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Two-stage cyclic enzymatic amplification method for ultrasensitive electrochemical assay of microRNA-21 in the blood serum of gastric cancer patients

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ABSTRACT

MicroRNA (miRNA) is riveting nowadays due to its close relevance to human malignancies. Exploiting a fast and convenient biosensor for sensitive and specific detecting miRNA is necessary and it has significant meaning for oncology. In this work, to understand the relationship between miRNA-21 and gastric cancer, we employ T4 RNA ligase 2 to initiate specific ligation reaction depending on the sequence of target RNA, and T7 exonuclease to catalyze the first stage cyclic enzymatic amplification method (CEAM) specifically, which also resting on the target RNA sequence, and the second stage CEAM to further amplify the response resulting from the initial target RNA. Our two-stage CEAM strategy can detect miRNA-21 as low as 0.36 fM with remarkable specificity, and most importantly, it can be used to inspect the expression level of miRNA-21 in blood serum of gastric cancer patients. This will offer new perspective for figuring out the pathways of miRNA-21 involving in cancer.

1. Introduction

MicroRNAs (miRNAs), a sort of short ribonucleic acids without encoding any proteins, have been authenticated playing crucial roles in gene regulations both in animals and plants (Carthew and Sontheimer, 2009). They can regulate gene expression level, in some kinds of particular situations, via inhibiting mRNA translation, repressing the translational process or other ways (Bartel, 2004). Researchers have found that they are critical in cell cycle, system balance and body health of human (Gurtan and Sharp, 2013). Their dysregulation is more or less, directly or indirectly related to human diseases, such as diabetes (Pandey et al., 2009), cardiovascular diseases (Vickers et al., 2014), neurodegeneration (Goodall et al., 2013) and cancer (Croce, 2009; Lu et al., 2005). Elucidating the relationship between miRNAs and cancer is more and more crucial and pressing for cancer diagnosis and curing, and figuring out the pathways of miRNAs participating in malignancies, explicitly. Then, the precise determination of microRNAs' expression level has become more and more important and urgent (Dong et al., 2013). According to some recent reports, some kinds of miRNAs are closely associated with human cancer, and miRNA-21 is one of them (Chen et al., 2008; Iorio et al., 2005; Jansson and Lund, 2012; Kumarswamy et al., 2011; Mitchell et al., 2008; Pan et al., 2010; Papagiannakopoulos et al., 2008). Especially, miRNA-21 level in gastric cancer patients' blood serum is elevated (Chan et al., 2008; Zhang et al., 2008). Thus, developing a specific method to sensitively detect miRNA-21, and unveiling the relationship between miRNA-21 and the clinicopathological features will help illustrating the roles of miRNA-21 playing in the cancer stages. And it could make contribution to clinic diagnosis and prognosis in cancer curing via using miRNA-21 as biomarker and therapeutic targets.

Owing to the advanced technology, methods of detecting miRNAs have been springing up rapidly in decades. Undoubtedly, microarray technology is an inexpensive, attractive, and high-throughput method; but it cannot offer absolute quantitative date, and its imperfect specificity largely limits its application (Ach et al., 2008; Bak et al., 2008; Git et al., 2010; Leshkowitz et al., 2013). Although northern blotting has been improved in its sensitivity, it is still not suitable for practical clinic tests due to its intrinsic complex steps and low-throughput. (Dangwal et al., 2012; Wark et al., 2008). The qRT-PCR is famous for its sensitivity and accuracy,

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however, it is costly in instrument and labors, and frail to contamination (Huang et al., 2011). Leong et al. use DNA nano-structure to monitor RNAs, but its fluorescent signal has a relative high background (Tay et al., 2015). At this point, electrochemical detection method is coming on the stage because it is more convenient and easier to operate. For instance, Leong et al. utilize DNA nano-pyramids to construct electrochemical biosensors for immunoassays with excellent performance (Giovanni et al., 2015; Yuan et al., 2014) Electrochemical method is a good choice to detect miRNA. Wu et al. fabricated an electrochemical biosensor to detect miRNA based on Pd nanoparticles (Wu et al., 2013). However, since miRNA is trace in cells, its detection range and limit is not sensitive enough to detect miRNA at low concentration. Gao et al. developed a ruthenium oxide nanoparticle-tagged miRNAs and then use the hybridized miRNA strands as template to guide the deposition of polyaniline under the catalyst of ruthenium oxide. The miRNA expression profiling is achieved by monitoring voltammetric peak current of polyaniline (Peng et al., 2010). Nevertheless, they need to modify target miRNA, which is very difficult because of the low abundance and vulnerability of miRNA. Employing cyclic enzymatic amplification method could overcome those disadvantages. Gao et al. developed another miRNA biosensor based on the digestion of duplex-specific nuclease towards deoxyribonucleotides in DNA/RNA duplex (Ren et al., 2013). Nonetheless, hybridization and enzyme hydrolyzing processes are happening on the electrode surface. This largely limits the reaction efficiency and thus affects its sensitivity. So does one of our groups' previous works (Wang et al., 2014). Herein, we designed three kinds of particular DNA to meet enzymatic specificity of T4 RNA ligase 2 (T4 Rnl 2) and T7 exonuclease (T7 Exo). Therefore, only in the present of target RNA, can the ligation reaction and the first stage cyclic enzymatic amplification method (CEAM) happen. And the two-stage CEAM significantly amplified the response to improve detection sensitivity. The expression level of miRNA-21 is successfully determinated both in non-cancer people and cancer patients' gastric serum.

