



Signal-on photoelectrochemical biosensor for microRNA detection based on Bi₂S₃ nanorods and enzymatic amplification

Mo Wang, Huanshun Yin*, Nannan Shen, Zhenning Xu, Bing Sun, Shiyun Ai*

College of Chemistry and Material Science, Shandong Agricultural University, 271018 Taian, Shandong, PR China

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ABSTRACT

In this work, a photoelectrochemical (PEC) biosensor was fabricated for sensitive and specific detection of microRNA based on Bi₂S₃ nanorods and enzymatic signal amplification. Using the catalytic effect of alkaline phosphatase on L-ascorbic acid 2-phosphate trisodium salt (AAP), ascorbic acid (AA) was *in situ* generated and used as electron donor. Based on this, a signal-on protocol was successively achieved for microRNAs detection due to the dependence of photocurrent response on the concentration of electron donor of AA. The results demonstrated that the photocurrent response enhanced with increasing the hybridized concentration of microRNA. Under the amplification of the immunogold labeled streptavidin (SA-AuNPs), a low detection limit of 1.67 fM was obtained. The fabricated biosensor showed good detection stability and specificity, and it could discriminate only one-base mismatched microRNA sequence. Moreover, the down-regulated expression of microRNA-21 in DF-1 chicken fibroblast cells infected with subgroup J avian leukemia virus (ALVs) was confirmed by the developed method, indicating that microRNA-21 might be a new biomarker for avian leukemia. This work opens a different perspective for microRNAs detection and early diagnose of avian leukemia.

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1. Introduction

MicroRNAs are a new class of small, non-coding single-stranded RNAs, which can regulate the expression of target genes in cell proliferation, differentiation, and tumorigenesis (He et al., 2005). Recently, microRNAs have been regarded as new biomarkers for early diagnosis of diseases including cancer (Esquela-Kerscher and Slack, 2006). However, their unique properties of small sizes, low abundance, and high sequence similarity have made a great challenge for quantitative analysis. The traditional detection methods for microRNAs are northern blotting (Válóczi et al., 2004), RT-PCR (Lao et al., 2006) and fluorescent microarray (Thomson et al., 2004). However, northern blotting is a semi-quantitative detection method with time- and sample-consuming. Moreover, this technique needs microRNA separation and enrichment, which increase the detection difficulty. RT-PCR generally requires microRNAs isolation, purification, and reverse transcription to cDNA prior to the amplification step. In addition, the instrument for RT-PCR is very expensive. Fluorescent microarray frequently suffer from poor reproducibility and inaccuracy. Therefore, it is important to develop new analytical methods for microRNAs detection with high sensitivity and good reliability.

Up to now, a great deal of efforts has been devoted and many detection methods have been developed for microRNAs detection,

such as fluorescence (Zhang and Zhang, 2012), spectrophotometry (Zhang et al., 2009), chemiluminescence (Bi et al., 2011), electrochemiluminescence (Wu et al., 2012), capillary electrophoresis (Khan et al., 2011), Raman spectroscopy (Abell et al., 2012) and mass spectrometry (Wambua et al., 2012). However, all of these methods suffer from the limitation of expensive instruments. Compared with these methods, the electrochemistry instrument was simple, low cost and easy to operate. Among various biosensors based on electrochemistry, the photoelectrochemical biosensors have attracted a great deal of attention and become an alternative to the conventional analytical technique due to the simple operation, inexpensive instrument, low background current and high sensitivity (Wang et al., 2013a). Moreover, the photoelectrochemical sensors have the advantages of both optical techniques and electrochemical sensors by coupling the light irradiation and electrochemical detection. Though many photoelectrochemical sensors have been developed to detect proteins (Liao et al., 2012; Wang et al., 2009b, 2013b; Zhang et al., 2011a), cells (Qian et al., 2010), DNA (Lu et al., 2008; Zhang et al., 2013, 2011b), small molecules (Huang et al., 2013b; Li et al., 2011; Wang et al., 2009a; Zhu et al., 2009) and metal ions (Liang et al., 2012; Wang et al., 2012; Zhang and Guo, 2012), no work have been done for microRNA detection using this technique.

