

Electron Transfer Mediated Electrochemical Biosensor for MicroRNAs Detection Based on Metal Ion Functionalized Titanium Phosphate Nanospheres at Attomole Level

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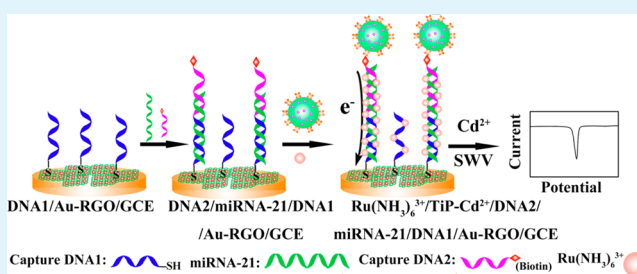
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S Supporting Information

ABSTRACT: MicroRNAs (miRNAs) have emerged as new candidates as diagnostic and prognostic biomarkers for the detection of a wide variety of cancers; thus, sensitive and selective detection of microRNAs is significant for early-phase cancer diagnosis and disease prevention. A novel and simple electrochemical miRNA biosensor was developed using Cd²⁺-modified titanium phosphate nanoparticles as signal unit, two DNA as capture probes, and Ru(NH₃)₆³⁺ as electron transfer mediator. Large quantities of cadmium ions were mounted in titanium phosphate spheres to output the electrochemical signal. Because of the presence of Ru(NH₃)₆³⁺ molecules that interacted with DNA base-pairs as electron wire, the electrochemical signal significantly increased more than 5 times. This approach achieved a wide dynamic linear range from 1.0 aM to 10.0 pM with an ultralow limit detection of 0.76 aM, exerting a substantial enhancement in sensitivity. Moreover, the proposed biosensor was sufficiently selective to discriminate the target miRNAs from homologous miRNAs and could be used for rapid and direct analysis of miRNAs in human serum. Therefore, this strategy provides a new and ultrasensitive platform for miRNA expression profiling in biomedical research and clinical diagnosis.

KEYWORDS: microRNAs, titanium phosphate, electron transfer, electrochemical biosensor



INTRODUCTION

miRNAs are a class of nonprotein-coding small RNA molecules with 17–25 nucleotides that regulate multiple genes associated with human cancers, neurological diseases, and viral infections via binding to corresponding messenger RNAs (mRNAs) to induce the mRNA degradation or translation blocking.¹ Distinct miRNA expression patterns are associated with various tumor types. miRNA-155, miRNA-21, miRNA-205, miRNA-17-92, etc. have been found overexpressed in many cancer types including hematopoietic cancers and breast, lung, and colon cancers.^{2–4} Therefore, miRNAs have been potential biomarkers that could serve for early disease diagnosis as well as for assessing the prognosis and monitoring the response to the treatment.

For the quantification of miRNAs, their small size, high sequence similarity, and low expression levels impose great challenges in the analysis using conventional techniques including Northern blotting,^{5,6} quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR),^{7,8} and microarrays.^{9,10} Numerous new strategies such as colorimetric measurement,^{11–13} fluorescence detection,^{14,15} electrochemical detection,^{16–18} surface plasmon resonance,¹⁹ bioluminescence technology,⁴ chemiluminescence technology,²⁰ electrochemiluminescence methods,²¹ and capillary electrophoresis assays²²

have been developed to improve the detection sensitivity and flexibility. Among these methods, electrochemical (EC) biosensors have attracted increasing attention because of their low cost, convenient operation, rapid detection, and good sensitivity.²³ EC methods usually involved either label-free or miRNA labeling.²⁴ Schiavo and co-workers published a label-free method based on G oxidation signal of G-rich miRNAs after hybridization with a G-free probe at carbon electrodes.²⁵ However, the use of a G-free probe limited the application in the detection of G-free miRNAs, and meanwhile it showed a lower sensitivity. To overcome these difficulties, labeling methods integrated with signal amplification strategies such as nanoparticles-based amplification,^{26,27} enzymatic signal amplification,²⁸ and rolling circle amplification^{29,30} have been developed. With the development of nanotechnology, a variety of nanoparticles including quantum dots,³¹ gold nanoparticles,^{32–34} and oxidase-like nanoparticles^{27,35,36} are widely used in the fabrication of the biosensor as signal sources. Wang et al. developed a miRNA biosensor by combining rolling circle amplification (RCA), quantum dots tagging, and anodic

