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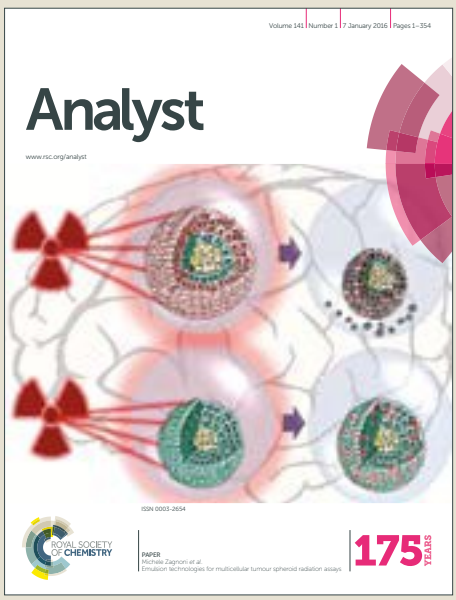

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ARTICLE

An isothermal electrochemical biosensor for sensitive detection of microRNA based on catalytic hairpin assembly and supersandwich amplification

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A novel isothermal electrochemical biosensor was proposed for the sensitive detection of microRNA (miRNA) based on the ingenious combination of the target catalyzed hairpin assembly (CHA) and supersandwich amplification strategies. Since miRNA-221 has been reported overexpression in cancers and it has been a potentially useful biomarker for diagnosis of related diseases, miRNA-221 was chosen as a model target miRNA. The target miRNA-221 triggered a toehold strand displacement assembly of two hairpin substrates, which led to the cyclicality of the target miRNA and the CHA products. Subsequently, the CHA products hybrided with a capture probe on the electrode, the exposed stem of the CHA products was further used to propagate the supersandwich. After that, the signal probe was modified with horseradish peroxidase (HRP) to form a supersandwich multiplex HRP-DNA label, which could achieve amplified electrochemical signal. Using the isothermal dual signal amplification strategies, as low as 0.6 pM (3σ) miRNA-221 could be detected. In addition, this biosensor showed high selectivity and could discriminate miRNA-221 from homologous miRNAs. Notably, human miRNA from cancer cells could also be detected, the results were in excellent agreement with the ones obtained using qRT-PCR. Given that the biosensor avoided nanoparticles introduction, the limitation of the nanoparticles was overcome. The proposed biosensor has great potential for the broad applications in the field of clinical analysis.

1. Introduction

MicroRNAs (miRNAs) are a class of evolutionally conserved small (18-25 nt) single stranded, endogenous, noncoding ribonucleic acid molecule.[1] Since the abnormal expression of certain miRNAs is closely related to a variety of diseases and disorders, especially cancers, miRNAs can be used as valuable biomarkers for cellular level research and related diseases.[2, 3] For example, miRNA-221 that is coded from chromosome X and functions as an oncogenic miRNA participates in a lot of forms of human cancers.[4] It has been reported to be overexpressed in many human cancers, such as human hepatocellular carcinoma (HCC),[5] colorectal cancer[6] and glioblastoma.[7] However, few papers have reported on the sensitive detection of miRNA-221.[8, 9] Therefore, it is urgently needed to detect miRNA-221 for diagnosis and pathogenesis of cancers.

The commonly used methods for miRNA detection are based on traditional molecular biology techniques such as quantitative reverse transcription polymerase chain reaction (qRT-PCR),[10] Northern blotting,[11] and microarrays.[12, 13] However, quantitative detection of miRNAs is quite challenging using these methods, due to the intrinsic characteristics of miRNA, including its short size, sequence homology among family members, low abundance in the total RNA samples, and susceptibility to degradation.[14, 15] For detecting low levels of miRNAs, some signal amplification strategies have been demonstrated. The signal amplification strategy can be simply categorized into two distinct types: target recycling strategy and signal molecules increasing strategy. On the one hand, the target cycling methods usually operated via nucleases (e.g., endonuclease,[16, 17] polymerase,[18, 19] and exonuclease[20]), to indirectly amplify the amount of target analytes. Among these target recycling strategies, CHA is an enzyme-free and isothermal signal amplification technique with negligible background.[21] Recently, CHA has been widely used in various techniques, such as fluorescence[22, 23] electrochemistry,[24, 25, 26] surface plasma resonance (SPR),[27] and colorimetry.[28, 29] On the other hand, the signal molecules increasing strategy can be achieved by nanoparticles and DNA nanostructures.[30] As a typical DNA nanostructure, the supersandwich amplification strategy can be implemented at constant temperature for signal amplification without enzyme or nanoparticles, which avoided complex material synthesis, probe conjugations and strict conditions.[31, 32]

