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Electrochemical determination of microRNA-21 based on bio bar code and hemin/G-quadruplet DNAenzyme

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A simple and novel microRNA (miRNA) biosensor was developed using DNA-Au bio bar code (DNA-Au) and G-quadruplex-based DNAenzyme. DNA-Au increased the amount of miRNA-21 participating in hybridization. Hemin/G-quadruplex DNAenzyme significantly improved the catalysis of H_2O_2 by oxidation of hydroquinone, resulting in an obvious reduction current of benzoquinone for miRNA-21 indirect detection. Under optimum conditions, the linear relationship between miRNA-21 concentration and reduction response was obtained with the detection limit of 0.006 pM, which showed a good sensitivity. Besides, selectivity of the biosensor was investigated by detecting the base mismatched miRNAs. This proposed method was further applied to detect miRNA-21 extracted from human hepatocarcinoma BEL-7402 cells and human mastocarcinoma MCF-7 cells. The influence of bisphenol A (BPA) on the expression of miRNA-21 in cells was also investigated. The biosensor performs well in practical applications, which suggests it may provide a new platform for gene diagnosis.

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1 Introduction

MicroRNA (miRNA), a kind of endogenous and noncoding RNA molecule (18–24 nucleotides), commonly exists in plants, animals and virus genomes.[1] As a marker of the classic epigenetic systems, miRNA plays an important role in gene expression.[2] Recently, some reports showed that miRNA was correlated with human cancerous tissues growth and cell differentiation.[3,4] Among these different miRNAs, microRNA-21 (miRNA-21) is regarded as a potential biomarker due to its over-expressed level in many cancer tissues, such as lung adenocarcinomas,[5] glioblastoma,[6] breast cancer,[7] and cervical carcinoma.[8] Thus, miRNA-21 detection has attracted great interest from researchers.

Nowadays, many techniques for miRNA analysis are explored, such as northern blot,[9] cation exchange-based fluorescence amplification,[10] microarrays,[11] real-time PCR[12] and electrochemical method.[13] Among these approaches, electrochemical sensors raise much interest due to its super advantages of good sensitivity, excellent selectivity, low cost and simple operation.[14] Considering the small size of miRNA, target miRNA was generally labeled with electrocatalytic nanoparticles to enhance the determination response in the biosensor's fabrication.[15,16] Although the detection limit is low, the procedure of miRNA modification needs professional operation and strict working conditions, which may cause some inconvenience for real sample determination. Peptide nucleic acid (PNA) capture probes, which have a much higher affinity for miRNA with neutral backbone, are widely applied in miRNA detection with low background noise.[17,18] However, the high thermal stability of PNA–nucleic acid complexes can result in the low selectivity, and the expense is relatively high.[19] Therefore, a simple, low-cost and reliable biosensor for miRNA detection is required to be developed.

Gold nanoparticles (AuNPs) have been received much attention in the filed of DNA or protein determination due to its special properties of easy preparation, low cost and good biocompatibility.[20,21] Recently, DNA-Au bio bar code (DNA-Au) was introduced in many electrochemical reports with good performance.[22,23] Because a large number of DNA sequences can be modified on the AuNPs, the detection sensitivity is significantly improved.[24] In order to enhance the determination selectivity, stem-loop structured hairpin DNA with its excellent specificity has been widely employed for discrimination testing.[25,26] Moreover, it is suitable to detect many biomolecules, such as miRNA,[27,28] DNA[29] and bacterium.[30]

Nowadays, enzyme amplification technology is regarded as the other effective method to enhance the detection signal and applied to biosensor fabrication.[31–34] Catalytic nucleic acids (DNAzymes) have been widely used for biorecognition and biosensing events as a kind of artificial enzyme because of their many advantages, such as ease of preparation, low cost to produce and stability of operation.[35,36] For example, hemin, as an active cofactor for many enzymes containing iron(III),[37] which can catalyze the peroxidation reaction because of its peroxidase-like ability.[38] When incubating with guanine-rich DNA aptamer,

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hemin can intercalate into a G-quadruplex structure, subsequently, a hemin/G-quadruplex DNAzyme is formed.[39]

In this research, we built a novel and simple biosensor based on the amplification of DNA-Au and hemin/G-quadruplex DNAzyme for miRNA-21 detection. MiRNA-21 could be determined indirectly by the reduction response of benzoquinone. Also, the miRNA-21 expression levels from different cells were investigated in this work. The method was applied to detect the influence of bisphenol A (BPA) on miRNA-21 expression. This biosensor may provide a new platform for miRNA practical analysis and in the diagnoses of some diseases.