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stripping voltammetric detection (ASV).³⁷ The method could specifically quantify miRNA-16 over a 6-decade dynamic range and reach an ultralow detection limit of 0.32 aM. However, in the control rolling circle amplification process, it may be complicated and difficult. Furthermore, quantum dots need to be dissolved in acid solution before electrochemical detection. Our group previously provided a novel multianalyte electrochemical immunoassay for ultrasensitive detection of human cardiopathy biomarkers cardiac troponin I (cTnI) and human heart-type fatty-acid-binding protein (FABP) by using metal ion functionalized titanium phosphate nanospheres (TiP) as labels.³⁸ The proposed immunoassay exhibited high sensitivity and selectivity for the detection. Most importantly, the TiP–Cd²⁺ conjugates could be detected directly without acid dissolution, which would greatly simplify the detection steps. Therefore, TiP–Cd²⁺ conjugates are potential labels for the electrochemical detection of miRNAs.

MiRNA detection is often performed using sandwich assays via nucleotide hybridization because it is difficult to label miRNAs directly in a biological matrix.²⁵ DNA-based genosensor is usually related to the electrical conductivity of DNA. At present, it is generally recognized that a π -stacked double-stranded (ds) DNA molecule is an efficient one-dimensional electrical conductor³⁹ and that the capability of transporting electrons is comparable to that of conventional conducting polymers^{40,41} and only 100 times less efficiently than a metal wire.⁴² However, in fact, arguments on the mechanisms of electron transfer (ET) properties of dsDNA still exist, and the electron transfer efficiency of DNA over long distances is not completely clear.^{43–45} Abi and Ferapontova reported that the ET communication between the electrode and anthraquinone, built in DNA through the acetylene linker, was achieved only when Ru(NH₃)₆³⁺ molecules were electrostatically attached to the DNA duplex to form the electronic wire.⁴⁶

Herein, an ultrasensitive electrochemical genosensor based on two DNAs as the target capture and Cd²⁺ functionalized titanium phosphate nanosphere (TiP–Cd²⁺) as a label is fabricated for the detection of miRNA-21. Large amounts of Cd²⁺ ions were incorporated into the TiP nanospheres, providing high electrochemical signals. Ru(NH₃)₆³⁺ molecules are introduced to interact with DNA base-pairs as an “electron bridge” for electron transport. The results indicated that Ru(NH₃)₆³⁺ greatly improved the electron transfer and significantly enhanced signals. Therefore, the proposed biosensor exhibited good sensitivity and selectivity with a low detection limit of 0.76 aM and could meet the requirements for direct detection of miRNA-21 in human serum, showing its great potential for cancer diagnosis.

EXPERIMENTAL SECTION

Materials and Reagents. Streptavidin protein (SA) was purchased from Beijing Biosynthesis Biotechnology Co., Ltd. (Beijing, China). Graphite powder (KS-10), bovine serum albumin (BSA), 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), poly(allylamine hydrochloride) (PAH), 6-mercapto-1-hexanol (MCH), chloroauric acid (HAuCl₄), hexamine ruthenium(III) chloride (Ru(NH₃)₆Cl₃), and Tween-20 were from Sigma-Aldrich. Glutaraldehyde (GLU) was obtained from Shanghai Reagent Company (Shanghai, China). Tetrabutyl titanate (TBOT), docusate sodium salt (AOT), and H₃PO₄ were from Nanjing Chemical Reagent Co., Ltd. (Nanjing, China). Agarose and diethyl pyrocarbonate (DEPC) were purchased from Beyotime Institute of Biotechnology (Nantong, China). DNA

oligonucleotides were purchased from Shanghai Sangon Biological Engineering Technology & Services Co. (China). miRNA-21 oligonucleotides were obtained from Shanghai Genepharma Co., Ltd. (Shanghai, China) (Table 1). Phosphate-buffered saline (PBS)

Table 1. Oligonucleotide Sequences Employed in the Present Work

	sequences (5'→3') ^a
miRNA-21	UAGCUUAUCAGACUGAUGUUGA
capture DNA1	SH-C3-TCAACATCAGT
capture DNA2	PO ₄ -CTGATAAGCTA-Biotin
1-base mismatch	U <u>A</u> ACUUAUCAGACUGAUGUUGA
3-bases mismatch	U <u>A</u> <u>A</u> CUUAUC <u>A</u> CACUGAUGU <u>C</u> GA

^aThe underlined letters refer to the mismatched bases.

solutions with different pH values were prepared by mixing the stock solution of NaH₂PO₄ and Na₂HPO₄ and then adjusting the pH with 0.1 M NaOH and H₃PO₄. HAC/NaAc solutions with different pH values were prepared by mixing the stock solutions of HAC and NaAc. Human serum is from Gulou Hospital (Nanjing, China). All other reagents were of analytical reagent grade and used without further purification. All aqueous solutions were prepared using DEPC-treated ultrapure water from a Milli-Q system (Millipore, Billerica, MA, U.S.A.).