Given the advantages of CHA and supersandwich strategies, herein, a sensitive isothermal electrochemical

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† Electronics supplementary information (ESI) available: Characterization of report probe.

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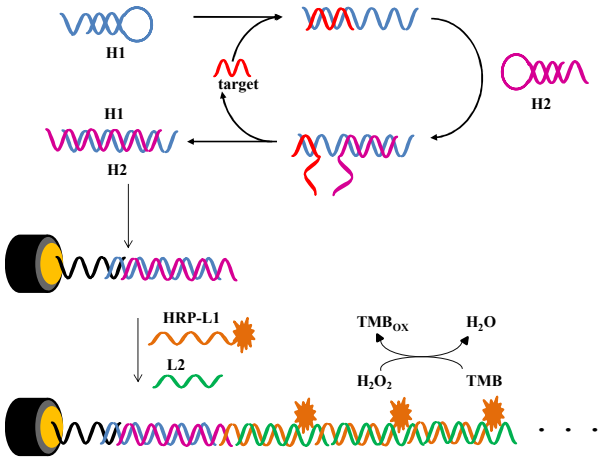
biosensor for miRNA-221 detection was proposed based on the ingenious combination of CHA and supersandwich amplification strategies. As shown in Scheme 1, the presence of target miRNA-221 triggered the dynamic assembly of H1 and H2 to produce H1-H2 complexes accompanying the release of miRNA-221. Meanwhile, the released miRNA-221 could further trigger the hybridization of additional pairs of H1 and H2. Then, H1-H2 complexes hybridized with a capture probe on the electrode, the newly emerging DNA fragment on the electrode was further used to propagate the supersandwich. After incubated with supersandwich products, HRP-DNA as signal tags was introduced to form a supersandwich complex on the electrode, which can catalyze the reaction of TMB/H_2O_2. Amperometric signals that related to target miRNA were finally generated by measuring the increase in reduction current. With the isothermal dual amplification strategies, this method demonstrated high sensitivity and selectivity, and it also had good performance in a complex biological matrix. Moreover, this biosensor avoided the use of nanoparticle for signal amplification.



Scheme 1 The schematic illustration of the electrochemical biosensor for the sensitive detection of miRNA based on the ingenious combination of CHA and supersandwich amplification strategies.

2. Experimental

2.1. Reagents

Diethylpyrocarbonate (DEPC) and all the DNA molecules were purchased from Sangon Biotech. Co., Ltd. (Shanghai, China). The RNA sequences were synthesized by TaKaRa Biotechnology Co. (Dalian, China). Horseradish peroxidase (HRP), tris (2-carboxyethyl) phosphine hydrochloride (TCEP), sulfosuccinimidyl-4- (N-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC) and 3, 3', 5, 5'-tetramethylbenzidine (TMB) were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). Serum samples were collected from healthy volunteers. All the chemical reagents were of analytical grade and used without further purification. Ultrapure water (18.2 MΩ cm) was used throughout. All the consumable items were treated by DEPC and sterilized three times. All DNAs were heated at 95 °C for 5 min and then gradually cooled down to room temperature before use. The sequences of the DNA and RNA were listed in Table 1.

Table 1. Sequences of DNA and RNA probes used.

