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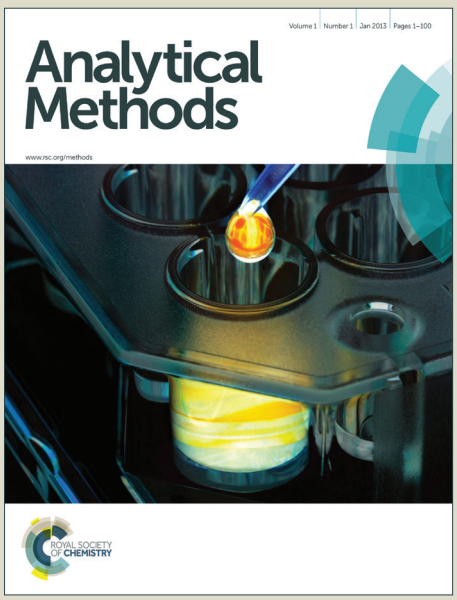

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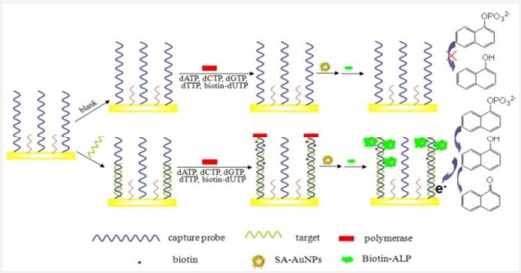
Title Text **A sensitive electrochemical biosensor for microRNA based on streptavidin-gold nanoparticles and enzymatic amplification**		An electrochemical method for microRNA has been proposed with dual signal amplification based on streptavidin-gold nanoparticles and enzyme

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ARTICLE

A sensitive electrochemical biosensor for microRNA based on streptavidin-gold nanoparticles and enzymatic amplification

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An electrochemical method for microRNA (miRNA) has been proposed with a dual signal amplification strategy. The method relies on polymerase extension and two-step signal amplification using streptavidin-gold nanoparticle biocomplexes and alkaline phosphatase. The target miRNA can hybridize with the capture DNA template, which can act as a primer and be extended along the template in the presence of DNA polymerase and dNTPs. Biotin group was introduced into the duplex due to the incorporated biotin-11-dUTP. Thus, biotinylated alkaline phosphatase would bind to the duplex using streptavidin–gold nanoparticles as linkers, which resulted in an amplified electrochemical signal. The electrochemical signal exhibited a linear correlation to the logarithm of miRNA concentration ranging from 100fM to 1nM, with the detection limit of 99.2fM. The specificity of the method allowed single-nucleotide difference between miRNA family members to be discriminated. The established biosensor displayed excellent analytical performance toward miRNA detection and might present a convenient tool for biomedical research and clinic diagnostic application.

Introduction

microRNAs (miRNAs)，a large class of short, endogenous, noncoding RNA of about 18−24 nucleotides (nt) found in nematodes, plants, insects, and mammals are play important roles in normal and pathologic processes. They regulate gene activity and act to promote or repress cell proliferation, migration, and apoptosis.[1−4] It has been reported that specific changes in miRNA expression profile are associated with human cancers, neurological diseases, and viral infection.[5] MiRNAs have been regarded as biomarkers and therapeutic targets in cancer treatment recently.[1,4,6−9] Therefore, sensitive, accurate and multiplexed miRNA detection has great significance for diagnosis and treatment of human cancers, as well as for better understanding of the mechanism of tumorgenesis and discovery of new targets for drugs.[10] However, their unique characteristics of small sizes, extremely low abundance in total RNA samples, and high sequence homology among family members have made a great challenge for quantitative analysis.[11] Northern blot is currently used as the gold standard method for miRNA analysis,[12-15] but its low sensitivity, labor-intensive steps, and the requirement of a large amount of RNA samples have limited its broad applications. The microarray-based method makes multiple miRNA analysis feasible,[12, 13] but its low sensitivity and poor specificity are still unavoidable.[16] The real-time PCR based method has been developed for its high sensitivity and specificity. However, it requires strict operation process and sophisticated person. It requires precise control of temperature cycling for successful amplification, and the short length of miRNAs makes their experimental design very sophisticated.[17-18] To resolve these problems, many convenient and sensitive methods have been developed in the recent years, such as: rolling circle amplification (RCA) [19], sequencing [20], surface plasmon resonance spectroscopy [21], surface enhanced raman spectroscopy,[22] bioluminescence,[23] fluorescence,[24] and electrochemistry,[25-26] etc.