2. Experimental section

2.1. Reagents and solutions

Hydrogen tetrachloroaurate trihydrate ($HAuCl_4 \cdot 3H_2O$), tris(hydroxymethyl)aminomethane (Tris), EDTA, dithiothreitol (DTT), and tris(2-carboxyethyl)phosphine hydrochloride (TCEP) were purchased from Aladdin (Shanghai, China). DNase I (RNase-free) was provided by Thermo Scientific (USA). T4 Rnl2 and T7 Exo were purchased from New England Biolabs (USA) and they were diluted in their corresponding store buffer to desired stock concentrations and stored at −20 °C according to the manufacturer's instructions. The HPLC-purified miRNAs and DNA oligonucleotides were supplied by Katara Biotechnology Co., Ltd. (Dalian, China) and Sangon Biotech Co., Ltd. (Shanghai, China), respectively. The base sequences used in the study are as follows: DNA1, 5′-AGTGCCGCATCATGCTGATCAA-CATCAGTCTGATAA-3′; DNA2, 5′-TCAGCATGATGCGGCACT-3′; DNA3, 5′-ferrocene-$(CH_2)_6$-TTATCAGACTGATGTTGATCAGCATGATGCGG-$(CH_2)_6$-SH-3′; microRNA-21, 5′-UAGCUUAUCAGACUGAUGUUGA-3′; one-base mismatch RNA1, 5′-UAGCUUAUCAGACUGAUGUUG<u>C</u>-3′; one-base mismatch RNA2, 5′-UAGCUUAUCAGA<u>A</u>UGAUGUUGA-3′; three-base mismatch RNA, 5′-UAGCU<u>G</u>AUCAG<u>C</u>CUGAU<u>A</u>UUGA-3′; non-complementary RNA, 5′-UUGGACUGAAGGGUGCUCCCCU-3′. The synthesized DNA and miRNA were diluted in TE buffer (containing 10 mM Tris–HCl and 1 mM EDTA, pH 8.0) to desired stock concentrations and stored at −20 °C according to the manufacturer's instructions.

The buffer solutions employed in this work are as follows. TE buffer: 10 mM Tris–HCl, 1.0 mM EDTA (pH 7.4). DNA3 immobilization buffer: 300 mM Tris–HCl, 1.0 mM EDTA, 1.0 M NaCl, 10 mM TCEP, 1.0 M $NaClO_4$ (pH 7.4). T4 Rnl 2 store buffer: 10 mM Tris–HCl, 35 mM $(NH_4)_2SO_4$, 0.1 mM EDTA, 0.1 mM DTT and 20% glycerol (pH 7.50). T4 Rnl 2 reaction buffer: 250 mM Tris–HCl, 10 mM $MgCl_2$, 5 mM DTT, 2 mM ATP (pH 7.5). T7 Exo store buffer: 10 mM Tris–HCl, 0.1 mM EDTA, 5 mM DTT, 20% glycerol (pH 8.0). NEBuffer 4 (T7 Exo reaction buffer): 50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM DTT (pH 7.9). Electrochemistry determination buffer: 10 mM phosphate buffered solution (PBS) containing 100 mM NaCl and 1 mM $NaClO_4$ (pH 7.4). And washing buffer: 10 mM Tris–HCl (pH 7.4). All the solutions and redistilled deionized water were treated with DEPC and autoclaved to protect from RNase degradation.