Name	Sequence (from 5' to 3')
Capture probe	SH-TTT TTG GTT GGT TGG TTG C
H1	GAA ACC CAG CAG ACA ATG TAG CTC CAT GTG TAG AAG CTA CAT TGT CTG CAA CCA ACC AAC CAA
H2	TGT AGC TTC TAC ACA TGG AGC TAC ATT GTC TGC CCA TGT GTA GAC CGA ATT AG
L1	SH-TGA CAT TTG CTT CAT TCC TAT ACG AGT GGC TAT CTT TGC TCT AAT TCG GTC TAC ACA TGG
L2	AGC AAA GAT AGC CAC TCG TAA TCA TCA CTG GAC CGA TAC GCC ATG TGT AGA CCG AAT TAG
miRNA-221	AGC UAC AUU GUC UGC UGG GUU UC
single-base mismatched RNA (smRNA)	AGC UAC AUU GUC UCC UGG GUU UC
three-base mismatched RNA (tmRNA)	AGC UAC AUU CUC UCC UGC GUU UC
deleted RNA	AGC UAC AUU GUC U_C UGG GUU UC
inserted RNA	AGC UAC AUU GUC A UGC UGG GUU UC
miRNA-222	AGC UAC AUC UGG CUA CUG GGU CUC
miRNA-221-5p	ACC UGG CAU ACA AUG UAG AUU U
miRNA-141	UAA CAC UGU CUG GUA AAG AUG G
Random miRNA	N_{22}

The underline bases were the mismatched bases.

2.2. Synthesis and characterization of HRP-DNA conjugate

The HRP-DNA conjugate was prepared using the bifunctional crosslinker sulfo-SMCC, according to the reported method with minor modifications.[33, 34] The DNA L1 was firstly incubated with TCEP (in phosphate buffer, pH=5.5) for 1 h at 37 °C to reduce any disulfide bonds formed upon storage of the oligonucleotide. The HRP was activated with sulfo-SMCC and purified by Amicon-30 K (Millipore, U.S.A.). The purified solution of sulfo-SMCC-activated HRP was mixed with the above solution of thiol-DNA. The resulting solution was kept at room temperature for 48 h. Then, it was purified by Amicon-50 K (Millipore, U.S.A.) using 100 mM PBS (pH=7.4). Finally, the HRP-DNA conjugate was characterized using a 12% SDS-PAGE experiment. This gel was stained by Coomassie brilliant blue. As shown in Fig. S1 in Electronic Supplementary Information (ESI), the migration of the HRP-DNA conjugate band was less than that of HRP. Presumably, the reason was that DNA L1 could conjugate with HRP and increased the molecular weight of HRP (each DNA is around 10 K). The results demonstrated that the HRP-DNA conjugate was formed.

2.3. Agarose gel electrophoresis

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To verify the CHA and supersandwich amplification, agarose gel electrophoresis was used. In the gel electrophoresis assay, a sample containing 2 μL 6 × loading buffer, 2 μL of SYBR Gold and 10 μL of each reaction sample was subjected to the 2% agarose gel electrophoresis. For the CHA reaction, 1 μM (H1) and 1 μM (H2) were incubated with various concentrations of target miRNA and reacted for 2 h at 37 °C. For the surpersandwich reaction, 1 μM (L2) was incubated with L1 and HRP-L1 for 1 h at 37 °C, respectively. All the gels were prepared using a 1 × TBE buffer and were run at 90 V for 40 min.

2.4. Electrochemical detection of miRNA-221 by dual amplification strategies

Electrochemical characterizations including amperometric measurements and electrochemical impedance spectroscopy (EIS) measurements were carried out on a CHI660C electrochemical workstation (Shanghai Chenhua Instruments Co., Ltd., China). All experiments were conducted on a conventional three-electrode system composed of platinum wire as the auxiliary, an Ag/AgCl electrode as the reference and a 2-mm diameter gold electrode as the working electrode.

A previously described pretreatment procedure[35] was applied to clean the gold electrode. After that, 20 μL of 1 μM thiolated capture probe was dropped on the pretreated gold electrodes surface and incubated overnight at 4 °C. After washed with the buffer, the electrodes were immersed into 20 μL of 1 mM MCH for 1 h to obtain a well-aligned DNA monolayer. The electrodes were further rinsed with the washing buffer and dried with nitrogen to obtain the electrochemical DNA biosensor.