Apparatus. All electrochemical measurements were performed on a CHI 660D workstation (Chenhua, Shanghai, China) with a conventional three-electrode system composed of a platinum wire as the auxiliary, a saturated calomel electrode as the reference, and the modified glass carbon electrode (GCE) as the working electrode. Transmission electron micrographs (TEM) were measured on a JEOL JEM 200CX transmission electron microscope using an accelerating voltage of 200 kV. Electrochemical impedance spectroscopy (EIS) was performed with an Autolab electrochemical analyzer (Eco Chemie, The Netherlands) in a 10 mM K₃Fe(CN)₆/K₄Fe(CN)₆ (1:1) mixture with 1.0 M KCl as the supporting electrolyte, using an alternating current voltage of 5.0 mV, within the frequency range of 0.01 Hz–100 kHz.

Preparation of Strepavidin–TiP–Metal Ion Probes. First, TiP nanospheres were synthesized according to the literature.^{38,47} Briefly, 5 g of AOT was dissolved into 32 mL of ethanol, and H₃PO₄ (6 mL) was added to get a turbid solution. Then, a mixture of TBOT with ethanol (1.75 g/32 mL) was dropped quickly into the AOT/ethanol solution and ultrasonically mixed to get a stable mixture solution. The mixture was stirred at 80 °C for 6 h. The solid product was washed with ethanol and deionized (DI) water for several times to remove the residual phosphoric acid and surfactant. For ion exchange, 1 mL of TiP nanospheres (40 mg mL^{−1}) was dispersed into 30 mL of 10 mM Cd(NO₃)₂ aqueous solution and stirred at 50 °C for 24 h. The resulting hybrid nanospheres were obtained by centrifugation and rinsed with water several times. The TiP–Cd²⁺ products were dispersed into 2 mL of DI water with the concentration of 20 mg mL^{−1}. Next, 2 mL of the TiP–Cd²⁺ hybrids were dispersed into 2 mL of PAH (2 mg mL^{−1}) aqueous solution and sonicated for 20 min. Then, the hybrids were washed with DI water, dispersed into 2 mL of GLU (0.25 wt %), and sonicated for 5 min. After washing with DI and PBS three times, 600 μ L of SA (0.01 mg mL^{−1}) solution was added into the TiP–Cd²⁺ hybrids and shaken for 6 h. After centrifugation, the obtained bioconjugates were further washed with PBS at least three times and resuspended in 8 mL of tris buffer (pH = 7.4) as the assay solution.

Sensor Fabrication. Graphene oxide (GO) was prepared by a modified Hummers method using graphite powder as the starting material.⁴⁸ Polyethylenimine (PEI)-modified graphene/Au composites (Au-RGO) were prepared by the following procedure. In brief, the mixture containing GO (10 mL, 0.05 mg mL^{−1}) and PEI (10 mL, 2 mg mL^{−1}) was sonicated for 30 min and reacted at 90 °C for 6 h in the round-bottomed flask. The as-prepared PEI-grafted graphene (RGO) was centrifuged at 22 000 rpm for 30 min, washed several times with

deionized water, and redispersed in water with a final concentration of 1.0 mg mL^{-1} for further use. Colloidal Au nanoparticles (NPs) with an average diameter of $\sim 10 \text{ nm}$ were prepared as reported previously.⁴⁹ Then the RGO dispersion was added into AuNPs solution (the volume ratios of RGO/Au = 1:3) and sonicated for 30 min to obtain PEI-modified graphene/Au composites (Au-RGO). Au-RGO composites were further washed with water three times and redispersed in water at a concentration of 1 mg mL^{-1} for further use. The glass carbon electrode (GCE) with a diameter of 3 mm was polished using 0.3 and $0.05 \mu\text{m}$ alumina slurry followed by rinsing thoroughly with water. After successive sonication in 1:1 nitric acid, acetone, and water, the GCE was dried with nitrogen. Ten μL of Au-RGO composite solution was dropped on the fresh pretreated GCE and allowed to dry at room temperature to obtain Au-RGO-modified GCE (Au-RGO/GCE).