2 Experimental

2.1 Reagents and materials

Tetra-chloroaurate(III) tetrahydrate ($HAuCl_4 \cdot 4H_2O$) was purchased from National Pharmaceutical Group Chemical Reagents Company (China). Hydroquinone (HQ) was obtained from Sigma-Aldrich (USA). Mercaptopropionic acid (MPA), tris(2-carboxyethyl) phosphine hydrochloride (TCEP), disodium ethylenediaminetetraacetic acid (EDTA) and hemin were purchased from Aladdin (Shanghai, China). Diethylpyrocarbonate (DEPC) was obtained from Amresco (USA). All reagents were of analytical pure grade.

The buffer solutions for electrochemistry were as follows. DNA immobilization buffer was prepared with 0.1 M Tris–HCl, 1.0 mM EDTA, 1.0 M NaCl and 1.0 mM TCEP (pH 7.4). MiRNA hybridization buffer was prepared with 1 × SSC (pH 7.4). DNA hybridization buffer was prepared with 0.1 M Tris–HCl (pH 7.4). Washing buffer solutions were 10.0 mM Tris–HCl buffer (pH 7.4) and 0.1 × SSC (pH 7.4). The hemin solution (100 μM) was prepared with DMSO and stored in the dark at −20 °C. The oligonucleotides were dissolved with TE buffer (10 mM Tris–HCl, 1.0 mM EDTA, pH 7.4). 0.1 M PBS (pH 7.4) was prepared by mixing stock solutions of 0.1 M Na_2HPO_4 and 0.1 M NaH_2PO_4. The pH of buffer solutions was adjusted by adding 0.1 M HCl or 0.1 mM NaOH. Piranha solution was prepared by 30% H_2O_2 and 98% H_2SO_4 with the volume ratio of 3 : 7. All other chemicals were of analytical grade. All of the solutions and distilled deionized water used throughout the experiments were treated by DEPC and autoclaved to avoid the degradation of RNase.

PAGE-purified DNA and HPLC-purified miRNA oligonucleotides were purchased from Sangon Biotechnology Co., Ltd (Shanghai, China) and TaKaRa Biotechnology Co., Ltd (Dalian, China), respectively. Their sequences were listed as follows:

probe DNA: 5′-TGGAGCCCTTCTC-SH-$(CH_2)_6$-3′;

target DNA: 5′-GAGAAGGGCTCCA-$(CH_2)_6$-SH-3′;

hairpin DNA:

5′-SH-$(CH_2)_6$-AAAGGGTTGGGCGGGATGGGCGTCAACATCAG TCTGATAAGCTACGCCCAT-3′;

MiRNA-21:

5′-UAGCUUAUCAGACUGAUGUUGA-3′;

one-base mismatched miRNA: 5′-UAGCUUAUCA*C*ACUGAU GUUGA-3′;

three-base mismatched miRNA: 5′-U*C*GCUUAUCA*C*ACUGAU GU*C*GA-3′;

non-complementary miRNA: 5′-ACCGCACAGGUGGAAUCU AACG-3′.

2.2 Electrochemical measurements

Differential pulse voltammetry (DPV) was performed with CHI832A electrochemical workstation (Shanghai Chenhua Co., China). Electrochemical impedance spectroscopy (EIS) analysis was carried out at CHI 660C electrochemical workstation (Shanghai Chenhua Co., China). The measurements were based on a conventional three-electrode system with a platinum wire auxiliary electrode, an modified Au working electrode (2 mm diameter) and a saturated calomel reference electrode (SCE) with saturated KCl solution. The concentration of miRNA was checked spectrophotometrically at 260 and 280 nm with NanoVue™ instrument (GE-Healthcare, Buckinghamshire, UK).