Among these methods, electrochemical analysis has gained increasing interest due to its inherent advantages such as simplicity, sensitivity and low cost as demonstrated in wide applications in different fields. In this paper, a simple and sensitive electrochemical method for miRNA was reported with signal amplification using streptavidin–gold nanoparticles (SA–AuNPs) and alkaline phosphatase (ALP), combined with polymerase extension. gold nanoparticles (AuNPs) have large surface to volume ratio and are biocompatible, which makes them very suitable to be used as carriers of biological molecules. In the present method, AuNPs were used to load a large amount of streptavidin to construct the SA–AuNPs complexes, which could amplify the detection signal. The target miRNA hybridized with the capture probe immobilized on the electrode and then triggered primer extension reaction. During the extension, the biotinylated nucleotide would be incorporated into the duplex, and then the biotinylated ALP (biotin-ALP) could be captured on the electrode surface using SA-AuNPs as linkers through the streptavidin-biotin interactions. The ALP catalyzed the conversion of an electrochemically inactive 1-naphthyl phosphate into an electrochemical active naphthol for generating an amplified electrochemical signal. The ability of SA-AuNPs on the signal amplification was also investigated comparing with streptavidin (SA). We found that the effect of SA-AuNPs on signal

amplification was significant and with the amplification using SA-AuNPs and ALP, the result for the detection of miRNA was satisfactory.

Experimental

Reagents and Apparatus

DNA oligonucleotides used in this work were synthesized by Shanghai Sangon Biotechnology Co., Ltd. (Shanghai, China). DNA sequences used were as follows:

Capture Probe: 5'-TCAGAGCTACTGAACTATACAACCT A C TACCTCATTTTTT-3'SH

RNA oligonucleotides used in this work were synthesized by Dalian TaKaRa Biotechnology Co. Ltd. (Dalian, China) DNA sequences used were as follows:

let-7a: 5'-UGAGGUAGUAGGUUGUAUAGUU

let-7b: 5'- UGAGGUAGUAGGUUGUGUGGUU

let-7c: 5'-UGAGGUAGUAGGUUGUAUGGUU

Bst DNA polymerase was received from New England BioLabs (U.K.). Streptavidin-gold nanoparticles (SA-AuNPs) and 1-naphthyl phosphate (1-NP) were obtained from Sigma–Aldrich (Shanghai, China) and biotinylated alkaline phosphatase (biotin-ALP) was purchased from Vector Laboratories. INC.(U. S.). The deoxynucleotide solution (dATP, dGTP, dCTP, dTTP, Biotin-11- dUTP) and RNase inhibitor were purchased from Shanghai Sangon Biotechnology Co., Ltd. (Shanghai, China). All other reagents were of analytical reagent grade and used as received without further purification. The solutions were prepared using ultrapure water which was obtained through a Millipore Milli-Q water purification system (Billerica, MA) and treated by DECP.

Differential Pulse Voltammetry (DPV) and electrochemical impedance spectra (EIS) experiments were carried out on a CHI760B electrochemical workstation (Shanghai Chenhua Apparatus, Shanghai, China). A conventional three-electrode system consisted of a gold electrode (2.0 mm diameter) as the working electrode, a platinum foil as the auxiliary electrode, and a saturated calomel electrode (SCE) as the reference. All experiments were performed in a 10 mL voltammetric cell at ambient temperature.

Electrode modification

The gold electrode was polished sequentially with 0.3 and 0.05 μm alumina powder followed by ultrasonic cleaning sequentially with ultrapure water, absolute alcohol, and ultrapure water for 5 min each. Then, the gold electrode was dipped in fresh piranha solution (70% H_2SO_4, 30% H_2O_2) for 30 min. Finally, the electrode was rinsed with ultrapure water and dried under nitrogen stream. An aliquot of 10 μL 1 μM capture solution was pipetted onto the surface of cleaned electrode which was kept overnight at 4℃. Afterwards, the electrode was rinsed with washing buffer (PBST, 10mM KH_2PO_4-Na_2HPO_4, 0.05% tween-20, pH 7.4) to remove the unbound DNA probes. And then, 10 μL of 1 mM MCH was dropped on the electrode surface kept for 30 min in order to block the nonspecific sites. Finally, the resulting electrode was rinsed with the washing buffer solution and ready for use.