2.2. Sample preparation

The serum sample is provided by the tumor center of Taian city central hospital. 15 μL serum of non-cancer human or gastric cancer patients' was uniformly mixed with 1 μL EDTA DNase reaction buffer, 1 μL $MgCl_2$ solution and 10 μL DNase, and incubated at 37 °C for 30 min. Afterwards, the reaction mixture was incubated at 65 °C for 10 min to inactive DNase. The reaction product was used for sample analysis.

2.3. Electrode preparation

The plane gold electrode was firstly polished to mirror-like surface using 0.3 μm alumina slurry, followed with ultrasonic cleaning in double-distilled deionized water and anhydrous alcohol for 3 min. The gold electrode was rinsed with double-distilled deionized water and dried under N_2 blowing. Then the gold electrode was immersed into 3 mM $HAuCl_4$ solution containing 0.1 M KNO_3 and the AuNPs were electrochemically deposited on the electrode surface using single-potential mode at −0.2 V for 200 s under stirring. Then note the electrode as AuNPs/Au and then stored in the humid chamber for further use.

2.4. Ligation and digestion reaction

Before the ligation reaction, 10 μL 10^{-8} M DNA1 and 10^{-10} M DNA2 were uniformly mixed with 10 μL 10^{-10} M miRNAs, and then the mixture was heated for 2 min at 65 °C and then incubated for 30 min at 39 °C for hybridization. After hybridization, 0.1 U T4 Rnl 2 and 5 μL its reaction buffer were added into the mixture to a final volume of 40 μL. Then the reaction was performed by incubation for 60 min at 39 °C, followed by heat inactivation of the ligase at 90 °C for 5 min. The ligation products were put on ice immediately, ready for next step. Then 10 μL NEBuffer containing 0.2 U T7 Exo was added in the ligation products and incubated at 37 °C for 1 hour. Afterwards, 10 μL 4×10^{-7} M DNA3 was added and then incubated at 37 °C for another 1 hour. Next, mix the same volume of the reaction product and DNA3 immobilization buffer and incubated the final mixture on the AuNPs/Au surface at ambient temperature under humid environment. The final electrode was noted as DNA1/RNA/T7/DNA3/AuNPs/Au.

2.5. Experiments

To prove our strategy, we did some parallel experiments as followings, and the results are shown in Fig. 1. 1. DNA1 in the ligation reaction was replaced by the same volume of TE buffer, and the other conditions were as same as the above details. This final electrode was noted as RNA/T7/DNA3/AuNPs/Au. 2. RNA in the ligation reaction was replaced by the same volume of TE buffer, and the other conditions were as same as the above details. This final

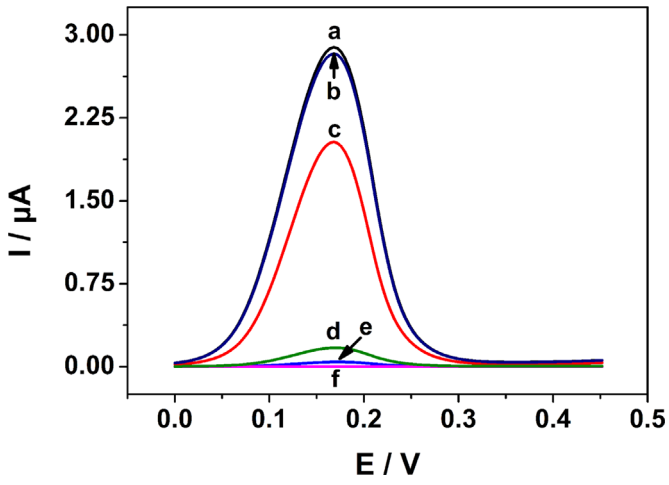


Fig. 1. Differential pulse voltammogram (DPV) of RNA/T7/DNA3/AuNPs/Au (a), DNA1/RNA/DNA3/AuNPs/Au (b), DNA1/RNA/T7/DNA3/AuNPs/Au with target miRNA-21 concentration of 10^{-10} M (c) and 10^{-15} M (d), DNA1/T7/DNA3/AuNPs/Au (e), and DNA1/RNA/T7/AuNPs/Au (f).

electrode was noted as DNA1/T7/DNA3/AuNPs/Au. 3. T7 Exo in the digestion reaction was replaced by the same volume of T7 Exo store buffer, and the other conditions were as same as the above details. This final electrode was noted as DNA1/RNA/DNA3/AuNPs/Au. 4. DNA3 in the digestion reaction was replaced by the same volume of TE buffer, and the other conditions were as same as the above details. This final electrode was noted as DNA1/RNA/T7/AuNPs/Au.