2.3 Preparation of DNA-Au bio bar code

DNA-Au was prepared according to the previous report.[23] First, the fresh colloidal gold nanoparticles solution were synthesized by citrate reduction of $HAuCl_4$ with the traditional method.[40] Second, the solution containing 2×10^{-7} μM hairpin DNA and 4×10^{-8} μM target DNA was activated with 2.5 μL of acetate buffer (pH 5.2) and 2.5 μL of TCEP for 1 h in dark condition. Third, 500 μL colloidal gold nanoparticles were added into the solution, and shaken gently overnight. The aging procedure was carried out with 10 μL of 10 mM acetate buffer for another 24 h. Last, the excess reagents were removed after centrifugation at 12 000 rpm for 30 min. This red precipitate was then washed and centrifuged three times. The obtained nanoparticles were dispersed into 500 μL redistilled deionized water and stored in refrigerator at 4 °C until further use.

2.4 Preparation of AuNPs modified Au electrode

Prior to use, Au electrode was polished with 0.03 μm alumina powder to a mirror-like finish. It was then washed with 95% ethanol and distilled deionized water for 3 min in an ultrasonic bath to remove the alumina powder and organic substance, respectively. After immersion in Piranha solution (a mixture with 3 : 7 (v/v) of 30% H_2O_2 and 98% H_2SO_4) for 13 min, the electrode was cleaned in distilled deionized water. Then, it was allowed to dry in a N_2 stream until further use. AuNPs were deposited in 3.0 mM $HAuCl_4$ solution containing 0.1 M KNO_3 by amperometry at −0.2 V with 200 s. The obtained electrode was named as AuNPs/Au.

2.5 DNA self-assembly and hybridization

5 μL DNA immobilization buffer containing 1×10^{-6} M probe DNA was dropped on the surface of AuNPs/Au. After reaction for 2 h at room temperature in a humidity chamber, the probe DNA was immobilized on the surface *via* bonding of Au–S. Then, 10.0 mM Tris–HCl (pH 7.4) was used as the cleaning solution to remove the unstable immobilized probe DNA. Subsequently, 5 μL of 1×10^{-7} M MPA diluted with immobilization buffer was dropped on the electrode to eliminate the influence of unspecific probe DNA adsorption. The electrode was defined as probe/AuNPs/Au. For the hybridization reaction, 5 μL DNA hybridization buffer containing DNA-Au (hairpin DNA, 1×10^{-7} M; target DNA, 2×10^{-8} M) was added on the

surface of probe/AuNPs/Au. The hybridization process was carried out at 37 °C in a humidity chamber. Two hours later, the electrode was cleaned by 10.0 mM Tris–HCl (pH 7.4) to remove any nonbonding DNA-Au. The obtained electrode was named as DNA-Au/probe/AuNPs/Au. MiRNA-21 solution diluted with 2 × SSC was dropped on DNA-Au/probe/AuNPs/Au at 37 °C under humid conditions. Following hybridization between hairpin DNA and miRNA-21 for 2.5 h, the loop stem structure of hairpin DNA was opened. This electrode was regarded as miRNA-21/DNA-Au/probe/AuNPs/Au.

2.6 Fabrication of G-quadruplex-based DNAenzyme

The electrode was rinsed with 0.1 × SSC to remove the unhybridized miRNA-21. Then, 5 μL of 20 mM Tris–HCl (10 mM DMSO, 0.03% Triton X-100 and 20 mM KCl, pH 7.4) was dropped on the electrode and incubated for 1 h at room temperature. Finally, an equal volume of 100 μM hemin was added on to the electrode surface. Due to the guanine-rich DNA sequences of hairpin DNA, G-quadruplex-based DNAenzyme was formed after reaction at room temperature for 0.5 h. This electrode was defined as hemin/miRNA-21/DNA-Au/probe/AuNPs/Au. The procedure of miRNA biosensor fabrication based on hemin/miRNA/DNA-Au/probe/AuNPs/Au is shown in Scheme 1.