CHA amplification system consisted of hairpin probes (H1 and H2) and target miRNA-221. After 2 h CHA reaction, the products hybridized with the capture probes immobilized on the gold electrode for 1 h at 37 °C. Following washed by PBS buffer containing 0.05% Tween-20, 20 μL of surpersanwich products was dipped onto the electrochemical biosensor at 37 °C for 60 min, and was washed thoroughly with PBS buffer containing 0.05% Tween-20. Amperometric detection was fixed at 100 mV, and the electroreduction current was measured 100 s after the HRP redox reaction reached a steady state.

2.5. Cell extractions and qRT-PCR analysis

The total RNA, including miRNA, was extracted from human cancer cell lines using Trizol reagent (Sangon Co. Ltd., Shanghai, China). The concentrated and pure RNA was divided into two equal parts for detection using qRT-PCR (Sangon Co. Ltd., Shanghai, China) and the proposed biosensor.

The cDNA samples were prepared by using the reverse transcription (RT) reaction with RevertAid Premium Reverse Transcriptase (Thermo Scientific, USA). The transcribed cDNA was then used as the template for qRT-PCR. qPCR analysis of miRNA was performed with SG Fast qPCR Master Mix(2X) (BBI), according to the indicated protocol on an LightCycler480 Software Setup (Roche).

3. Results and discussion

3.1. Investigation of the CHA and supersandwich amplification strategies

The feasibility of CHA and supersandwich amplification strategies was first investigated by PAGE. As shown in Fig. 1A, lane 1 and lane 2 showed the band of H1 and H2, respectively. There was almost no H1-H2 hybrid formed when the H1 was incubated with the H2 for 2 h (lane 3), indicating no hybridization occurred between the two hairpin DNAs. When target miRNA was added into the mixture of H1 and H2, a new band corresponding to H1–H2 complexes appeared at a short electrophoresis distance, while the bands corresponding to H1 and H2 almost completely disappeared (lane 4), which could be attributed to the successful dynamic assembly catalyzed by target miRNA.

The formation of the supersandwich structures was also verified by agarose gel electrophoresis. As seen in Fig. 1B, the mixture of L1 and L2 could hybridize with each other and form longer DNA fragments (lane 8). At the same time, the mixture of HRP-L1 and L2 also produced a ladder of different lengths of the supersandwich structure (lane 9), which was longer than L1-L2. The gel electrophoresis results confirmed the formation of the multiplex HRP-DNA supersandwich nanostructures in the process.

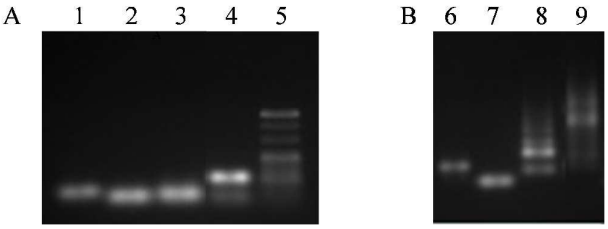

Fig. 1 Analysis of CHA (A) and Supersandwich (B) product using 2 % agarose gel electrophoresis: lane 1, 1 μM H1; lane 2, 1 μM H2; lane 3, 1 μM H1 + 1 μM H2; lane 4, 1 μM H1 + 1 μM H2 + 1 μM target miRNA; lane 5, marker; lane 6, 1 μM L1; lane 7, 1 μM L2; lane 8, 1 μM L1 + 1 μM L2; lane 9, HRP- L1 + 1 μM L2.