Then we recorded the differential pulse voltammogram (DPV) responses of RNA/T7/DNA3/AuNPs/Au, DNA1/RNA/DNA3/AuNPs/Au, DNA1/RNA/T7/DNA3/AuNPs/Au with miRNA-21 concentration of 10^5 and 1 fM, DNA1/T7/DNA3/AuNPs/Au, and DNA1/RNA/T7/AuNPs/Au electrode. And their final currents are shown in Fig. 1(a–f), respectively.

To test the specificity of this biosensor, we replace the target miRNA-21 of 100 pM with two one-base mismatch RNAs, one three-base mismatch RNA and non-complementary RNA at the same concentration. Other experimental conditions were the same. And the results of target miRNA-21, two one-base mismatch RNAs, one three-base mismatch RNA and non-complementary RNA of 100 pM are shown in Fig. 3Da–e. The miRNA-21 expression in non-cancer and gastric cancer patients have already deduct background.

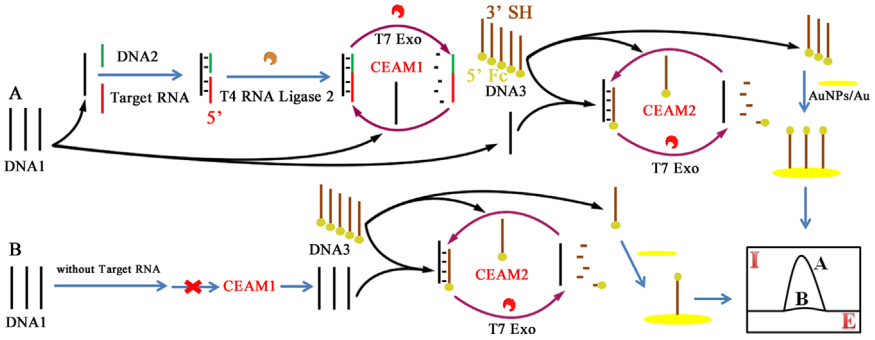
2.6. Differential Pulse Voltammetry (DPV) measurement

After the final electrode was rinsed by washing buffer, it was immersed in the determination buffer for DPV measuring. DPV was performed at CHI830D electrochemical workstation (CH Instrument, Inc. Austin, USA) in the potential range of −0.2–0.6 V. The parameters of increment potential, 0.004 V; pulse amplitude, 0.05 V; pulse width, 0.05 s; sample width, 0.0167 s; pulse period, 0.2 s; and quiet time, 2 s. The plane Au (d=2 mm, geometric area is 0.0314 cm^2, Gaoss Union®, China) or modified Au electrode was used as the working electrode, saturated calomel electrode as the reference electrode and platinum wire as the counter electrode.

3. Results and discussion

3.1. Detection strategy

RnI 2 can efficiently ligate a 3′-OH RNA to a 5′-PO_4 RNA or DNA, but fails to join a 5′-OH RNA to a 3′-PO_4 RNA or DNA.(Nandakumar and Shuman, 2004) T7 Exo is a sequence-independent nuclease, and it can hydrolyze mononucleotides from blunt or recessed 5′ termini of DNA or DNA/RNA duplex, but is inactive towards single strand DNA and RNA, or duplex RNA.(Cui et al., 2014; Wang et al., 2013, 2012) As shown in Scheme 1A, DNA1 (black), DNA2 (green), and miRNA-21 (red) can form a duplex strand with a nick between DNA2 and miRNA-21, and at this point, T4 RnI 2 can effectively seal the nick and thus a DNA1/DNA2-RNA duplex is formed, successfully. There is one point here that the 5′ termini of RNA extends out with four extra ribonucleotides, which is longer than the 3′ termini of DNA1. So, after the addition of T7 Exo into the reaction system, instead of DNA2-RNA, DNA1 will be hydrolyzed from its 5′ termini because of the special enzymatic activity of T7 Exo. Then the single strand of DNA2-RNA ligated by T4 RnI 2 would be released to hybridize with excess fresh DNA1 and initiate the next hydrolyzing cycle. Thus, part of DNA1 will be broken down. This is the first-stage cyclic enzymatic amplification method. At this moment, the reaction system is mixed up with a fixed number of DNA3 (brown), which can hybridize with DNA1 to form a DNA1/DNA3 duplex strand. Importantly, a similar point here is that the 5′ termini of DNA1 is longer than the 3′ termini of DNA3 with four extra deoxyribonucleotides; therefore, T7 Exo will digest DNA3 rather than DNA1. Thus, a portion of DNA3 would be consumed. This is the second-stage cyclic enzymatic amplification method. Labeled with a thiol group and a ferrocene (Fc) molecule at its 3′ and 5′ termini, respectively, DNA3 could be immobilized on Au nanoparticles (AuNPs) modified Au electrode surface and generate voltammetric signal response. One target miRNA-21 can trigger thousands of hydrolyzing DNA1 and thus prevent lots of DNA3 from digestion to generate a strong electrochemical response. Consequently, the more target miRNA-21, the more DNA1 will be digested, the more DNA3 would be left, and the stronger final electrochemical response will be gotten. Precise determination of miRNA-21 thus can be achieved by recording the oxidation current of Fc. On the other hand, without target miRNA-21, DNA1 would not be hydrolyzed by T7 Exo, as shown in Scheme 1B. Then,