2.7 Electrochemical determination

DPV was performed in a certain volume of 0.1 M PBS (pH 7.4) containing 1.0 mM H_2O_2 and 0.2 mM HQ. EIS was performed in 5.0 mM $[K_3Fe(CN)_6]/[K_4Fe(CN)_6]$ (1 : 1) solution containing 0.1 M KCl with the frequency from 10^{-1} to 10^5 Hz. The electrochemical measurements were carried out at room temperature.

2.8 Total RNA extraction

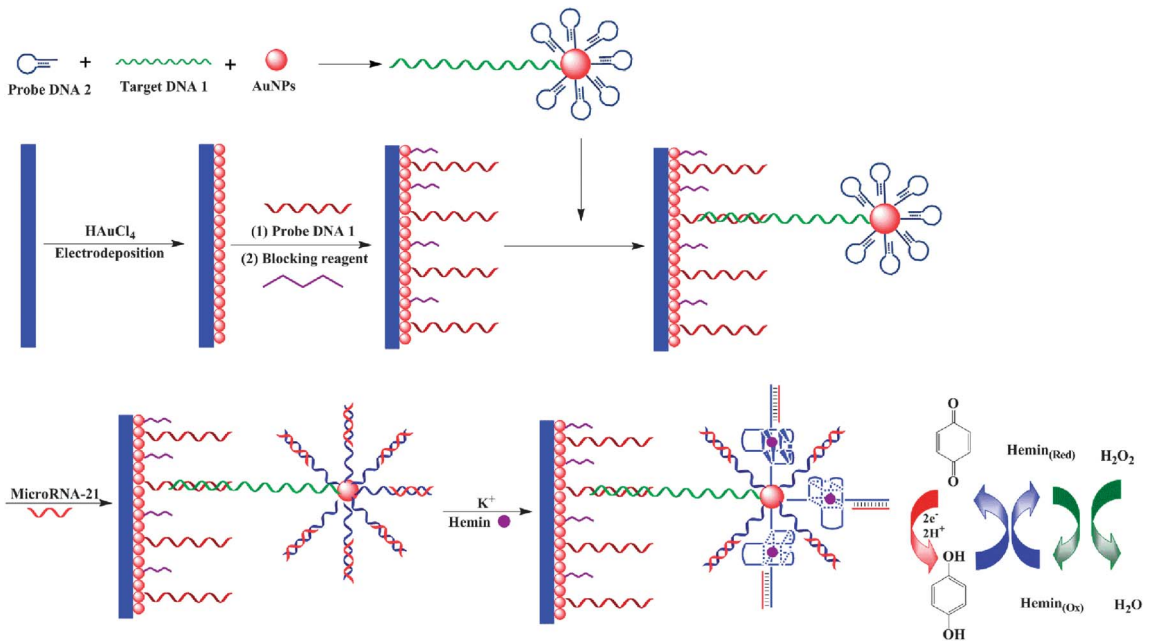
Normal human hepatic L-02 cells, human hepatocarcinoma BEL-7402 cells and human mastocarcinoma MCF-7 cells were obtained from College of Life Science, Beijing Normal University (China). Cells were grown in Dulbecco's modified Eagle's medium (DMEM, purchase from GIBICO, USA) with 10% fetal calf serum (FCS) (Invitrogen, New Zealand) at 37 °C in a 5% CO_2 humid chamber. Total RNA was isolated from the cells using TRIzol reagent according to the manufacturer's instructions. UV-vis spectrophotometry was used to determine RNA concentration. The integrity of RNA was investigated by agarose gel electrophoresis.

Normal human hepatic L-02 cells were incubated with 100 μM BPA solution at same concentration for three days and five days, respectively. The other procedures for miRNA-21 extraction were carried out as mentioned above. These cells were cultured to test the influence of BPA on miRNA-21 expression.

