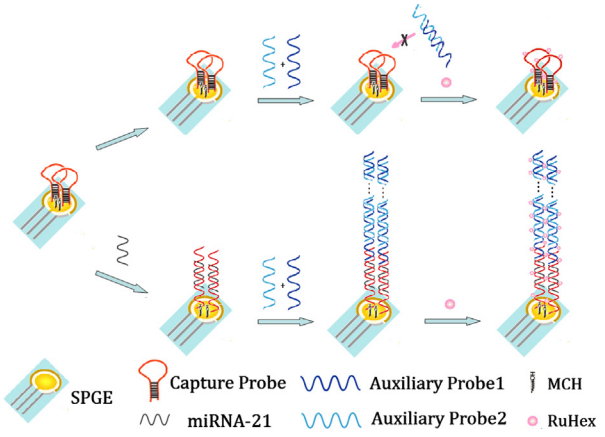

Fig. 1. Schematic representation of the concatamer-based ultrasensitive electrochemical miRNA biosensor for the detection of target miRNA-21.

DNA concatamers. After finishing the assembly, the resulting electrode was again thoroughly rinsed and dried before electrochemical characterization. For miRNA expression analysis of the real samples, 8 μL of the human serum was directly dropped on the CP/MCH modified electrode surface for 120 min. Next, DNA concatamers self-assembly and hybridization with CP were carried out as described above.

2.4. Detection method and apparatus

All electrochemical measurements were carried out on a CHI 660C electrochemical working station (CH Instrument Company, USA). SPCEs were used for the measurements. Various concentrations of target miRNA-21 standards or samples were prepared by using I-buffer solution. All measurements were carried out at room temperature. Differential pulse voltammetry (DPV) parameters were as follows: potential scan range from −0.6 to 0.2 V (versus Ag), modulation amplitude was 0.05 V, pulse width was 0.05 s, and sample width was 0.02 s in V-buffer solution, which was degassed with nitrogen for 15 min. The DPV peak current was registered as the sensor signal. Electrochemical impedance spectroscopy (EIS) experiments were performed in E-buffer, with the biasing potential of 0.14 V (versus Ag), the amplitude of 5 mV and the frequency range from 1 Hz to 10^5 Hz.

Human serum samples of breast cancer patients and healthy donors (confirmed by pathological examinations) were obtained from Fujian Medical University (Fujian, China). Signed informed consent was obtained from each patient participating in the study before surgery.

3. Results and discussion

3.1. EIS of different modified electrodes

Different modified electrodes are characterized by electrochemical impedance spectroscopy (EIS). In the impedance spectra, the increase in semicircle diameter indicates the increase in the electron transfer resistance (R_{et}) at the interface (Zhang et al., 2008). The bare SPGE exhibits a nearly straight line (Fig. 2a), reflecting excellent electrochemical conductivity. Compared with the bare SPGE, the SPGE modified with capture probe (CP) and MCH shows a larger electron transfer resistance (Fig. 2b), mainly due to the electrostatic repulsion between negative charges of the DNA phosphate backbone and the $[Fe(CN)_6]^{3-/4-}$ ion, which also demonstrated that CP had been successfully immobilized on the electrode. When CP hybridized with target miRNA-21, some more phosphate backbone anions blocked electron transfer and a larger semicircle diameter was obtained (Fig. 2c). If there was only one auxiliary probe (AP1) in the solution, AP1 could hybridize with the CP. The monolayer of DNA on the electrode surface became denser, and the negative charges on the electrode surface increased. Thus the electron-transfer resistance was enhanced further, and the value of R_{et} increased (Fig. 2d). When the solution containing AP1 and AP2 was dripped on the surface of the SPGE, the DNA concatamers, self-assembled by AP1 and AP2, were firmly immobilized on the SPGE via target miRNA-21 and CP. The R_{et} increased significantly (Fig. 2e) because more DNA were captured on the electrode surface, which made the electron transfer more difficult. This was the direct evidence of successful binding of DNA concatamers on the electrode surface.