Electrochemical assay for miRNA

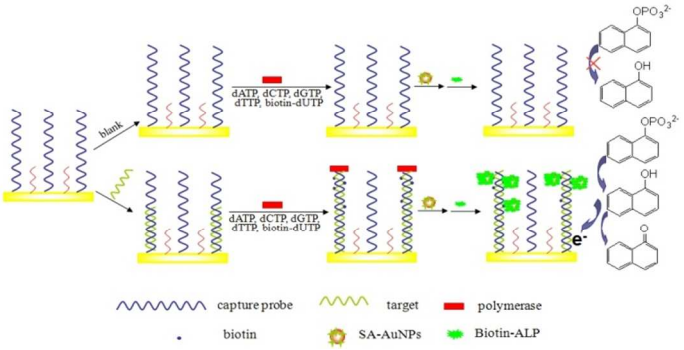
The mixture solution containing 1μL of varied concentration of miRNA, 1μL of 10× Bst polymerase buffer (200mM Tris-HCl, 100mM NaCl, 100mM $(NH_4)_2SO_4$, 20mM $MgSO_4$, 1% Triton X-100, pH 8.8), 1μL of Bst polymerase, 1μL of dNTP mixtures (0.25μL dATP, 0.25μL dCTP, 0.25μL dGTP, 0.25μL of the mixture of dTTP and Biotin-dUTP), 1μL of RNase inhibitor and 5μL H_2O was pipetted on the electrode and incubated for 1h at 55℃. And then it rinsed with 1× Tris-HCl (2.5 mM Tris-HCl, pH 7.9, 5mM NaCl, 0.5 mM $MgCl_2$) buffer containing 0.05 % Tween 20. Blocked with 1% BSA, 10 μL SA-AuNPs with proper concentration were dropped on the electrode. And then, 10 μL of biotin-ALP was dropped on the electrode.

Finally, the electrode was immersed into 3 mL of measuring buffer (100 mM tris-HCl , 1 mM Mg^{2+} and 4 mM 1-NP, pH 9.8) for 3 min. DPV voltammogram was recorded at a potential range from 0 to 0.6 V (vs. SCE) with a 100 mV/s scan rate.

Results and discussion

Principle of miRNA assay

The working principle of miRNA assay is illustrated in Scheme 1. The capture probe with a thiol group at the 3' terminus was immobilized on the surface of the gold electrode via Au-SH binding. Part of the capture probe was complementary to the target miRNA. The specific hybrization of miRNA with the capture probe resulted in the formation of a DNA/RNA heteroduplex. In the presence of DNA polymerase (Bst polymerase) and dNTPs including dATP, dGTP, dCTP, dTTP, Biotin-11- dUTP, the miRNA could act as a primer and be extended along the capture DNA template. Biotinylated nucleotides could then be incorporated by the polymerase in the downstream extention of the miRNA. Thus, SA-AuNPs could bind with the biotin group in the downstream extention of the miRNA, which allowed the capture of biotinylated ALP on the electrode surface via the SA-AuNPs linker due to the specific streptavidin-biotin interaction. As a result, ALP catalyzed the conversion of an electrochemically inactive 1-NP into an electrochemical active naphthol, generating an amplified electrochemical signal.



Sheme 1. Principle of the signal-amplified electrochemical assay for miRNA

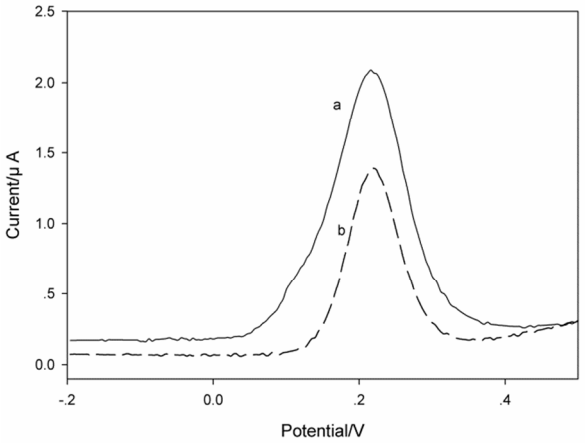


Fig. 1 Effect of SA-AuNPs on signal amplification (a) in the presence of SA-AuNPs (b) in the presence of SA

Effect of SA-AuNPs on the signal amplification

In this study AuNPs were employed as a carrier for SA. Because of its large ratio of surface to volume, several straptavidin molecules would be loaded on one nanoparticle with more biotin-ALP captured, resulting in the signal amplification. In this study, we studied the ability of SA-AuNPs on the signal amplification comparing with SA in the presence of 10pM of miRNA. As shown in Fig. 1, when using SA-AuNPs, the electrochemical signal （curve a）was much larger than obtained with SA alone（curve b）, indicating a strong ability of SA-AuNPs on the signal amplification.