3 Results and discussion

3.1 Characterization of different electrodes

EIS was a valuable and sensitive method to characterize the surface of electrode.[41] Fig. 1 showed the Nyquist diagrams of different electrodes in 5.0 mM $[K_3Fe(CN)_6]/[K_4Fe(CN)_6]$ (1 : 1) solution containing 0.1 M KCl. The electro-transfer resistance value (R_{et}) of bare Au (curve a) was about 216 Ω. After deposition of AuNPs, the R_{et} (curve b) decreased significantly with a nearly straight line due to the good conductivity of AuNPs. A small semicircle was obtained at probe/AuNPs/Au and R_{et} value was 96 Ω (curve c), which illustrated the probe DNA was immobilized on the surface. The R_{et} increased to 251 Ω (curve d) after hybridization with DNA-Au. The reason for this was that the more negatively charged phosphate of DNA limited the electron



Scheme 1 Schematic representation of miRNA-21 biosensor fabrication.

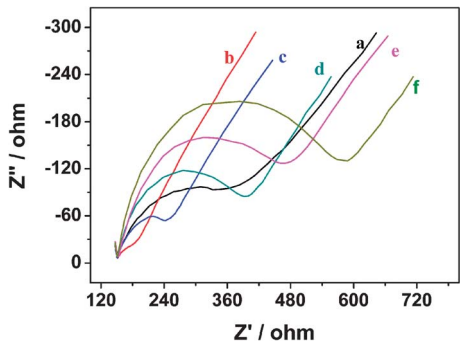


Fig. 1 Nyquist plots of the bare Au electrode (a), AuNPs/Au (b); probe/AuNPs/Au (c); DNA-Au/probe/AuNPs/Au (d); miRNA/DNA-Au/Probe/AuNPs/Au; (e) hemin/miRNA/DNA-Au/Probe/AuNPs/Au in 5.0 mM $[K_3Fe(CN)_6]/[K_4Fe(CN)_6]$ (1 : 1) solution containing 0.1 M KCl at the potential of 0.2 V with the frequency ranged from 10^{-1} to 10^5 Hz. The concentration of miRNA-21 and hemin was 5×10^{-10} M and 1×10^{-4} M, respectively.

transfer of $Fe(CN)_6^{3-/4-}$, which in turn led to the increase of R_{et}. When miRNA-21 hybridized with hairpin DNA modified on DNA-Au, a big semicircle with 313 Ω (curve e) was observed due to the immobilization of miRNA-21 with negative charges. After incubating with hemin solution, the R_{et} significantly increased to 430 Ω (curve f), which was attributed to the formed hemin/G-quadruplex structure preventing the transferring of $Fe(CN)_6^{3-/4-}$ to the electrode surface and that the hemin was non-conducting. This phenomenon demonstrated that the biosensor was successfully fabricated.

3.2 Electrochemical behaviors of hemin/miRNA-21/DNA-Au/probe/AuNPs/Au

DPV was selected to investigate the electrochemical behaviors of different electrodes. Fig. 2 shows the DPV responses of miRNA-21/DNA-Au/probe/AuNPs/Au (curve a) and hemin/miRNA-21/DNA-Au/probe/AuNPs/Au (curve b) in 0.1 M PBS (pH 7.4) containing 1.0 mM H_2O_2 and 0.2 mM HQ from −0.18 to 0.2 V. The reduction current was about 0.029 μA at miRNA-21/DNA-Au/probe/AuNPs/Au. When hemin was modified on the electrode the response (2.46 μA) significantly increased, to 85 times higher than that of miRNA-21/DNA-Au/probe/AuNPs/Au. This phenomenon demonstrated that hemin could improve the detection sensitivity. Meanwhile, the reduction potential of benzoquinone was about −0.096 V at the modified electrode without the assembly of hemin. When treated with hemin, the potential shifted to −0.008 V, illustrating that the use of hemin effectively enhanced electron transfer. This result indicated that the hemin/G-quadruplex had been successfully built. In addition, the improved sensitivity and catalytic ability exhibited by the biosensor was suitable for the determination of miRNA-21 according to the reduction response of HQ.