Scheme 1. Schematic representation of two-stage cyclic enzymatic amplification method detecting target miRNA-21 with target (A) and without target (B).

facilitated by lots of left DNA1, DNA3 would be largely digested, resulting in a weak output in the end.

3.2. Detection feasibility assay

To prove our strategy, we constructed five parallel reaction systems (see experimental section). As shown in Fig. 1, without DNA1, the system results in the maximal peak current at 0.168 V (curve a). It can be ascribed to that in the absence of DNA1, T7 Exo cannot digest DNA3 and the left DNA3 is at its maximal level with modified Fc to generate the highest peak current value. Without T7 Exo (Fig. 1, curve b), the final electrode will give the electrochemical response nearly as strong as the system without DNA1 do. The tiny difference can be explained as that most DNA1 would hybridize with DNA3, to impede DNA3 immobilization, and this results in the decreased ferrocene density on the electrode surface. Therefore, DNA1 and T7 Exo are the two main factors of hydrolyzing process to lower the final response, and alter the response along with the varied target RNA concentrations. Since only in the present of DNA3, the reaction system can generate electrochemical response, the system gets no signal without DNA3 (Fig. 1, curve f). This proves that the final current signal originate from Fc labeled DNA3. The systems with target miRNA-21 of 10^{-10}, 10^{-15} and 0 M produce a lower, a much lower and an ignorable response, respectively (Fig. 1, curve c, d and e), which is reasonable according to the principle. The more target RNA the stronger final response and they are weaker than those systems' without any main hydrolyzing factor, and when the concentration of target RNA is 0 M, the result signal will approach 0. Thus the concentration of RNA can be detected through this biosensor.

3.3. Optimum of detection conditions

In order to obtain a reasonable response current to sensitively determinate target miRNA-21, the experimental conditions were optimized. The performances of the reaction systems with different ligation reaction time, first-stage T7 Exo cyclic reaction time, second-stage T7 Exo cyclic reaction time and immobilization time of DNA3 are investigated. As results are exhibited in Fig. 2A, after 50 min, the response current is level off, indicating that the ligation reaction is almost done in 50 min. So, to be efficient, 50 min is chosen as the ligation reaction time. As shown in Fig. 2B and C, after 70 min and 60 min, the result peak currents are almost unchanged inferring that after 70 min and 60 min, almost all DNA1 and DNA3 are consumed. In order to ensure that these step reactions are going adequately and the remaining quantity of DNA1 or DNA3 could activate the next step reaction, we should preserve some DNA1 and DNA3 but not too little. So, the first and second T7 Exo cyclic reaction times are chosen as 60 min and 50 min, when these two stages of cycle just hit the point but not go entirely. As shown in Fig. 2D, the electrochemical response enhance as the immobilization time of DNA3 increase because as the time goes longer, the more Fc labeled DNA3 would be immobilized on the Au electrode surface. However, after 13 h, the enhancement is not obvious. Then, 13 h is selected as DNA3 immobilization time for the following further exploration of this strategy.