Thiol-capturer DNA1 (probe 1, $5 \mu\text{L}$, $10 \mu\text{M}$) was activated with tris(2-carboxyethyl)phosphine (TCEP) ($5 \mu\text{L}$, 10 mM) for 0.5 h to prevent the terminal cysteine from forming disulfide bonds and then mixed with $10 \mu\text{L}$ of tris buffer solution (TBS, $\text{pH} = 7.4$, 10 mM). The mixture was dropped on the Au-RGO/GCE electrode and reacted at 4°C for 16 h in humidified surroundings. Finally, the electrode was washed with TBS several times, immersed into $10 \mu\text{L}$ of 1 mM MCH for 1 h at room temperature to block the nonspecific binding sites, and then washed with TBS.

RNA Hybridization Assay and Measurement Procedure. To carry out the RNA hybridization assay, the modified electrode was first incubated with a $12 \mu\text{L}$ drop containing the target RNA (miRNA-21 with different concentrations or serum samples, $5 \mu\text{L}$), capturer DNA2 (probe 2, $5 \mu\text{L}$, $10 \mu\text{M}$), T4 DNA ligase ($1 \mu\text{L}$, 1000 U mL^{-1}), and $10\times$ TBS ($1 \mu\text{L}$, 100 mM) for 4 h at 37°C to perform the hybridization reaction, followed by washing with TBS three times. Next, it was further incubated with $10 \mu\text{L}$ of TiP-Cd²⁺-SA bioconjugate solution for 60 min at 37°C and then washed thoroughly with TBS (containing 1% BSA) to remove nonspecifically bound conjugates. $\text{Ru}(\text{NH}_3)_6^{3+}$ ($10 \mu\text{L}$, 1 mM) was dropped on the electrode and followed by washing with TBS three times. After treatment with $\text{Ru}(\text{NH}_3)_6^{3+}$, the electrochemical measurement was carried out in 3 mL of HAC/NaAc ($\text{pH} 4.6$, 0.2 mol L^{-1}) containing Bi^{3+} ion at a final concentration of $10 \mu\text{g mL}^{-1}$.⁵⁰ The analytical procedure involved an electrodeposition at -1.2 V for 120 s and square wave voltammetry (SWV) scanning from -1.0 to -0.4 V with a pulse amplitude of 25 mV , a pulse frequency of 15 Hz , and a quiet time of 2 s .⁵¹ The electrochemical responses were recorded at about -0.68 V for quantitative measurement of miRNA-21.

Polyacrylamide Gel Electrophoresis Analysis. Polyacrylamide gel (8%) was employed to verify the binding of miRNA-21 with DNA1 and DNA2. First, $2 \mu\text{L}$ of $10 \mu\text{M}$ DNA1 and $2 \mu\text{L}$ of $10 \mu\text{M}$ DNA2 were incubated with $2 \mu\text{L}$ of $10 \mu\text{M}$ miRNA-21 in Tris-HCl buffer ($\text{pH} = 7.4$, 10 mM) at 37°C for 4 h to form stable DNA hybrid by a base-pairing hybridization. Electrophoresis was carried out at 100 V for 45 min at room temperature. After separation, the gel was stained with ethidium bromide (EB) and imaged by a Bio-Rad imaging system (Hercules, CA, U.S.A.).

RESULTS AND DISCUSSION

Characterization of TiP-Cd²⁺/SA. TiP nanoparticles with uniform morphology, size distribution, and good dispersion were synthesized. As shown in Figure 1A, PAH was modified on TiP-Cd²⁺ via the covalent reaction between the phosphate group of TiP-Cd²⁺ and the amine group of PAH. Then SA was attached to TiP-Cd²⁺ using the glutaraldehyde as a cross-linking agent. The average diameter of TiP was $\sim 100 \text{ nm}$ (Figure 1B). After ions changing with Cd^{2+} , no size difference was observed between TiP and TiP-Cd²⁺ (Figure 1C). As shown in Figure 1D, SA layers could be observed on the surface of TiP-Cd²⁺, indicating the successful decoration of SA. Furthermore, the reaction concentration of SA was investigated. As shown in Figure S1, Supporting Information, with the