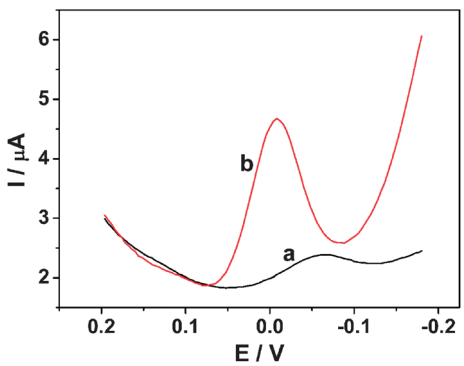


Fig. 2 DPV responses of miRNA/DNA-Au/PDNA/AuNPs/Au (a) and hemin/miRNA/DNA-Au/PDNA/AuNPs/Au (b) in 0.1 M PBS (pH 7.4) solution containing 1.0 mM H_2O_2 and 0.2 mM HQ. The concentration of miRNA-21 and hemin was 5×10^{-10} M and 1×10^{-4} M, respectively.

3.3 Optimization of hybridization time

In order to optimize the experimental conditions, the hybridization time was investigated over the range 1.5 to 3.5 h. The influence of DNA-Au hybridization time is shown in Fig. 3A. The reduction signal increased from 1.5 to 2 h due to the enhancement of DNA-Au amount hybridized with probe DNA. While, the response value decreased when the hybridization time exceeded 2 h, suggesting that more DNA-Au on the modified electrode could prevent the reaction between miRNA-21 and hairpin DNA due to steric hindrance. Therefore, the maximum amount of DNA-Au was obtained at 2 h. In addition, Fig. 3B demonstrated the effect of miRNA-21 hybridization time. As can be seen, the current increased significantly from 1.5 to 2.5 h. Then, the current decreased slightly from 2.5 to 3.5 h. The result indicated that more hemin/G-quadruplex might hinder electron transfer from benzoquinone to the electrode

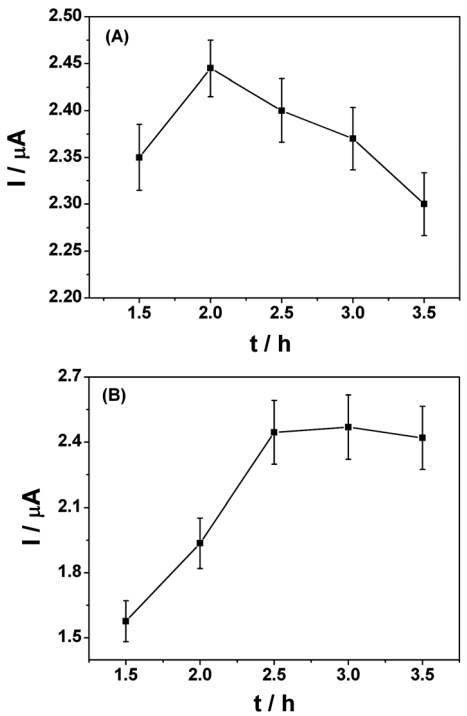


Fig. 3 Effects of DNA-Au hybridization time (A) and miRNA-21 hybridization time (B) on the reduction peak current in 0.1 M PBS (pH 7.4) solution containing 1.0 mM H_2O_2 and 0.2 mM HQ.

surface. Thus, to ensure the efficiency of the experiment, 2 h and 2.5 h were chosen as the optimum hybridization time for DNA-Au and miRNA-21, respectively.

3.4 Detection sensitivity and selectivity for miRNA-21

The detection sensitivity for miRNA-21 based on hemin/miRNA-21/DNA-Au/probe/AuNPs/Au was investigated by DPV in 0.1 M PBS containing 1.0 mM H_2O_2 and 0.2 mM HQ. As can be seen in Fig. 4A, the reduction response increased with increasing miRNA-21 concentration ranged between 0.01–500 pM. The linear relationship between the logarithm of miRNA-21 concentration and the reduction currents was obtained as $I = 1.404 + 0.430\log c$ ($R = 0.991$) and the detection limit was calculated as 6 fM (see inset Fig. 4A). This result demonstrated that the biosensor had good sensitivity for miRNA-21 determination.