3.2. Electrochemical characterization of the modified electrode

RuHex cations can bind to the phosphate backbone of DNA strands via electrostatic interaction. The more the RuHex binding to DNA, the greater the signal. In the absence of target miRNA-21,

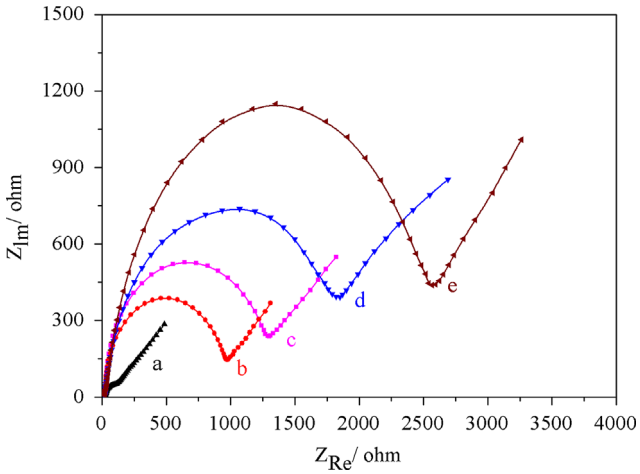

Fig. 2. Impedance spectra (Nyquist plot) of the SPGE modified with various oligonucleotides in the presence of 5 mM $[Fe(CN)_6]^{4-/3-}$: (a) bare SPGE, (b) CP/MCH, (c) CP +miRNA-21/MCH, (d) CP+miRNA-21+AP1/MCH, and (e) CP+miRNA-21+AP1+AP2/MCH. The frequency range was 1–10^5 Hz and the amplitude was 5.0 mV.

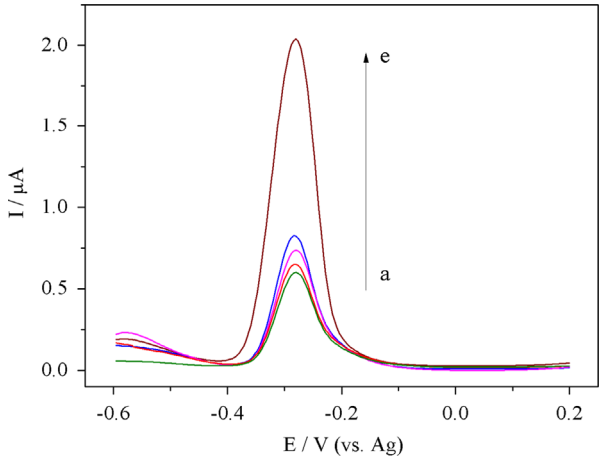

Fig. 3. Differential pulse voltammetry (DPV) responses of the SPGE modified with various oligonucleotides: (a) CP, (b) CP+miRNA-21, (c) CP+AP1+AP2, (d) CP +miRNA-21+AP1, and (e) CP+miRNA-21+AP1+AP2. The concentration of miRNA-21 is 10 pM. The concentrations of AP1 and AP2 are both 1 μM.

there was only a small reduction peak observed (Fig. 3a), due to the small amount of RuHex electrostatically binding to CP. After hybridization with target miRNA-21, some more RuHex cations were binding to CP and target miRNA-21, and a slightly larger peak was obtained (Fig. 3b). Although auxiliary probe 1 (AP1) and auxiliary probe 2 (AP2) can self-assemble to be DNA concatamers, the DNA concatamers cannot link to the electrode surface in the absence of target miRNA-21. Thus another small reduction peak was observed (Fig. 3c), due to a small amount of nonspecific adsorption. In the presence of target miRNA-21, if there was only AP1 and no AP2 in the solution, still only a small reduction peak was observed (Fig. 3d). When the solution containing 1 μM AP1 and 1 μM AP2 was dripped on the surface of the SPGE, the DNA concatamers, self-assembled by numerous AP1 and AP2, were firmly immobilized on the SPGE via target miRNA-21 and CP. Thus the large number of RuHex binding to DNA concatamers produced a remarkable amplified electrochemical signal (Fig. 3e).

3.3. Electrochemical detection of target miRNA

As can be seen in Fig. 4A, a dramatic increase in the electrochemical signals was observed on increasing the concentrations of target miRNA-21. The practical detection limit was 100 aM.