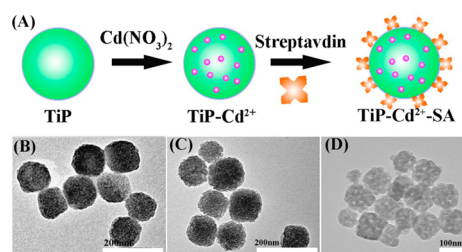
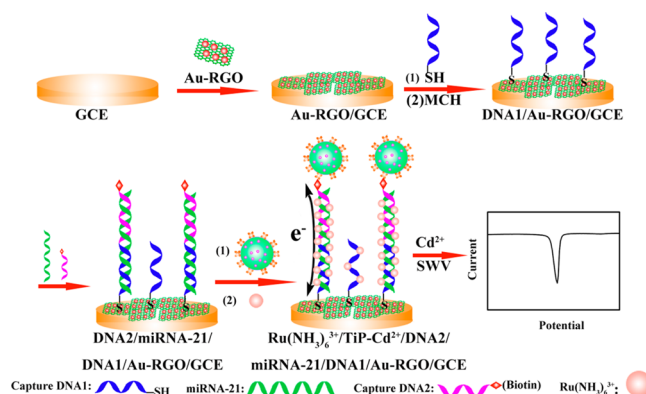


Figure 1. (A) Preparation procedures of SA-functionalized TiP-Cd²⁺. TEM images of TiP (B), TiP-Cd²⁺ (C), and TiP-Cd²⁺-SA (D).

increase of SA concentration, the coating amount of SA increased. But when the concentration of SA increased to 0.1 mg mL^{-1} , TiP-Cd²⁺-SA aggregated. Thus, the concentration of SA at 0.01 mg mL^{-1} is chosen in the following experiments.

Principle of the Sensor. The principle of the TiP-Cd²⁺ probes for electrochemical miRNA biosensing is outlined in Scheme 1. Gold nanoparticle modified graphene (Au-RGO) is

Scheme 1. Schematic Illustration of the Stepwise Sensor Construction Process



immobilized on a glass carbon electrode (GCE) surface. Thiol-DNA1 is attached to Au-RGO via Au-S bonds. DNA1 and DNA2 both own 11 bases that are complementary to the half part of miRNA-21, respectively. Only in the presence of target miRNA (miRNA-21) and biotin-labeled reporter DNA2 can the sandwich structure form. Then, streptavidin-modified TiP-Cd²⁺ (TiP-Cd²⁺-SA) is further attached to DNA2 via biotin-avidin conjugation as signal tags. Finally, $\text{Ru}(\text{NH}_3)_6^{3+}$ molecules are incorporated into the duplex DNA via the electrostatic interaction to accelerate the electron transfer. The corresponding electrochemical current responses of Cd^{2+} are used to quantify the concentration of miRNA-21.

Characterization of the Genosensor. To facilitate the immobilization of the capture DNA1, gold nanoparticle (AuNP)-functionalized PEI-modified graphene nanosheet (Au-RGO) hybrids with good conductivity and biocompatibility were fabricated via a simple sonication-induced assembly and used as biosensor platforms. The obtained Au-RGO hybrid architecture was confirmed by the TEM image as shown in Figure 2A. AuNPs of 10 nm were distributed homogeneously on the graphene sheet surface. EIS was an effective and sensitive method to characterize the conductivity of the electrode surface. The semicircle diameter at higher frequencies is related to the electron-transfer resistance (R_{et}), and the linear part at lower frequencies corresponds to the diffusion process. The stepwise construction process of the microRNA

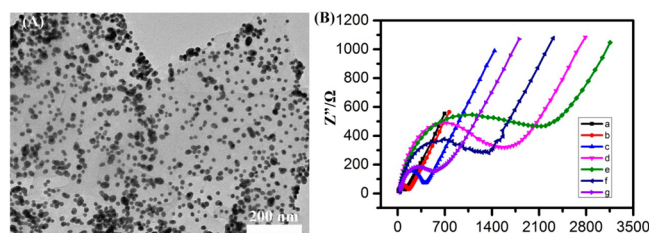


Figure 2. (A) TEM image of Au-RGO; (B) Nyquist diagram of electrochemical impedance spectra recorded from 0.01 to 10^5 Hz for $[\text{Fe}(\text{CN})_6]^{3-/4-}$ (10 mM, 1:1) in 1.0 M KCl using a bare GCE (a), Au-RGO/GCE (b), and DNA1/Au-RGO/GCE without (c) or with the modification of MCH (d), DNA2/miRNA-21/DNA1/Au-RGO/GCE (e), and the TiP- Cd^{2+} -SA/DNA2/miRNA-21/DNA1/Au-RGO/GCE in the absence (f) or in the presence of $\text{Ru}(\text{NH}_3)_6^{3+}$ (g).