3.2. Characterization of electrode surface modification

As a powerful tool to analyse the interface properties of electrodes, EIS was employed to investigate the stepwise assembly process of electrochemical sensing platform. The EIS curves were obtained in 100 mM PBS containing 0.5 mM $[Fe(CN)_6]^{3-/4-}$ and the semicircle diameter was equal to electron-transfer resistance (Ret). Fig. 2 showed the impedance spectra of modified electrodes formed in different construction steps. Compared to 680.5 Ω for the bare the gold electrodes (curve a), Rct value increased to 895.7 Ω (curve b) with capture probe assembled on the electrode. After blocked by MCH (curve c), Rct value risen to 1034 Ω owing to the low conductivity of MCH. Besides, when the hybridization of the capture probe and H1-H2 complexes occurred, Rct value increased significantly to 1290 Ω owing to the steric hindrance effect (curve d). Then, with the supersandwich products assembled on the Au electrode, the value of Rct increased significantly to 2390 Ω (curve e). It suggested that the

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proposed biosensor was constructed as expected, providing a sensitive sensing platform for miRNA detection.

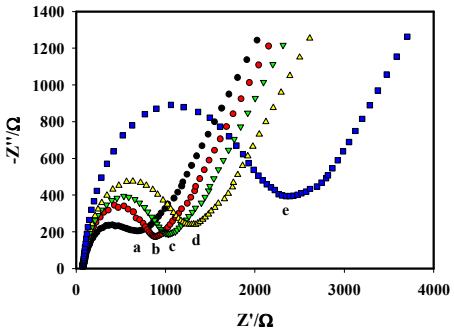

Fig. 2 EIS in 10 mM PBS containing 0.1M KCl and 0.5 mM $[Fe(CN)_6]^{3-/4-}$ at bare electrode (a), capture probe modified electrode (b), MCH immobilized on the electrode surface (c), capture probe modified electrode after hybridized with H1–H2 complexes with target miRNA (d), and reaction with supersandwich products (e), respectively.

3.3. Optimization of experiment conditions

Different experimental parameters, such as the surface density of capture probe on the Au electrode, the concentration of H_2O_2, the incubation time of CHA and supersandwich, and the temperature, could influence the performance of the detection system. To achieve optimal sensing performance, thus, the effects of these factors were investigated.

3.3.1. Effect of surface density. The effect of Au electrodes with different surface density of capture probe was first investigated (Fig. 3A). The surface density of capture probe on Au electrodes was quantitatively measured with chronocoulometry as previously described.[36] As shown in Fig. 3A, different surface coverage (3.14×10^{12} molecules/cm^2, 3.48×10^{12} molecules/cm^2, 4.58×10^{12} molecules/cm^2, 5.75×10^{12} molecules/cm^2) was obtained through incubation of electrodes with 0.5 μM, 1 μM, 2μM, 5 μM capture probe, respectively. As shown in Fig. 3A, with the increasing concentrations of capture probe, current response was enhanced. Further increasing of capture probe concentration led to a slight decrease of current, indicating that the efficiency of DNA hybridization became lower at high concentration of capture probe owing to its steric hindrance. The largest ΔI could be obtained at 1 μM capture probe (3.48×10^{12} molecules/cm^2). Presumably, low-density DNA surface captured few target strands and high-density DNA surface decreased the hybridization efficiency, resulting in the decrease of ΔI. Therefore, 1 μM capture probe was chosen in the following experiments.

3.3.2. Effect of H_2O_2 concentration. As the co-reaction reagent of the TMB, the H_2O_2 concentration has great influence on the reaction efficiency. Thus, the effect of H_2O_2 concentration was investigated. As displayed in Fig. 3B, the current response generated by TMB/H_2O_2 reaction was investigated by changing the concentration of H_2O_2 in the electrolyte solution. The current increased with the increasing of H_2O_2 concentration up to 1 mM and then reached a platform with further addition of H_2O_2. Thus, 1 mM H_2O_2 was employed for the next following experiments.

3.3.3 Effect of the reaction time and incubation time of CHA. The reaction time of CHA which influences the stability and interaction of the hairpins was the key factor for the proposed biosensor, so the reaction time of CHA was studied. With the time increasing, ΔI increased and tended to be saturated when the reaction time of CHA was 120 min (shown in Fig. 3C). The 120 min was chosen as the optimal reaction time, the result was in good accordance with the published works.[21]

Then, the incubation time of CHA products and capture probe was investigated (shown in Fig. 3D). The current increased with the increasing of incubation time and then reached platform until 60 min. Therefore, the 60 min was chosen for the subsequent experiments.