For photoelectrochemical biosensors, photocurrent response efficiency is an important parameter because it can greatly influence the detection sensitivity. It is well-known that the photocurrent response efficiency of biosensor is mainly depended

* Corresponding authors. Tel.: +86 538 8249248; fax: +86 538 8247660.

E-mail addresses: yinhs@sdau.edu.cn (H. Yin), ashy@sdau.edu.cn (S. Ai).

on the photoactive materials immobilized on substrate electrode. Therefore, development of suitable photoactive materials is necessary. Bi_2S_3 is a kind of narrow band gap semiconductor with band gap of 1.3–1.7 eV, which has received great attentions due to its wide application in electronic devices, optoelectronic devices, thermoelectric devices, hydrogen storage materials and sensors (Bao et al., 2008; Li et al., 2012; Ma et al., 2011a, 2011b). More importantly, Bi_2S_3 has also been considered as one of the most promising photoactive materials due to its low band gap, high absorption coefficient and reasonable energy conversion efficiency (~5%) (Peter et al., 2003; Tahir et al., 2010). However, to the best of our knowledge, photoelectrochemical biosensors based on Bi_2S_3 nanomaterials have not been reported.

In this work, we developed a signal-on photoelectrochemical method for microRNA detection based on photoactive material of Bi_2S_3 nanorods and an elaborate *in situ* production of electron donor of ascorbic acid (AA) under alkaline phosphatase (ALP) catalytic chemistry. For confirming the applicability of the fabricated biosensor, the expression level of microRNA-21 in DF-1 chicken fibroblast cells infected with subgroup J avian leukemia virus (ALVs) was also investigated.

2. Experimental

2.1. Reagents and instruments

L-Ascorbic acid 2-phosphate trisodium salt (AAP) and mercapto-propionic acid (MPA) were purchased from Sigma (USA). Ascorbic acid (AA), hydrogen tetrachloroaurate trihydrate ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$), tris(hydroxymethyl)aminomethane (Tris) and tris(2-carboxyethyl) phosphine hydrochloride (TCEP) were purchased from Aladdin (Shanghai, China). Diethylenetriamine (DEPC) and PEG-3350 were from Solarbio (China). AuNPs were synthesized according to the previous report with the average diameter of 13 nm (Liu and Lu, 2006), which were characterized by transmission electron microscopy (TEM). The SA-AuNPs was prepared according to the previous report with some modifications (see Supporting information) (Li and Cui, 2013; Zhao et al., 2013). Indium tin oxide (ITO) was purchased from Zhuhai Kaivo Electronic Components Co., Ltd. (Zhuhai, China, ITO coating 180 ± 25 nm, sheet resistance $< 15 \Omega/\text{cm}^2$). Streptavidin, bovine serum albumin (BSA), biotin-ALP and all DNA sequences were purchased from Shanghai Sangon Biotechnology Co. (Shanghai, China) and used without further purification. MicroRNA-21 was synthesized and HPLC-purified by TaKaRa Biotechnology Co., Ltd. (Dalian, China). The oligonucleotides sequences were shown as follows. Probe DNA S1: 5'-SH $(\text{CH}_2)_6$ -GGC CGT CAA CAT CAG TCT GAT AAG CTA AAC ATG ATA CGG CC-3'; microRNA-21: 5'-UAG CUU AUC AGA CUG AUG UUG A-3'; Capture DNA S2: 5'-biotin-GGC CGT ATC ATG TT-3'; single-base mismatched microRNA: 5'-UAG CUU AAC AGA CUG AUG UUG A-3'; and three-base mismatched microRNA: 5'-UAG CAU AUC ATA CUG AUG CUG A-3'. The nucleotide mismatches were indicated as italic and bold letters. Synthetic DNA and miRNA sequences were dissolved in TE buffer (pH 8.0) according to the manufacturer's recommendations.

The buffer solutions employed in this study are as follows. Probe immobilization buffer: 10 mM Tris-HCl, 1.0 mM EDTA, 1.0 M NaCl, and 1.0 mM TCEP (pH 7.0). MicroRNA hybridization buffer: $1 \times$ SSC. DNA hybridization buffer: 10 mM Tris-HCl, 1.0 mM EDTA, and 1.0 M NaCl (pH 7.0). PEC determination buffer, 0.1 M Tris-HCl (pH 9.8) containing 10 mM AAP. All of the solution and redistilled deionized water were treated with DEPC and autoclaved to protect from RNase degradation.