Characteristics of the modified electrode

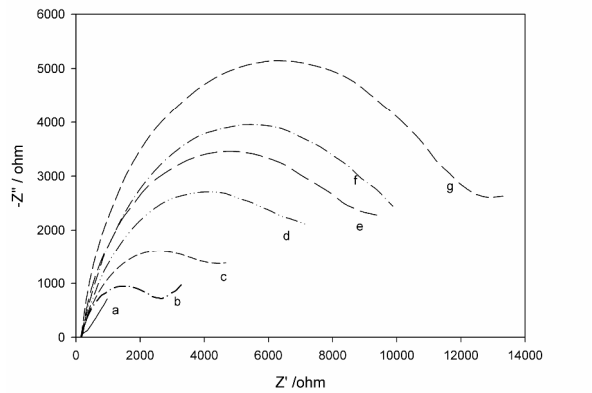


Fig. 2 Nyquist diagrams of electrochemical impedance spectra (in the frequency range of 0.1–10 kHz) after different steps of modification. All the measurements were performed in 5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ solution containing 100 mM KCl.

Electrochemical impedance is an effective and convenient technique for studying the feature of the modified electrode. Each step of self-assembled process was characterized by electrochemical impedance spectroscopy, as shown in Fig. 2. Curve a showed the electrochemical impedance of the bare electrode. When the capture probe was immobilised on the electrode (curve b) through the Au-SH bond and MCH blocked the modified elctrode (curve c), the electrochemical impedance increased obviously. After miRNA bound with capture probe and was extended along the template to form a double-stranded DNA (dsDNA) duplex, the electrochemical impedance increased continuously(curve d). The impedance increased when the electrode was blocked with BSA (curve e). The electrochemical impedance continued increasing with the introduction of the SA-AuNPs (curve f) and biotin-ALP (curve g). Due to the electron obstruction of the hydrophobic proteins or target miRNA assembled on the surface of electrode, the electrochemical impedance would increase step by step, which was in a good agreement with the EIS measurements and also indicated that the biosensor was constructed successfully.

Optimization of experiment conditions

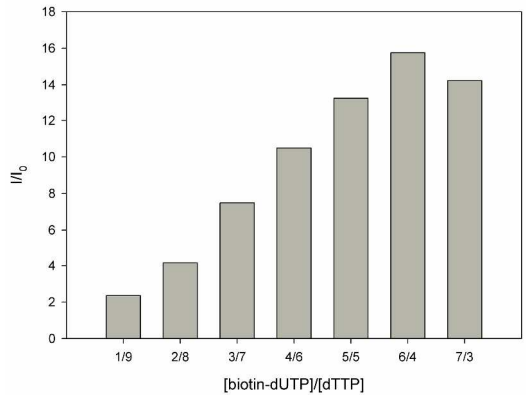


Fig. 3 Effect of the ratio of biotin-dUTP to dTTP at the condition: 1:50 dilution ratio of SA-AuNPs, 0.6U/mL Bst polymerase.

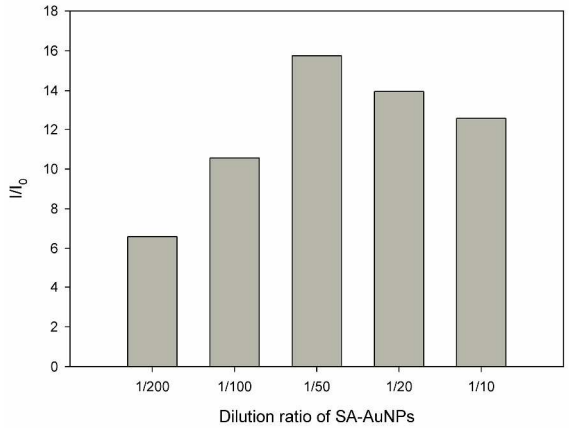


Fig. 4 Effect of the concentration of SA-AuNPs at the condition: the ratio of biotin-dUTP to dTTP is 6:4, 0.6U/mL Bst polymerase.