3.3.4 Effect of the reaction time and incubation time of supersandwich. In addition, the reaction time of supersandwich could influence the formation of supersandwich structures, and hence the optimum of hybridization time of supersandwich structure was also studied. As shown in Fig. 3E, with the increasing hybridization time of supersandwich structure, ΔI increased and tended to be saturated when the reaction time was up to 60 min. Subsequently, the best hybridization time of supersandwich structure was obtained, which was the same as the result of our previous work.[37]

Next, the incubation time of supersandwich products was investigated. As shown in Fig. 3F, with the time increasing, ΔI increased and then reached platform until 60 min. Therefore, 60 min was selected as the optimal incubation time of supersandwich products.

3.3.5 Effect of temperature. Temperature is a key parameter of reaction kinetics and determines the probability of collisions between molecules.[38] Because temperature is interrelated with stability hairpin probes, the specificity of this platform would be debased by high reaction temperature. Conversely, at low temperature, the hairpin probes could not get a sufficient collision probability that greatly reduced the formation of H1–H2 complexes. The incubating temperature of proposed biosensor was also optimized. As shown in Fig. 3G, the peak value of ΔI was obtained at 37 °C. Hence, 37 °C was designated as the optimum incubating temperature.

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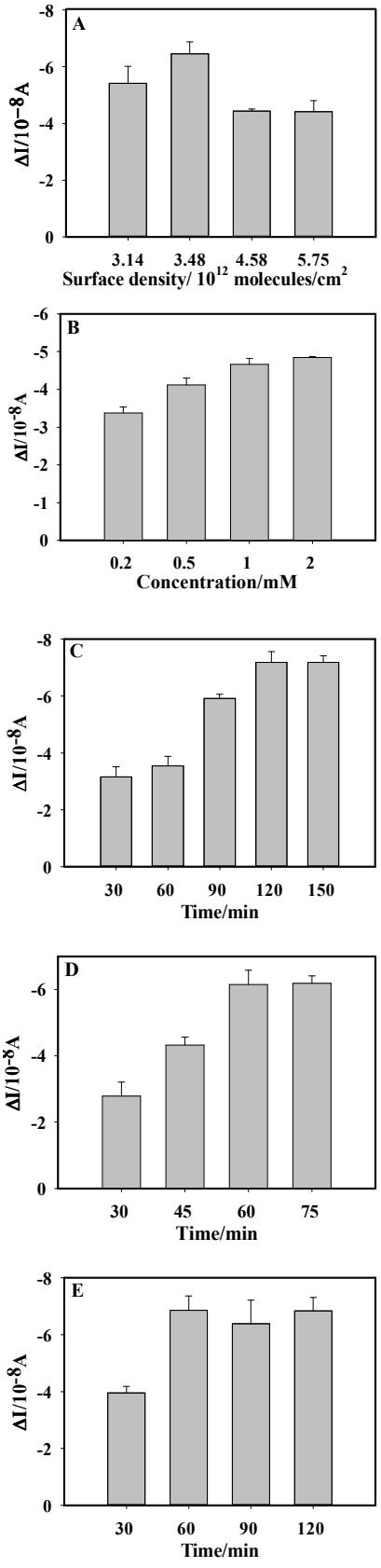

Fig. 3 Optimizations of experimental conditions. Effects of (A) surface density, (B) H_2O_2 concentration, (C) the reaction time of CHA, (D) the incubating time of CHA products and capture probe, (E) the reaction time of supersandwich, (F) the incubating time of supersandwich products, and (G) temperature on the amperometric responses of the biosensor for the presence of miRNA-221 (20 nM). $\Delta I = I - I_0$, where I and I_0 was the currents detected of the proposed biosensor in the presence and absence of miRNA-221, respectively. The error bars represented the standard deviation of three independent measurements. When one parameter changed, the others were under their optimal conditions.