Scanning electron microscopic (SEM) images were recorded by a Hitachi S4800 scanning electron microscope (Hitachi Corporation, Japan). Powder X-ray diffraction (XRD) was performed on a

Bruker D8 Advance X-ray diffractometer (Germany) with $\text{CuK}\alpha$ radiation ($\lambda = 0.1542$ nm).

2.2. Synthesis of Bi_2S_3 nanorods

Bi_2S_3 was synthesized according to the previous report with some modifications (Ge et al., 2012). In brief, 1.82 g $\text{Bi}(\text{NO}_3)_3 \cdot 5\text{H}_2\text{O}$ (0.00375 mol) was firstly added into 25 mL ethanediol and stirred for 20 min. The obtained solution was noted as solution A. Meanwhile, 1.35 g $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ (0.005625 mol) was added into 30 mL water and stirred for 1 h. The preparation solution was noted as solution B. 1.92 g Carbamide ($\text{CO}(\text{NH}_2)_2$, 0.032 mol) was added into 20 mL water and named as solution C. Then the solution B was added into the solution A drop by drop. A large number of black suspended matters were produced in the mixed solution. After that, solution C was poured into the mixed solution. Subsequently, the mixed solution was transferred into a Teflon-lined stainless steel autoclave (100 mL capacity), sealed and maintained at 180°C for 24 h. The resultant black solid product was filtered, washed with deionized water and ethanol, and finally dried in air.

2.3. PEC biosensor fabrication

The ITO slices were used as the working electrode, which were cleaned by immersing into ethanol/NaOH mixed solution (v/v, 1:1) and acetone successively with sonication for 30 min, followed by washing copiously with double distilled deionized water and dried at 60°C .

4 mg Bi_2S_3 powder was weighed accurately and dispersed ultrasonically in 1 mL double distilled deionized water, and then 40 μL of the suspension was dropped onto a piece of ITO slice with fixed area of 0.196 cm^2 . After drying in air, $\text{Bi}_2\text{S}_3/\text{ITO}$ was rinsed with double distilled deionized water for three times. Then, 40 μL of AuNPs were further dropped on the electrode surface and dried under an infrared lamp, followed by rinsing with double distilled deionized water for three times (the electrode was noted as AuNPs/ $\text{Bi}_2\text{S}_3/\text{ITO}$). Subsequently, AuNPs/ $\text{Bi}_2\text{S}_3/\text{ITO}$ was incubated with 20 μL probe immobilization buffer containing 0.5 μM probe DNA S1 for 2 h under humid conditions. After rinsed with 10 mM Tris-HCl (pH 7.4) for three times, 20 μL probe immobilization buffer containing 5 μM MPA was dropped on electrode surface and incubated for 1 h. The obtained electrode was noted as DNA1/AuNPs/ $\text{Bi}_2\text{S}_3/\text{ITO}$.

MicroRNA hybridization was performed by incubating probe/AuNPs/ $\text{Bi}_2\text{S}_3/\text{ITO}$ electrode with 20 μL microRNA hybridization buffer containing various concentrations of microRNA-21 for 2 h at 37°C (the electrode was noted as microRNA-DNA1/AuNPs/ $\text{Bi}_2\text{S}_3/\text{ITO}$). After that, the 3'-end of probe DNA S1 was further hybridized with 0.5 μM capture DNA S2 for 2 h, which was dissolved in 20 μL DNA hybridization buffer. The obtained electrode was named as DNA2/microRNA-DNA1/AuNPs/ $\text{Bi}_2\text{S}_3/\text{ITO}$. Afterwards, the electrode was incubated with 20 μL SA-AuNPs for 1 h under humid conditions (the electrode was noted as SA/DNA2/microRNA-DNA1/AuNPs/ $\text{Bi}_2\text{S}_3/\text{ITO}$). Following that, 20 μL 0.1% PEG-3350 was dropped on the electrode surface to block any non-specific reaction sites. Finally, the electrode was incubated with 20 μL biotin-ALP solution for 75 min, followed by rinsing with 10 mM Tris-HCl (pH 7.4). The electrode was indicated as ALP/SA/DNA2/microRNA-DNA1/AuNPs/ $\text{Bi}_2\text{S}_3/\text{ITO}$.