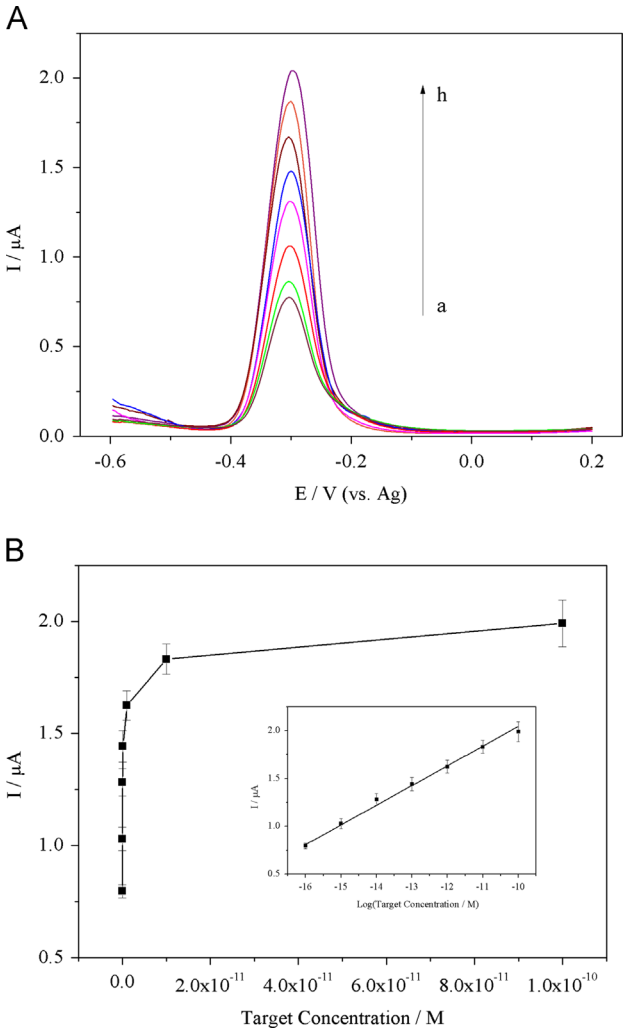

Fig. 4. (A) DPV responses for different target miRNA-21 concentrations: (a) 0 M, (b) 100 aM, (c) 1 fM, (d) 10 fM, (e) 100 fM, (f) 1 pM, (g) 10 pM, and (h) 100 pM. (B) The plots of peak currents versus the target miRNA-21 concentrations. Inset: the linear fit plot of peak current and the logarithm of the target miRNA-21 concentration. The illustrated error bars represent the standard deviation of five repetitive measurements.

This high sensitivity is due to large amount of RuHex cations electrostatically binding to the long-range DNA concatamers, which leads to the remarkably amplified electrochemical signals. As shown in Fig. 4B, a proportional relationship was observed between the logarithm of target miRNA-21 concentration and the DPV peak currents of the sensing platform in a linear range from 100 aM to 100 pM (i.e., over 7 orders of magnitude) with a calibration function of $I=0.1976\log C+3.9964$ ($R^2=0.9951$), where I is the peak current (μA), and C is the concentration of target miRNA-21 in M. The sensitivity of our biosensor exceeds those measured by northern blotting, microarray, or quantitative PCR, and compares well with those of the reported electrochemical amplification methods (Gao and Yang, 2006; Pöhlmann and Sprinzl, 2010; Dong et al., 2012; Gao et al., 2013). The relative standard deviation (RSD) of the sensor for five replicate determinations of 100 pM and 100 fM was 3.85% and 5.89%, respectively, which demonstrated the good reproducibility of the method.

3.4. Selectivity, stability of the biosensor

To examine the selectivity of our proposed concatamer-based amplified biosensor, we performed a comparison study on

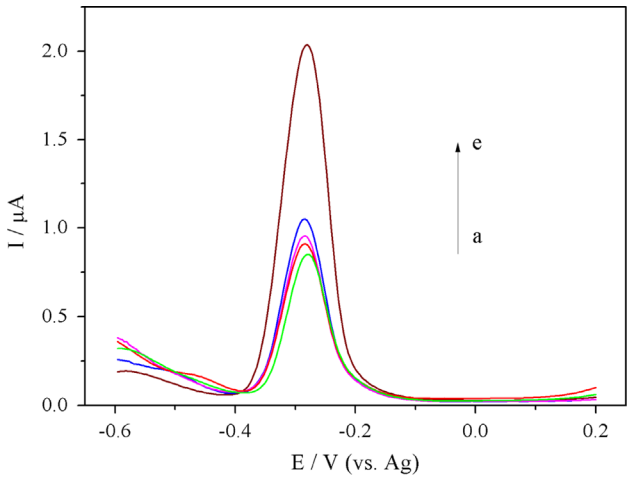

Fig. 5. DPV responses of the blank and the four various RNA sequences: (a) blank, (b) NC, (c) 2 MT, (d) 1 MT, and (e) target miRNA-21. The concentrations of 1 MT, 2 MT, and NC were 1 nM. The concentration of target miRNA-21 was 100 pM. The illustrated error bars represent the standard deviation of five repetitive measurements.