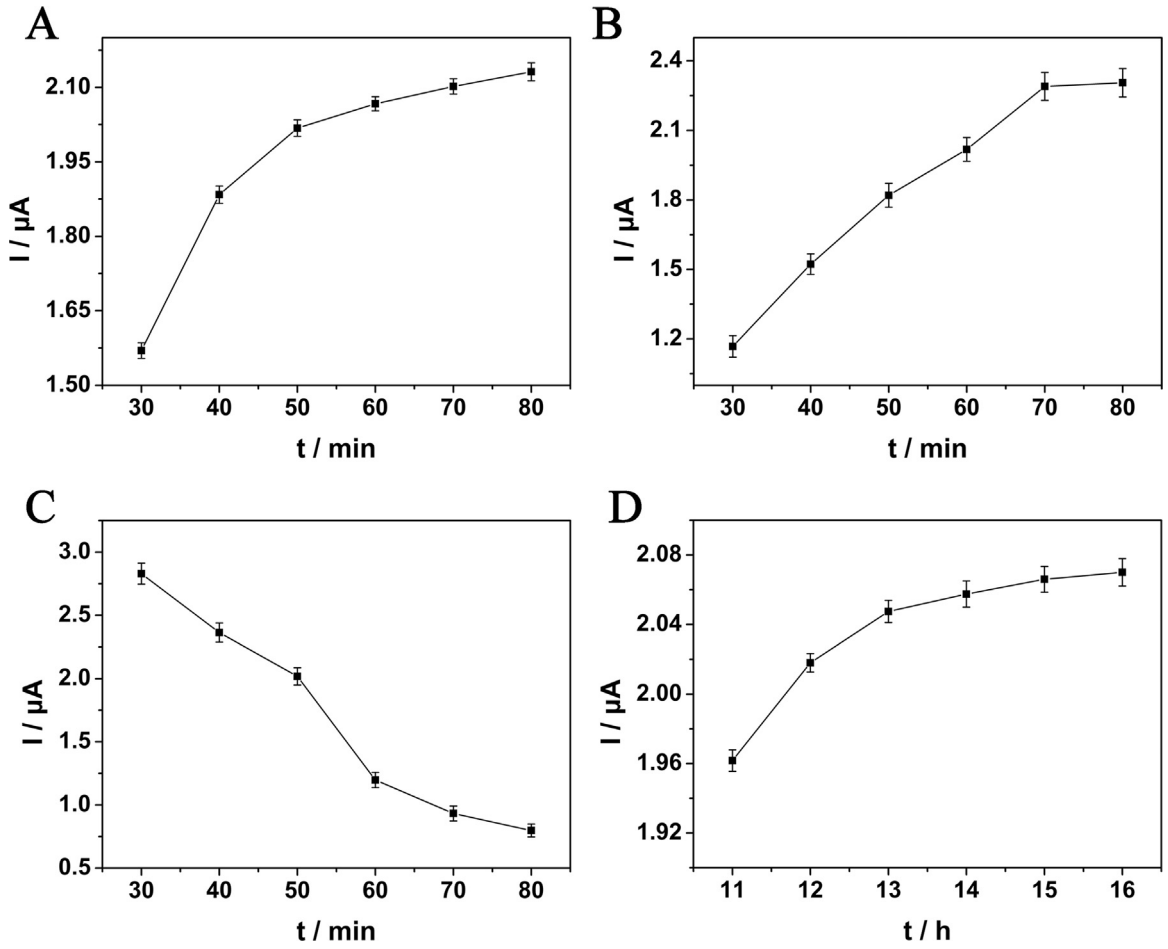


Fig. 2. Effects of ligation reaction time (A), the first-stage T7 Exo cyclic digestion reaction time (B), the second-stage T7 cyclic digestion reaction time (C) and the DNA3 immobilization time(D) on the electrochemical response of the biosensor.