biosensor was characterized by EIS as shown in Figure 2B. The bare GCE (curve a) and Au-RGO-modified GCE (Au-RGO/GCE, curve b) exhibit nearly straight lines, reflecting excellent conductivity. When DNA1 was loaded on the surface of Au-RGO/GCE, the R_{et} increased (curve c) to 180 Ω , which suggested that DNA1 formed an additional barrier and further prevented the redox probe to the electrode surface due to the electrostatic repulsion between negative charges of the DNA1 phosphate backbone and the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ ions, as well as demonstrated that DNA1 had been successfully immobilized on the electrode. After nonconductive MCH was used to block nonspecific sites and DNA1 hybridized with miRNA-21 and DNA2, larger semicircles (curve d is $\sim 700 \Omega$ and curve e is 1047 Ω) were observed due to the insulating properties of MCH and the increased negative charges of the nucleotide. However, when TiP- Cd^{2+} -SA and $\text{Ru}(\text{NH}_3)_6^{3+}$ were attached onto the electrode surface, the R_{et} significantly decreased to 620 Ω (curve f) and 292 Ω (curve g), respectively, which might be attributed to the introduction of positively charged metal ions that improved the electrostatic interaction with $[\text{Fe}(\text{CN})_6]^{3-/4-}$, and meanwhile $\text{Ru}(\text{NH}_3)_6^{3+}$ accelerated electron transfer as a conductor wire. These results indicated that the biosensor was effectively and successfully fabricated.

The hybridization reaction between the target and the capture probes was further analyzed by polyacrylamide gel electrophoresis. In Figure S2, Supporting Information, the first four lanes showed the marker, DNA1, DNA2, and miRNA-21, respectively. No bands were observed in lanes 2 and 3, because DNA1 and DNA2 are single-stranded sequences, while EB usually stains DNA duplex via its intercalation with the stacked base pairs. Lane 4 displayed a band at 20 and 25 bp compared with lane 4, indicating the formation of DNA1/miRNA-21 complex.

In lane 6, the band is brighter than that in lanes 3 and 4, demonstrating the formation of DNA2/miRNA-21 complex. The band in lane 7 has no significant difference comparing to lane 5. However, in the presence of T4 DNA ligase (lane 8), a wide band appeared at the position of >200 bp because T4 DNA ligase is a polypeptide so that DNA1/miRNA-21/DNA2/T4 complex exhibited a relatively low mobility, indicating the successful formation of DNA1/miRNA-21/DNA2/T4 complex.

Optimization of Conditions. The comparison of the performances of the assay with or without $\text{Ru}(\text{NH}_3)_6^{3+}$ is shown in Figure 3A. As we predicted, in the presence of $\text{Ru}(\text{NH}_3)_6^{3+}$, the peak current is ~ 5 times higher than that in the absence of $\text{Ru}(\text{NH}_3)_6^{3+}$, indicating that $\text{Ru}(\text{NH}_3)_6^{3+}$ molecules were electrostatically attracted to the DNA duplex; as a result, the electronic wire mediated ET between the electrode and TiP- Cd^{2+} formed, leading to great enhancement of electron transfer. For RNA hybridization, hybridization time is an important parameter. In Figure 3B, the current increased along with the increase of hybridization time from 1 to 4 h, but leveled off at longer time. Moreover, the dependence of the SWV peak current under different pH values of the detection solution was investigated (Figure 3C). The highest electrochemical signal was obtained at a pH of 4.6. Therefore, the incubation time of 4 h was selected as the optimal reaction time and 0.2 M HAc/NaAc (pH 4.6) was selected as the electrolyte for the electrochemical assay in all subsequent work.

Electrochemical Detection of Target miRNA. Under optimal conditions, a series of different concentrations of miRNA-21 were measured to confirm the ability of the designed electrochemical biosensor to detect microRNAs. As shown in Figure 4A, the electrochemical signal increased with

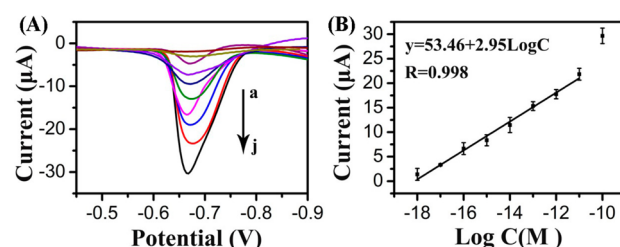


Figure 4. (A) SWV of the sensor recorded on Au-RGO/GCE with increasing miRNA-21 concentration from (a) to (j): 0, 10^{-18} , 10^{-17} , 10^{-16} , 10^{-15} , 10^{-14} , 10^{-13} , 10^{-12} , 10^{-11} , and 10^{-10} M. (B) The linear relationship between the peak current and the logarithm of the target miRNA-21 concentration from 10^{-10} M to 10^{-18} M. Each value is the average of six measurements.