mismatch targets and perfect complementary target. Fig. 5 shows the voltammograms for the different kinds of targets including the perfect complementary target miRNA-21 at a concentration of 100 pM and single-base mismatch target (1MT), two-base mismatch target (2MT), and non-complementary sequence (NC), each at a concentration of 1 nM. As expected, single-base mismatch target resulted in 16.9% increase in electrochemical signal (0.997 μA) when compared with blank treatment (0%, 0.795 μA) and perfect complementary target miRNA-21 (100%, 1.992 μA). Additionally, using the two-base mismatch target caused an 8.4% increase in the electrochemical signal (0.896 μA), whereas the non-complementary sequence caused only a 3.7% increase in the electrochemical signal (0.839 μA). These results demonstrate that our proposed biosensor shows high selectivity for the perfect complementary target miRNA-21. In this work, the stability of the biosensor was also investigated. The modified electrode, which was immersed in H-buffer and stored at 4 °C for 24 h, could still be used for the measurement of target miRNA-21 without significant change of current response (Fig. S1). Thus, the developed biosensor shows good stability within 24 h.

3.5. Detection of target miRNA in real samples

The expression of cancer-associated miRNA-21 in serum can be used as a biomarker of cancer progression (Resnick et al., 2009; Tsujiura et al., 2010; Asaga et al., 2011; Liu et al., 2012). To demonstrate the applicability of our biosensor for analyzing circulating miRNAs in real samples, the human serum samples from 7 newly diagnosed breast cancer patients and 7 healthy donors were tested. As shown in Fig. 6, the electrochemical signal changes were calculated according to the following equation: $\Delta I=I_{\text{hybrid}}-I_{\text{blank}}$, where I_{hybrid} and I_{blank} are the electrochemical signal of the biosensor with serum samples and biosensor without serum samples, respectively. It turned out that the breast cancer patients serum generated an increasing DPV signal (0.711 ± 0.082 μA) about 1.9 times higher than that obtained from the healthy donors serum (0.381 ± 0.059 μA), suggesting an up-regulation of miRNA-21 expression in the breast cancer human serum. This result is in good agreement with reported literatures (Wang et al., 2010; Asaga et al., 2011). The levels of target miRNA-21 in about 14 different human samples measured by our proposed biosensor are shown in Fig. S2. As references, expression levels of the serum miRNA-21 were simultaneously quantified by a commercial qRT-PCR kit. The results obtained with the biosensors

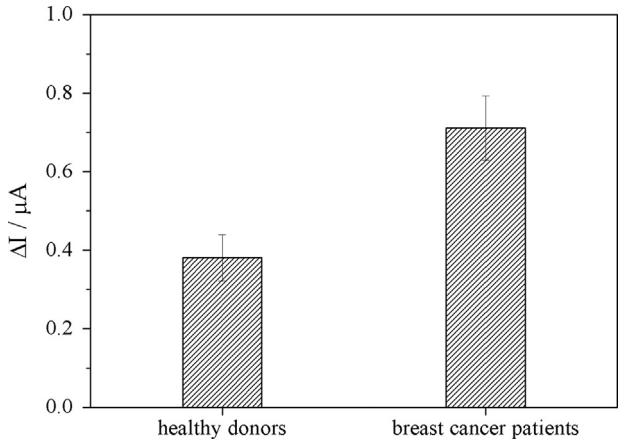

Fig. 6. DPV responses comparison of target miRNA-21 in the serum of breast cancer patients and healthy donors. Error bars represent the standard error in data collected from seven samples.

are in good agreement with those obtained by qRT-PCR on the same samples (Fig. S3). Accordingly, the proposed electrochemical biosensor can directly measure the expression levels of miRNA in human serum without pretreatment and provide great potential in the clinic diagnosis.

4. Conclusions

We have developed an ultrasensitive electrochemical miRNA biosensor for specific detection of cancer-associated miRNA-21 in serum samples. The self-assembled DNA concatamers can carry numerous RuHex, which results in the significantly enhanced electrochemical signals. This DNA concatamer-based biosensor permits detection of as low as 100 aM miRNA-21, without any enzyme, label, or expensive apparatus. Furthermore, it is gratifying that our biosensor can be applied to the direct determination of miRNA-21 in complex biological sample without miRNAs prior extraction and purification. In addition, the biosensor also displays high selectivity for target miRNA-21. Based on the advantages given above, this ultrasensitive electrochemical miRNA biosensor has a promising future in the area of miRNA diagnostics and clinical analysis.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2013.06.040.

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