2.4. PEC detection

Photoelectrochemical measurements were performed with a homemade photoelectrochemical system with different optical filters (the transmission wavelengths were 420, 435, 450, 475, 500, 520 and 550 nm). The light intensity was about $20 \text{ mW}/\text{cm}^2$,

which was measured by a radiometer (Photoelectric Instrument Factory of Beijing Normal University, Beijing, China). Photocurrent was recorded on a CHI832A electrochemical workstation (Austin, USA) with a three-electrode system. A modified ITO electrode with an area of 0.195 cm^2 was used as the working electrode, a Pt wire as the counter electrode and an SCE as the reference electrode.

2.5. Cell culture and total RNA extraction

DF-1 chicken fibroblast cell line was seeded in 75 cm^2 well at a density of approximately 1×10^6 cells per well. ALV-J China strain (NX0101) was inoculated onto DF-1 cells and incubated at 37°C for 2 h. Then the cells were cultured with fresh medium contained 1% fetal bovine serum (FBS, Invitrogen, CA, USA). Observed daily, on the seventh day of the post-inoculation, cells were collected and RNA was extracted from infected DF-1 cells using the RNA extraction kit (TaKaRa, Dalian, China) according to the manufacturer's recommended protocol. Total RNA extracted from uninfected DF-1 cells was used as control. RNA concentration was determined by UV-vis spectrophotometry. RNA integrity was checked by TBE agarose gel electrophoresis analyzing integrity and relation of the 28S rRNA, 18S rRNA, and 5S rRNA bands.

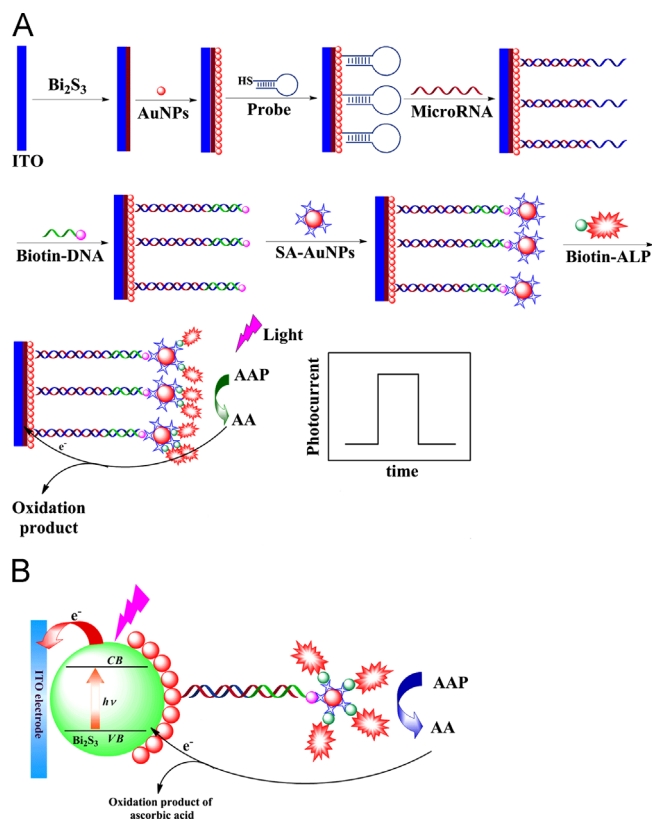
3. Results and discussion

3.1. Detection strategy

The fabrication process and detection mechanism of photoelectrochemical biosensor are shown in Scheme 1. As seen in Scheme 1A, the Bi_2S_3 nanorods and AuNPs were successively immobilized on ITO surface through physical adsorption (Huang et al., 2013a). Then, the thiol modified probe DNA with hairpin structure was assembled on AuNPs/ Bi_2S_3 /ITO surface through Au–S bond. Afterwards, the stem-loop structure of probe DNA was unfolded through microRNA hybridization event, and biotin was immobilized on the electrode via the hybridization reaction between the un-hybridized fragment of probe DNA at its 3'-end and biotin tagged DNA (biotin-DNA). Subsequently, the immunogold labeled streptavidin (SA-AuNPs) was captured through the specific reaction between biotin and streptavidin. Finally, biotin labeled ALP (biotin-ALP) was introduced to the photoelectrochemical system and catalyzed the hydrolysis of L-ascorbic acid 2-phosphate trisodium salt (AAP) to generate AA (Zhao et al., 2012). It is clear that the immobilization of biotin-ALP could not achieve without the hybridization of microRNA with probe to unfold its hairpin structure. More importantly, in the present detection system, the increased microRNA concentration could lead to the enhanced loading of SA-AuNPs and thus boost the immobilization amount of biotin-ALP, which could improve the photocurrent response due to the increased concentration of AA generated from the catalytic reaction (Scheme 1B). Based on the high selectivity of hairpin-structure DNA probe, signal amplification of immunogold labeled streptavidin, photoactive material of Bi_2S_3 nanorods, the proposed system could achieve the sensitive and selective microRNA detection.