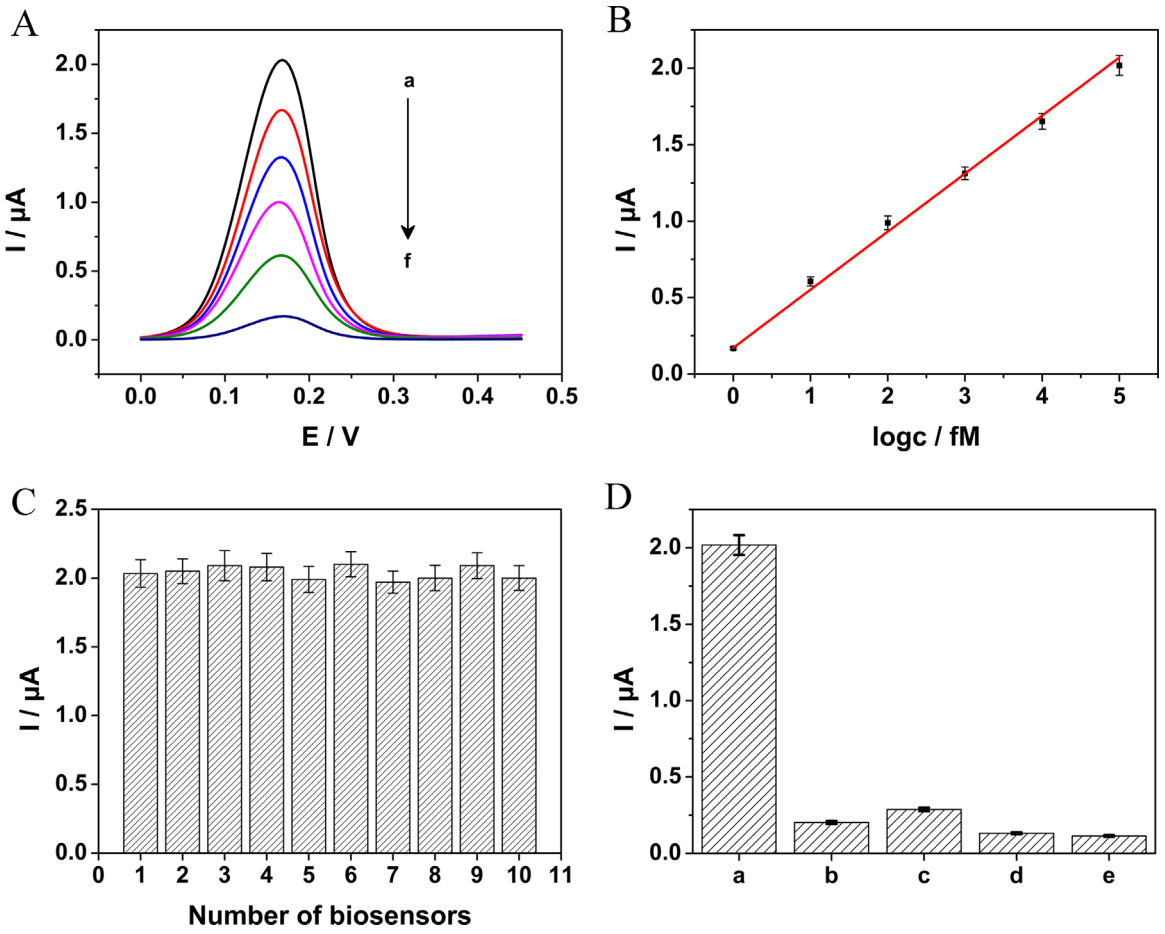


Fig. 3. The DPV response of the final electrodes with different miRNA-21 concentrations (a–f: 10^5, 10^4, 10^3, 10^2, 10, and 1 fM) (A), and the calibration curve (B). The reproducibility of 10 biosensors towards miRNA-21 of 100 pM (C). And The DPV response (D) of this biosensor detecting miRNA-21 (a), one-base mismatch RNA1 (b), one-base mismatch RNA2 (c), three-base mismatch RNA (d), and non-complementary RNA (e) of 100 pM.

3.4. Analytical performance

Since the feasibility of this strategy has been proved and the experimental conditions were optimized, this biosensor can be utilized to determinate target miRNA-21 sensitively ranging from 100 pM to 1 fM. The relationship between the peak current and the logarithm of miRNA-21 concentrations is plotted out (Fig. 3A), and is found out to be in good linear accordance with calibration equation of I_{pa} (μA)=0.17+0.38log$c$ (fM) (R^2=0.997). The detection limit is estimated to be 0.36 fM (S/N=3). The RSDs of 1 fM and 100 pM miRNA-21 are 3.20% and 4.63%, respectively. Comparison of this work's detection range and limit of with previous relevant analytical methods' is shown in Table 1, which illustrates the excellent sensitivity of this biosensor.

To estimate the reproducibility of this strategy, 10 biosensors were constructed to detecting 100 pM miRNA-21, independently. And the results are exhibited in Fig. 3C. 10 biosensors show similar DPV responses with the RSD of 2.4%. This substantiates the excellent reproducibility of this biosensor. To test the stability, after the DPV measurement was done, the final electrodes were put in the 4 °C and then, after 48 h, the electrochemical responses of these electrodes were tested. The result is 72.1% of the first time responses. This decrease is largely due to the instability of ferrocene.