In addition, the selectivity of this biosensor was discussed using four kinds of miRNA hybridized with DNA-Au at the same concentration. Fig. 4B shows a comparison of the currents of complementary miRNA-21 (curve a), one-base mismatched miRNA (curve b), three-base mismatched (curve c) and non-complementary miRNA (curve d) after background subtraction. The reduction signals were obtained as 0.505 μA for one-base mismatched miRNA, 0.255 μA for three-base mismatched miRNA and 0.129 μA for non-complementary miRNA, respectively. However, the reduction current of 1 pM complementary miRNA-21 was 1.332 μA, which was obviously higher than that of the other three mismatched miRNAs. Therefore, this biosensor could be used for miRNA sequence discrimination with good selectivity.

In order to test the reproducibility of this miRNA biosensor, five parallel electrodes of hemin/miRNA/DNA-Au/PDNA/AuNPs/Au incubated with 5×10^{-10} M miRNA-21 and 1×10^{-4} M hemin were prepared for this determination. The proposed electrodes demonstrated similar electrochemical signals and an acceptable relative standard deviation (RSD) of 8.2% ($n = 5$), indicated the good reproducibility of the miRNA biosensor.

3.5 Detection miRNA-21 of different cells

In order to investigate the performance of the biosensor with real samples, the content of miRNA-21 extracted from human hepatocarcinoma BEL-7402 cells, human mastocarcinoma MCF-7 cells and normal human hepatic L-02 cells was detected. As shown in Fig. 5A, the expression profile of miRNA-21 in BEL-7402 was 1.415-fold higher than that of normal L-02 cells. Similarly, the miRNA-21 expression enhancement of 1.468-fold from MCF-7 cells was obtained. The phenomenon illustrated that the miRNA expression levels of cancer cells were upregulated compared to those of normal cells, which correspond to previous reports.[42,43] To verify the validity of our developed method, qRT-PCR was performed and the results are shown in

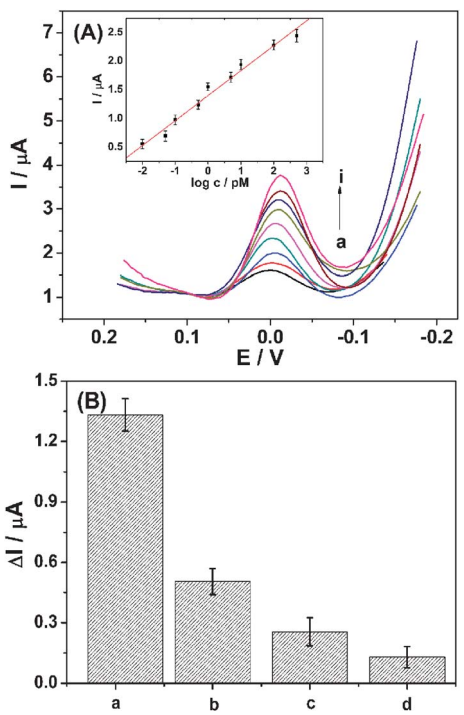


Fig. 4 DPV responses at hemin/miRNA/DNA-Au/PDNA/AuNPs/Au for different concentrations of miRNA-21 (from a to i): 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 100, 500 pM (A). The comparison of DPV signals (B) after background subtraction for modified electrodes hybridized with 10^{-12} M miRNA-21 (a), signal-base mismatched miRNA (b), three-base mismatched miRNA (c) and non-complementary miRNA (d). The electrolyte was 0.1 M PBS (pH 7.4) solution containing 1.0 mM H_2O_2 and 0.2 mM HQ.