3.2. Characterization of Bi_2S_3

The synthetic Bi_2S_3 was characterized by SEM and XRD. As seen in Fig. 1A, the morphology of Bi_2S_3 presented nanorod structure with 100–200 nm of length and 50–100 nm of width. Fig. 1B shows the XRD pattern of Bi_2S_3 nanorods. Almost all peaks in the pattern could be indexed to a pure orthorhombic phase bismuth sulfide, which is in good agreement with the literature (JCPDS File no. of 17-0320).



Scheme 1. (A) Schematic diagram of the fabrication process of the photoelectrochemical biosensor. (B) The photocurrent generation mechanism and microRNA detection mechanism of the photoelectrochemical biosensor.

3.3. Characterization of PEC biosensor

The fabrication of the photoelectrochemical biosensor was monitored by photocurrent in 0.1 M PBS (pH 7.4) containing 0.1 M AA as electron donor. As illustrated in Fig. 2A, the bare ITO electrode showed no photocurrent response (curve a). After Bi_2S_3 nanorods were immobilized on ITO surface, a strong photocurrent was observed (curve b), indicating that Bi_2S_3 was a promising photoelectric conversion material. However, the photocurrent decreased after AuNPs were dropped on Bi_2S_3 /ITO electrode surface (curve c). This decrease could be attributed to three factors. Firstly, the donated electron by AA was captured by AuNPs, which increased the recombination probability of photogenerated electrons and holes. As a result, the photocurrent decreased. Secondly, the vibration relaxation resulted from the plasmon resonance effect at the surface of AuNPs might also decrease the photocurrent. Thirdly, there are many citrate anions adsorbed on AuNPs surface, which repelled the negative charge controlled AA. Though AuNPs caused the photocurrent decrease, the main aim for immobilizing AuNPs was assembling thiol functionalized probe DNA on electrode surface through Au–S bond. Subsequently, the photocurrent decreased successively with the assembly of hairpin structure probe DNA S1 (curve d), microRNA hybridization (curve e) and DNA S2 hybridization (curve f). These decreases could be ascribed to two factors. One was the effect of steric hindrance resulting from immobilized nucleic acid, which blocked the electron donor of AA to capture the photogenerated holes and increased the probability of the recombination of holes and electrons. The other factor was the effect of electrostatic repulsion. It was well-known that the pK_a of AA was 4.1 (Li et al., 2010), AA was controlled by negative charge in 0.1 M PBS with pH 9.8. As a result, the diffusion of AA towards electrode surface could be blocked by the negative charged DNA and microRNA.

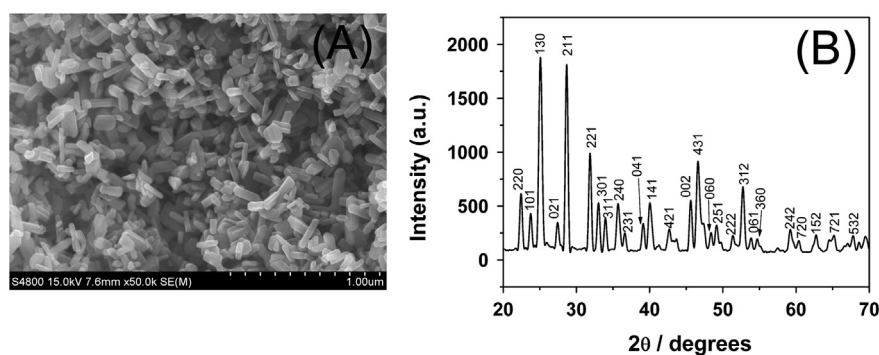


Fig. 1. SEM image (A) and XRD (B) of Bi_2S_3 nanorods.