To evaluate the specificity of this biosensor, the target miRNA-21 is replaced by four different RNAs (two one-base mismatch RNAs, one three-base mismatch RNA and one non-complementary RNA). As the Fig. 3D depicts, the electrochemical responses are too small to be noticed with the non-complementary RNA and three-base mismatch RNA. Even for the one-base mismatch RNAs, the resulting currents are only about 1/10 and 1/7 of the current from the biosensor with target RNA, respectively. Because of the mismatch base at 3′ of RNA, T4 Rnl 2 cannot seal the nick between RNA and DNA2. Afterwards, T7 Exo cannot hydrolyze DNA1 completely, and there will be no single-strand DNA2-RNA cooperating with T7 Exo to digest DNA1. Thus there will be lots of DNA1 left to largely digest DNA3, following a very small DPV response. With a base mismatch in the middle of RNA, the efficiency of T7 Exo hydrolyzing DNA1 will mostly be weakened. Then most DNA1 would be left. And so as the same principle, the DPV response would be very weak, too. This clearly verifies that this strategy can successfully discriminate miRNA with similar bases due to its delicate design.

Table 1
The comparison of detection performance of the electrochemical biosensor with other relevant methods.

Methods	Linear range (fM)	LOD(fM)	Refs.
Electrochemistry	300–200000	80	Gao and Yang, 2006
Fluorescence	100–5000000000	35	Li et al., 2009
Electrochemistry	6–2000	3	Peng and Gao, 2011
Electrochemistry	20000–10^8	3400	Cai et al., 2013
Electrochemistry	5–1000	2	Shen et al., 2013
Electrochemistry	100–10^4	45	Xia et al., 2013
Electrochemistry	20–5000	5.36	Yu et al., 2014
Electrochemistry	1–100000	0.36	This work

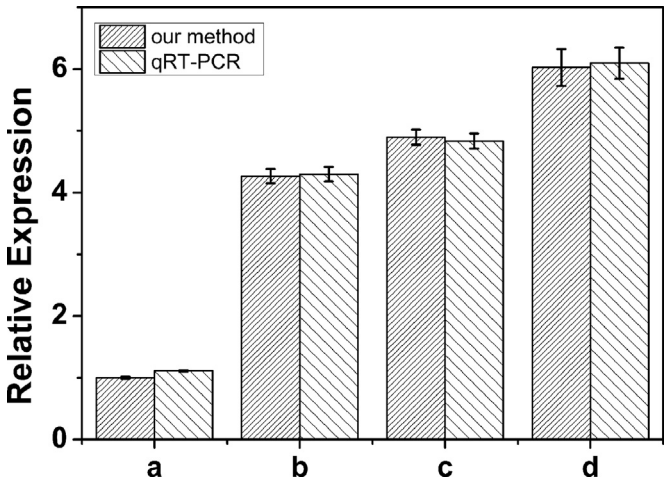


Fig. 4. The relative expression of miRNA-21 in serum of a non-cancer human (a) and three cancer gastric patients (b-d).

3.5. Serum miRNA detection

Furthermore, the relative expression level of miRNA-21 in non-cancer people and cancer patients' gastric serum were investigated via this strategy. After deducted the background, as shown in Fig. 4, the experiment results from our method and the qTR-PCR are in good accordance. The miRNA-21 concentration detected by this method in the serum of gastric cancer patients are 0.781, 0.896, and 1.10 pM, which is higher than it is in the serum of people without cancer, 0.183 pM. The miRNA-21 expression in gastric cancer patients' serum is higher than it in normal human beings, which are 4.26, 4.90 and 6.03 times of the normal one. This confirms that miRNA-21 is up-regulated in human gastric cancer serum and thus authenticates the practical ability and value of this biosensor. Employing this rapid and convenient method is able to help medical researchers clarify miRNA-21 target network and find out the way of using miRNA-21 as biomarker for cancer prediction and treatment. There will be significant meaning for gene functional study and drug development.

4. Conclusion

In summary, we carry out a two-stage cyclic enzymatic amplification method to determinate miRNA-21 in serum successfully, and optimized experimental conditions are investigated. Then the relationship between electrochemical responses with miRNA-21 concentrations is worked out. At last, the relative expression level of miRNA-21 in normal and cancer humans' gastric serum is revealed. Two-stage cyclic enzymatic amplification method endows this biosensor with excellent sensitivity, which can be further improved. Due to the unique functions of T4 Rnl 2 and T7 Exo, miRNA-21 can be specifically detected even with one-base mismatch. Additionally, most of the reactions are happened in the solution, which is far more efficient, and only the last step is taken place on the electrode surface. Our strategy will spend much less time and is very convenient for point-of-care testing and, significantly, if we can improve the immobilization step by shrinking its reaction time, our strategy will be a fine and rapid biosensor. Therefore, using this biosensor, miRNA-21 in serum can be easily, sensitively and specifically determinated without any expensive instrument or complicated procedures. This is an improvement for miRNA analysis.

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