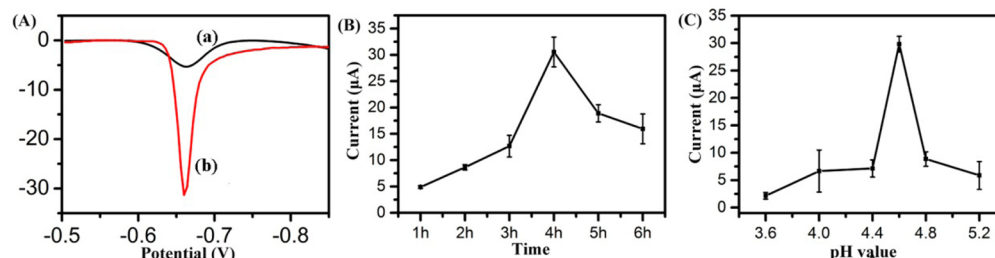


Figure 3. (A) Effects of the absence (a) or presence (b) of $\text{Ru}(\text{NH}_3)_6^{3+}$, (B) hybridization time, and (C) pH value of buffer solution in the RNA sensor. The miRNA-21 concentration is 10^{-10} M.

Table 2. Detection Limits of Various Methods for miRNA Detection

method	analyte	LOD	linear range	readout	ref
cross-catalyst strand-displacement network (CC-SDR)	let-7a	0.68 fM	1.0 fM to 10.0 fM	chemiluminescence	20
exponential amplification reaction (EXPAR)	let-7a	0.1 zmol	0.1 zmol to 1.0 fmol and 1.0 fmol to 1.0 pmol	fluorescence	53
cascade RCA-NESA-DNAzyme amplification	let-7d	2.0 aM	100 fM to 1 fM	colorimetry	11
horseradish peroxidase catalysis	let-7c	2.0 fM	5.0 fM to 2.0 pM	electrochemistry	18
RCA and quantum dots amplification	miRNA-16	0.32 aM	10.0 aM to 10.0 pM	electrochemistry	37
amplified voltammetric detection by Fc-capped gold nanoparticle amplification	miRNA-182	10.0 fM	10 fM to 2.0 pM	electrochemistry	34
p19 protein binding and displacement methods	miRNA-32	5.0 aM	10 aM to 1 μ M	electrochemistry	17
triple signal amplification of multifunctional gold nanoparticles, enzymes, and redox-cycling reaction	miRNA-21	3.0 fM	10 fM to 5 pM	electrochemistry	54
electron transfer mediated and nanoparticles-tagged amplification	miRNA-21	0.76 aM	1.0 aM to 10.0 pM	electrochemistry	present work

the increasing concentration of target used for hybridization and showed a good linear correlation versus the logarithm of target miRNA concentrations in the range from 1.0 aM to 10.0 pM (Figure 4B) with the limit of detection (LOD) of 0.76 aM, corresponding to 3.8 ymol of target in 5 μ L of sample solution. The regression equation is $I = 2.95 \log C_{\text{miRNA-21}} + 53.46$ with a correlation coefficient of 0.998, where I and C represent the current intensity and the miRNA-21 concentration, respectively. The typical levels of circulating microRNAs in serum are from 200 aM to 20 pM,⁵² which is a subset of the detection range of our proposed method. The limit of detection was estimated from three times the current value of the blank sample detection (free of target miRNA). The low detection limit was attributed to the signal amplification enabled with Cd^{2+} -functionalized TiP nanospheres and $\text{Ru}(\text{NH}_3)_6^{3+}$. Table 2 summarizes the detection limits of some recent reported signal amplification nucleic acids assays. This comparison clearly highlighted the remarkable enhanced LOD of electrochemical biosensor strategy. Although the detection limit of the electrochemical biosensor is not the lowest, the procedure is simpler via sandwich assays than other methods such as fluorescence, chemiluminescence, and colorimetry. Moreover, the linear range is widest with 8 orders of magnitude via electrochemical detection, which is a benefit for future practical application in biomedical fields.