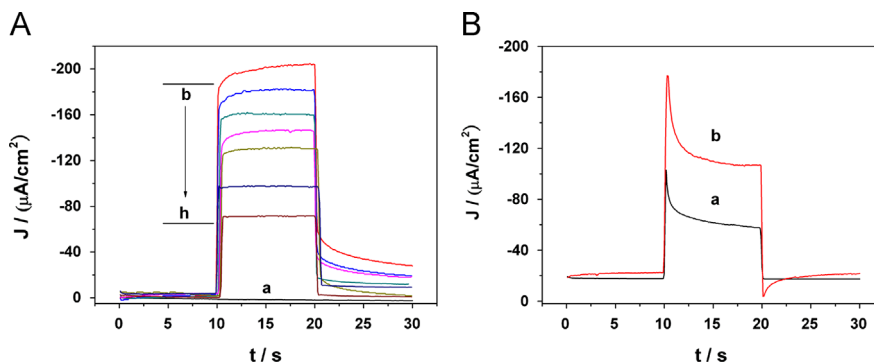


Fig. 2. (A) The photocurrent response of ITO (a), Bi_2S_3 /ITO (b), AuNPs/ Bi_2S_3 /ITO (c), DNA1/AuNPs/ Bi_2S_3 /ITO (d), microRNA–DNA1/AuNPs/ Bi_2S_3 /ITO (e), DNA2/microRNA–DNA1/AuNPs/ Bi_2S_3 /ITO (f), SA/DNA2/microRNA–DNA1/AuNPs/ Bi_2S_3 /ITO (g) and ALP/SA/DNA2/microRNA–DNA1/AuNPs/ Bi_2S_3 /ITO (h) in 0.1 M PBS (pH 7.4) containing 0.1 M AA. (B) The photocurrent response of Bi_2S_3 /ITO in 0.1 M PBS (pH 9.8) containing 10 mM AAP before and after treatment with ALP/SA/DNA2/microRNA–DNA1/AuNPs/ Bi_2S_3 /ITO for 30 min. Light wavelength, 450 nm. Applied potential, 0 V.

After capturing SA–AuNPs by biotin at the 3'-end of DNA S2 (curve g), the photocurrent decreased. This could be explained as the fact that the immobilization of large volume of SA–AuNPs on the electrode surface blocked the diffusion of AA to electrode surface. Afterwards, with immobilization of biotin–ALP through the specific interaction between biotin and the residual recognized sites of streptavidin, the photocurrent further decreased (curve h), which might be attributed to the increased steric hindrances for AA to electrode surface due to the immobilization of enzyme. These results also demonstrated the successively fabrication of PEC biosensor.

3.4. The feasibility for PEC detection of microRNA-21

In this work, we designed a signal-on photoelectrochemical biosensor for microRNA-21 detection. For testifying the detection feasibility, the photocurrent response of Bi_2S_3 /ITO was recorded in 0.1 M Tris–HCl (pH 9.8) containing 10 mM AAP before and after treatment with ALP/SA/DNA2/microRNA–DNA1/AuNPs/ Bi_2S_3 /ITO for 30 min. As shown in Fig. 2B, the photocurrent of Bi_2S_3 /ITO was about 7.86 μA (40.31 $\mu\text{A}/\text{cm}^2$) in 0.1 M Tris–HCl (pH 9.8) containing 10 mM AAP without ALP catalysis treatment (curve a). As expected, the photocurrent response of Bi_2S_3 /ITO increased significantly after the Tris–HCl solution was treated with ALP/SA/DNA2/microRNA–DNA1/AuNPs/ Bi_2S_3 /ITO for 30 min (curve b). This result indicated that ALP had been successively immobilized on the electrode surface, and AAP in Tris–HCl solution was successfully hydrolyzed to generate AA with the catalytic effect of ALP. Then, the produced AA acted as electron donor to capture photogenerated holes and blocked the recombinant of electron–hole pairs. Thus, the photocurrent increased. Based on these results, we think that the fabricated photoelectrochemical biosensor could be used to detect microRNA.