3.4. Analytical performance of miRNA detection

Under the optimal experimental conditions, the sensitivity of the proposed electrochemical biosensor for miRNA-221 was confirmed. Fig. 4A showed that the current from the elecrocatalytic reaction increased as the miRNA-221 concentration increased from 0 to 20 nM. Fig. 4B showed the relation between ΔI and the concentration of miRNA-221 of two methods (i.e. CHA amplified electrochemical biosensor and the proposed biosensor), it was estimated that the detection limit of CHA amplified electrochemical biosensor and the proposed biosensor were 0.16 nM (3σ) (curve b of Fig. 4B) and 0.6 pM (3σ) (curve a of Fig. 4B). This is to say, the detection limit of the proposed biosensor was 3 orders of magnitude lower than that of CHA amplified electrochemical biosensor. It indicated that the amplification efficiency of the proposed biosensor was more evident than that of by CHA amplified electrochemical biosensor. In addition, it could be comparable with or even much lower than other previously reported biosensors.[20, 39-41]

To evaluate the selectivity of the designed biosensor, the effects of nine kinds of miRNA sequences on current changes, including miRNA-221, miRNA-222, miRNA-221-5p, miRNA-141, smRNA, tmRNA, deleted RNA, inserted RNA and random miRNA were investigated. Among them, miRNA-221 and miRNA-222 belong to the same family, and miRNA-221,

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miRNA-222, miRNA-221-5p are associated with HCC.[8, 9] MiRNA-141 shows overexpression in various cancers.[42] As shown in Fig. 4C, the system only showed a remarkable amperometric response in the presence of miRNA-221. Nevertheless, in the presence of tmRNA, miRNA-141 and random miRNA, the amperometric response was negligible. For miRNA-222, miRNA-221-5p, smRNA, deleted RNA, inserted RNA, ΔI increased slightly as these RNA concentrations were increased. The results demonstrated that the biosensor had excellent selectivity for target miRNA against homologous miRNAs.

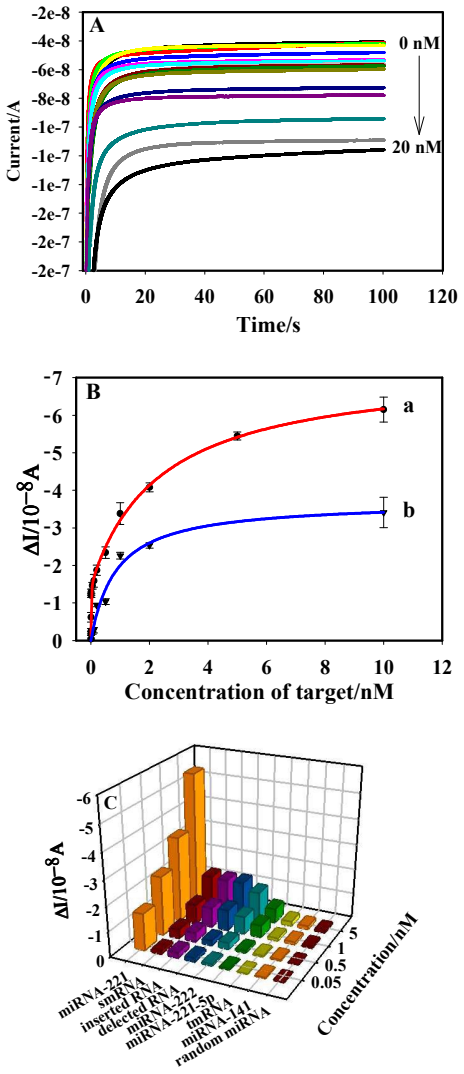

Fig. 4 (A) Amperometric responses of the biosensor with different concentrations of target miRNA-221. (B) The relationship between ΔI and miRNA-221 concentrations by using the proposed biosensor (a) and CHA amplified electrochemical biosensor (b). (C) Selectivity investigation of the proposed biosensor. The error bars represented the standard deviation of three independent measurements.