Specificity. Sequence specificity in miRNAs detection is an important consideration because miRNA families often possess closely homologous sequences. The specificity of the miRNA biosensor was investigated through a comparison assay on mismatch targets and perfect complementary target, including complementary target, single-base mismatched strand, and three-base mismatched strand at a same concentration of 100 pM. As Figure 5A illustrates, the current of a perfectly complementary target was 10.6 times higher than the single-base mismatch sequence, and the response of the three-base mismatch strand was almost ignored. These results suggested that the sensor owned high sequence specificity and excellent discrimination for similar miRNAs.

MicroRNA Detection in Serum Samples. miRNA-21 is overexpressed in a wide variety of cancers. Therefore, the direct detection of the expression level of miRNA-21 in the human blood serum extracted from patients was explored to validate the practicality of this method. miRNA-21 in the human blood serum from three types of cancer patients, including cervical cancer, breast cancer, and leukemia patients, was measured.

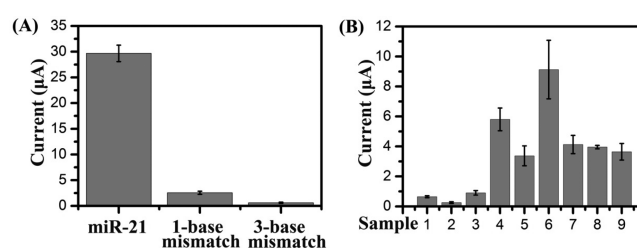


Figure 5. (A) SWV signals in the presence of miRNA-21, 1-base mismatch miRNA, and 3-base mismatch miRNA (all at a concentration of 10^{-10} M). Each value is the average of three measurements. (B) SWV signals of miRNA-21 in human blood serum obtained by electrochemical biosensor. Samples 1–3 are from three healthy persons. Samples 4 and 5 are from two cervical cancer patients. Samples 6 and 7 are from two breast cancer patients. Samples 8 and 9 are from two leukemia patients. Each value is the average of six measurements.

The electrochemical signal of miRNA-21 in cancer patients is significantly higher (>5 times) than that in the healthy group as shown in Figure 5B, suggesting an upregulation of miRNA-21 in cancer patient human blood serum.^{55,56} The miRNA-21 concentration in sample 6 (breast cancer patient, $\sim 9.2 \times 10^{-16}$ M, which is consistent with the previous report⁵⁷) is even 900-fold compared with sample 2 (healthy person, $\sim 1.0 \times 10^{-18}$ M). These results demonstrated that this method could be applied to the direct determination of miRNA-21 in serum samples without separation and enrichment. However, although patients can be discriminated from the healthy people, the type of disease cannot be clearly distinguished because of the complication with cancer that there are different subtypes of the same disease. It will be a huge challenge to design a gene biosensor for the diagnosis of disease types in future research.

To confirm the reliability of the method, a series of synthetic miRNA-21 at concentrations of 0, 10.0 aM, 0.1 fM, and 1.0 fM were spiked into healthy human blood serum (sample 2), respectively, to obtain the electrochemical signals. In Table S1, Supporting Information, the electrochemical response of miRNA-21 could reflect the real situation of the miRNA sample, indicating great potential for the miRNA analysis in clinical diagnosis.

CONCLUSION

In summary, an ultrasensitive and highly specific electrochemical biosensor for miRNA assay was developed based on

Cd²⁺-modified TiP nanoparticles amplification and enhanced electron transfer using Ru(NH₃)₆³⁺ as electronic wires. A proportional relationship was observed between the SWV peak currents and the logarithm of target miRNA-21 concentration in a linear range from 1.0 aM to 10.0 pM with a detection limit of 0.76 aM. At the same time, this proposed biosensor is sufficiently selective to discriminate the target miRNA from homologous miRNAs. Subsequently, the proposed strategy has successfully achieved the detection of human miRNAs from cancer patients' serum without separation and enrichment. Therefore, this biosensor is an attractive candidate for the development of an accurate, selective, and ultrasensitive method for miRNA expression profiling and clinical diagnostics.

■ ASSOCIATED CONTENT

Supporting Information

TEM images of TiP–Cd²⁺ nanoparticles modified with SA of different concentrations (Figure S1), polyacrylamide gel electrophoresis analysis of the RNA hybridization process (Figure S2), and reliability of the proposed method (Table S1). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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