In this study, biotin group was introduced into the duplex through incorporating biotin-dUTP. However, if only biotin-dUTP was used in the polymerase reaction instead of the mixture of dTTP and biotin-dUTP, the polymerase extension reaction could not proceed continuously because the incorporated biotin-dUTP would create huge steric hindrance at the polymerase active center and prevent the extension reaction.[27] So the mixture of biotin-dUTP and dTTP was used in this study and the ratio of biotin-dUTP to dTTP was optimized. The corresponding results are shown in Fig. 3, where I denotes the peak current in the presence of 10pM of miRNA and I_0 denotes the peak current in the absence of miRNA. The value of I/I_0 increased with the increasing ratio below 6:4. However when the ratio continued to increase, the peak current would be decreased, so the ratio of biotin-dUTP to dTTP was selected as 6:4 in the following experiments.

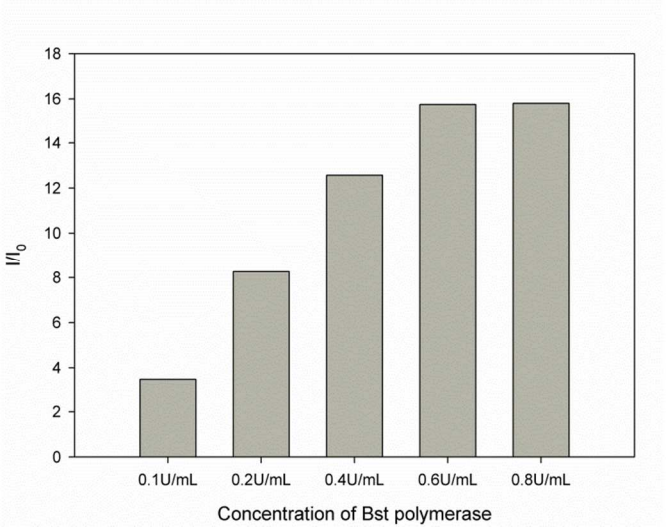


Fig. 5 Effect of the concentration of Bst polymerase at the condition: the ratio of biotin-dUTP to dTTP is 6:4, 1:50 dilution ratio of SA-AuNPs.

The concentration of the SA-AuNPs was a critical factor for the performance of this biosensor. With increasing concentration of SA-AuNPs, the electrochemical signal increased gradually either in the absence of or in the presence of microRNA. And the ratio of I to I_0 is plotted versus SA-AuNPs concentration in Fig. 4. When the dilution ratio of SA-AuNPs is 1:50, the ratio of I to I_0 reached maximum. When the concentration of SA-AuNPs increased continuately, the value of I/I_0 decreased slightly probably due to the non-specific adsorption of the SA-AuNPs. Therefore, the dilution ratio of 1:50 was selected in the following experiments. The concentration of Bst polymerase was also optimized. The result shows that with the increase of the concentration of Bst polymerase, the value of I/I_0 increases until the concentration reaches 0.6U/mL, as seen in Fig. 5. So the concentration was selected as 0.6U/mL in next experiments.

Analytical Performance of the Designed Biosensor

In order to elucidate the analytical performance of the established miRNA assay in this work, experiments were carried out with different concentration of synthetic target miRNAs under the optimal conditions. In Fig. 6A, the DPV signal increased with the increasing concentration of target miRNA. The plot of the DPV peak current was linear with the logarithm of target concentration in the range from 100 fM to 1 nM, and the limit of detection was calculated to be 99.2 fM according to the rule of three times standard deviation over the blank response, as shown in Fig. 6B. Compared to Northern blot[15], the proposed electrochemical biosensor showed a much lower limit of detection due to two-step signal amplification using SA-AuNPs and biotin-ALP, and moreover, the proposed method was more convenient and simple. The performance of this biosensor was also comparable to or more excellent than those of fluorescence sensor[28], electrochemical sensor based on graphene[26], and SPR imaging [29] etc.