3.5. Real sample analysis

To investigate the capacity of the proposed biosensor for detection of real sample, the RNA samples extracted from cell lines as model matrix were employed. Three human cancer cell lines, including HCC cell lines (Bel-7404, HepG-2) and human normal liver cell lines (L02) were chosen to measure the expression of miRNA-221 in total RNA from cells lines. In the literature,[8, 9] it was reported that miRNA-221 is overexpressed in HCC cell lines, as compared with human normal liver cell lines. The relative expression levels based on the expression ratio of miRNA-221 in target cell lines versus that in HepG-2 cell lines were used to characterize miRNA-221 levels. Herein, the relative expression levels of miRNA-221 in total RNA were specifically determined using qRT-PCR and the proposed biosensor. As shown in Fig. 5A, the proposed biosensor generated a result similar to that of qRT-PCR. Both methods showed that miRNA-221 had different expression levels in three cancer cell lines. It was observed that the total RNA from Bel-7404 cell lines had a higher concentration of miRNA-221 than that of L02 cell lines, which was in good accordance with a previous report[8] of up-regulation of miRNA-221 in liver cancer. The results indicated that this proposed biosensor would hold a great promise for practical application in miRNA detection.

The proposed biosensor was also used to detect miRNA-221 in human serum. The human normal serum (which does not contain miRNA in detectable quantity) was first treated using the RNase inhibitor.[43] Then different concentrations of miRNA-221 were introduced into the serum samples for obtaining miRNA-221 spiked serum samples. Next, after the miRNA-221 spiked serum samples were centrifuged to remove macromolecules using centrifugal filtration devices (30 K), such spiked serum samples were detected. As shown in Fig. 5B, the signal caused by miRNA-221 in human serum was similar to that in buffer, suggesting that the other components of the serum did not influence the measuring properties of the proposed biosensor.

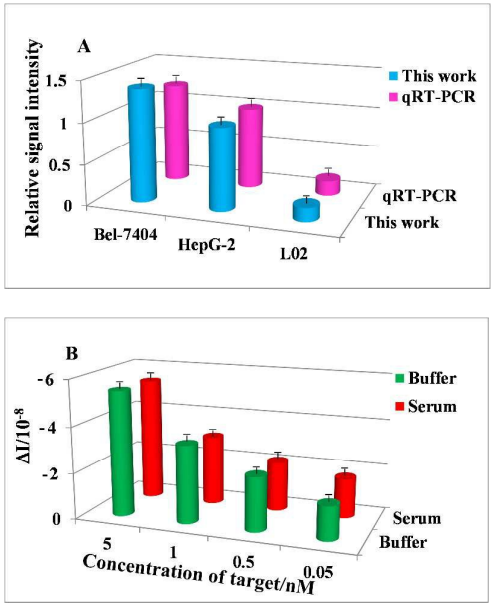

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Fig. 5 (A) Paired comparison of the miRNA-221 levels detected in total RNA isolated from three kinds of human cancer cell lines using the proposed biosensor (blue histogram) or qRT-PCR (pink histogram). Relative signal intensity was based on the expression ratio of miRNA-221 in target cell lines versus that in HepG-2 cell lines. (B) Detection of miRNA-221 in buffer (green histogram) and human serum (red histogram).

4. Conclusions

In summary, a novel isothermal electrochemical biosensor for sensitive quantitative detection of miRNA was successfully constructed based on CHA and supersandwich amplification strategies. On the basis of dual signal amplification strategies, the proposed biosensor exhibited a superior sensitivity for miRNA-221. Moreover, the prepared biosensor showed high specificity and could be applied to monitor miRNA-221 in real sample. The results obtained using the proposed biosensor were in excellent agreement with the ones obtained using qRT-PCR. In addition, the biosensor eliminated the need of nanoparticles. The proposed biosensor provides a powerful tool for signal amplification and has a wide potential application in bioanalysis. For prospective development, it is worthwhile to integrate with the microfluidic chip for further shortening the detection time and decreasing the reagent consumption.

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