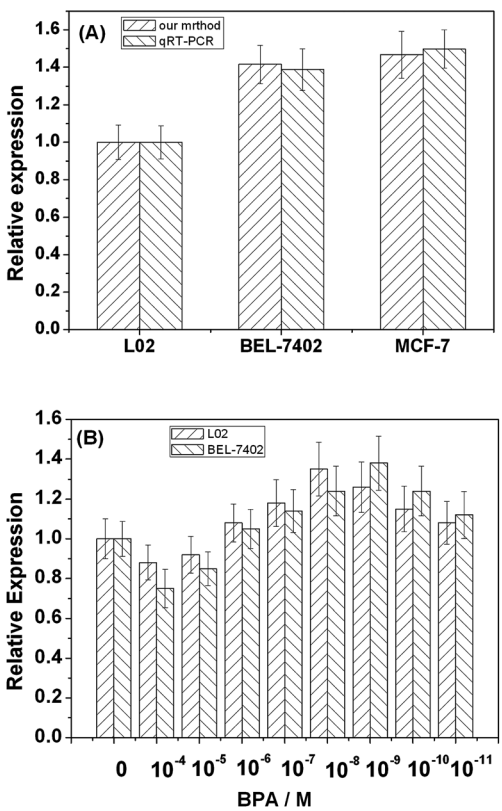


Fig. 5 Expression profiles of miRNA-21 in normal cells (L-02) and cancer cells (BEL-7402, MCF-7) (A). The expression profiles of miRNA-21 from human L-02 cells and BEL-7402 cells with 3 days of exposure to BPA (B).

Fig. 5A. The results indicated that the relative expression level of miRNA-21 obtained by our method was in accordance with the results obtained by qRT-PCR, which demonstrated the validity of our developed method. These results demonstrated that this biosensor can be used for the determination of miRNA-21 in real samples, providing a new platform for clinical diagnosis.

3.6 Influence of BPA on miRNA-21 expression

BPA, an environmental toxin, is representative of endocrine-disrupting chemicals. BPA has been widely used in the chemical industry during the production of polycarbonate and epoxy resin, which are extensively used in the plastics of nursing bottles, in food can linings, and beverage containers. BPA can leach into food from these containers.[44] It has been reported that BPA exposure can lead to specific miRNA alterations in placental cells[45] and breast carcinoma cells.[46] More importantly, research into the association between BPA and increased cancer risk suggests that BPA may elicit epigenetic effects.[47,48] In addition, DNA methylation patterns in cell signaling genes can be altered with BPA exposure, which illustrates that BPA can exert its function through epigenetic mechanisms.[49] Therefore, an investigation of dose-effect relationships between miRNA and BPA is essential. To achieve this aim, human L-02 cells and BEL-7402 cells were cultured for 3 days in the presence of different concentrations of BPA. The total RNAs were then extracted and the content of miRNA-21 was detected by the developed method. As can be seen in Fig. 5B, the relative expression level of miRNA-21 extracted from human L-02 cells increased with decreasing BPA concentration from 100 μM to 10 nM. The same conclusion can be obtained by detecting miRNA-21 extracted from BEL-7402 cells, in which, the relative expression level of miRNA-21 increased with decreasing BPA concentration from 100 μM to 1 nM. These results indicate that low-dose BPA can improve the expression level of miRNA-21 in both normal and carcinoma cells, and high-dose BPA may inhibit the expression level of miRNA-21 in the two kinds of cells.

4 Conclusion

In this work, a simple miRNA biosensor based on DNA-Au bio bar code and G-quadruplex-based DNAenzyme was fabricated. The bio bar code significantly increased the amount of hairpin DNA immobilized on the electrode. Hemin/G-quadruplex structure was formed after the hybridization between miRNA-21 and hairpin DNA, resulting in the improved signal owing to its excellent catalytic ability. The linear relationship between the logarithm of miRNA-21 concentration and current was obtained with a detection limit of 6 fM. Different base mismatched miRNAs were distinguished in this experiment. The biosensor showed that miRNA-21 expression in cancer cells was higher than those of normal cells. Moreover, the influence of BPA on miRNA-21 expression was investigated, illustrating that increasing the incubation time of BPA leads to increased miRNA-21 expression. The results demonstrated that this approach can be applied to practical determination with good sensitivity and selectivity.

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