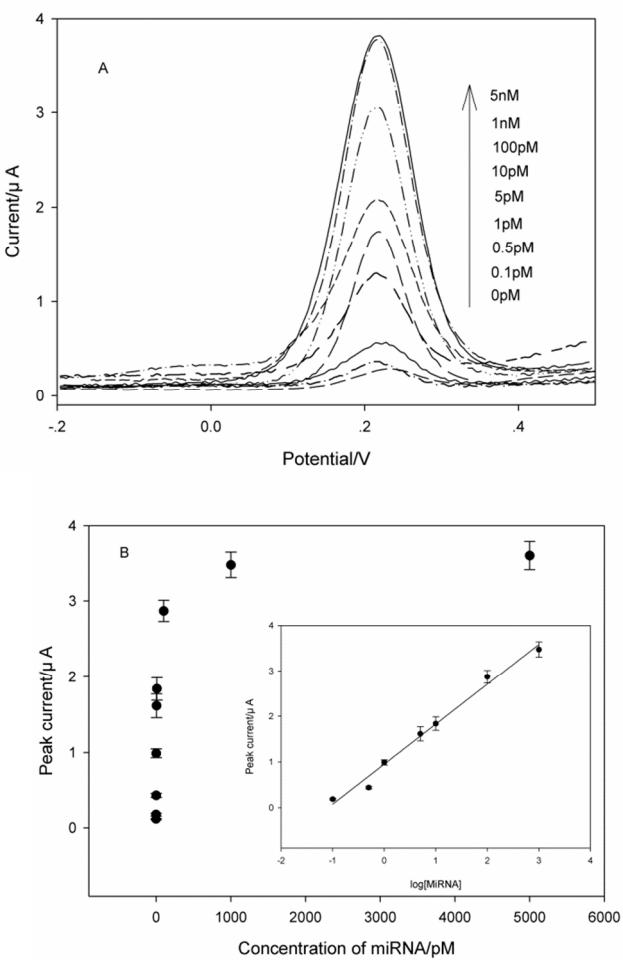


Fig. 6 (A) DPV response of different concentrations of miRNA the curves from bottom to top were 0, 0.1, 0.5, 1, 5, 10，100 pM, 1, 5nM. (B) The response curve obtained with different

concentrations from 0 to 5 nM. Error bars represent standard deviations (n=3). The insert shows the calibration curves of miRNA (1-1000 pM).

Specificity of the Strategy

A great challenge of the miRNA assay is to distinguish the miRNA family members with high similarity. To evaluate the selectivity of the proposed method, three members of let-7 family (let-7a, let-7b, and let-7c), with only one- or two-nucleotide difference between them, are selected as an experimental model system. Under the same experimental conditions, the sensing systems with different miRNA targets at 10 pM were compared upon the peak current. As shown in Fig. 7, the remarkable response difference between perfect-matched and mismatched miRNA targets indicated that the established miRNA assay readily discriminated a single mismatch in miRNA family members with excellent specificity. The simple, highly sensitive and specific method allowed accurate quantitation of miRNA let-7a at low concentrations, which is of great significance in the early diagnosis of lung diseases and medical research.[30]

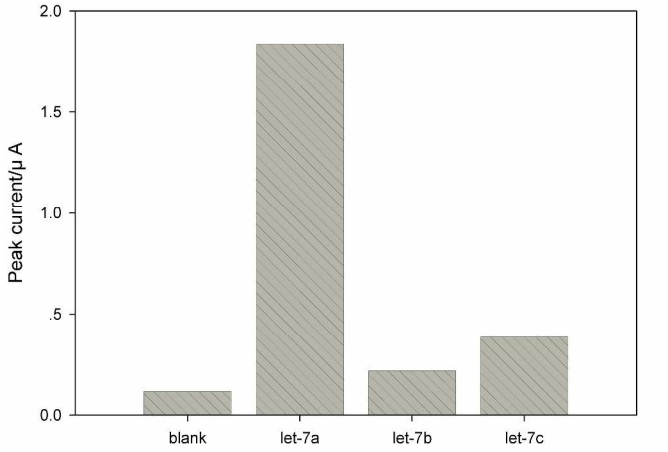


Fig. 7 The selectivity of the proposed strategy for miRNA assay. The concentrations of all miRNAs were 10 pM.

Conclusions

Based on streptavidin-gold nanoparticles and alkaline phosphatase amplification, an electrochemical miRNA detection method has been successfully developed. The linear range of this method is from 100fM to 1nM, with the detection limit of 99.2fM. The simple, highly sensitive and specific method can be expanded readily to other miRNA detection and might provide a promising tool for the basic research and clinical application.

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Notes and references

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