3.5. Optimization of detection conditions

The film thickness of Bi_2S_3 nanorods is an important influencing factor for the sensitivity of the photoelectrochemical biosensor. Thus Bi_2S_3 concentrations of 1, 2, 3, 4, 5, 6, 7 and 8 mg/mL (the Bi_2S_3 dispersion with different concentrations was prepared by dispersing different amounts of Bi_2S_3 in redistilled deionized water with the aid of ultrasonication) were investigated for their effect on the photocurrent response of the biosensor in 0.1 M Tris–HCl (pH 9.8) containing 10 mM AAP. As shown in Fig. S(5A see Supporting information), the photocurrent increased until 4 mg/mL Bi_2S_3 was modified on ITO electrode surface and then decreased. It can be explained as the fact that the greater the immobilized amount of Bi_2S_3 , the greater the amount of photo-generated electrons that can be captured by the ITO electrode to enhance the photocurrent. However, the thicker Bi_2S_3 film could block the electronic migration from the outer Bi_2S_3 to electrode surface. So the photocurrent decreased with further increasing Bi_2S_3 concentration. Considering the detection sensitivity, 4 mg/mL Bi_2S_3 was chosen for the following experiments.

In this work, the fabricated photoelectrochemical biosensor for microRNA-21 detection was based on visible light irradiation. Therefore the effects of light wavelength on the photocurrent response of biosensor were investigated with 420, 435, 450, 475, 500, 520 and 550 nm light irradiations. According to the results shown in Fig. S(5B see Supporting information), it could be concluded that the maximum photocurrent was obtained at 450 nm light irradiation. Therefore, this wavelength light was selected as the optimal irradiation source.

MicroRNA-21 hybridization time was also investigated and the photocurrent was recorded in 0.1 M Tris–HCl (pH 9.8) containing 10 mM AAP. As seen in Fig. S(5C see Supporting information), the photocurrent increased rapidly when extending the hybridization

to 120 min. Then the photocurrent increased slowly with further prolonging the hybridization time. It can be explained as the approximate saturated hybridization between microRNA-21 and probe at 120 min. And only a little microRNA-21 could be further hybridized with hairpin structure probe and unfold the stem-loop structure to capture ALP, which led to a weak increase in photocurrent. Therefore, the hybridization time of 120 min was selected.

In order to achieve the “signal on” strategy, ALP was immobilized on the biosensor to catalyze AAP hydrolysis to produce electron donor of AA in 0.1 M Tris–HCl (pH 9.8) containing 10 mM AAP. Thus, the catalytic reaction time of ALP towards AAP hydrolysis has great influence on the sensitivity of the fabricated biosensor. Fig. S(5D see Supporting information) showed the effect of ALP catalysis time on the photocurrent response of Bi₂S₃/ITO in 0.1 M Tris–HCl (pH 9.8) containing 10 mM AAP. The photocurrent increased quickly with extending catalysis time to 30 min, then tends to level off. With the extension of catalysis time, more and more electron donor of AA was generated to capture holes and improved the photocurrent response. Considering the detection efficiency and sensitivity, 30 min was selected as the optimal enzyme catalysis time.

3.6. MicroRNA-21 detection performance

MicroRNA-21 concentration detection was carried out using Bi₂S₃/ITO as the working electrode. The photocurrent signal was recorded in 0.1 M Tris–HCl (pH 9.8) containing 10 mM AAP after incubating with ALP/SA/DNA2/microRNA–DNA1/AuNPs/Bi₂S₃/ITO for 30 min to catalyze the hydrolysis of AAP. Under the optimum conditions, the photocurrent increased with microRNA-21 hybridization concentration from 5 to 5000 fM (Fig. 3A). As shown in Fig. 3B, the photocurrent was proportional to the logarithm value of microRNA-21 concentration. The linear regression equation could be expressed as J ($\mu\text{A}/\text{cm}^2$) = $-3.4317.79 \log c$ (fM) – 28.69 ($R=0.9912$) and the detection limit was estimated to be 1.67 fM ($S/N=3$). The detection performance was compared with other reported methods and the results are listed in

Table S1 (see Supporting information). The results indicated that the developed method showed reasonable linear range and acceptable detection limit.

For microRNAs biosensor, detection specificity is an important parameter. In order to prove the detection specificity of the developed method, the photocurrent change ($\Delta J = J_2 - J_1$) of Bi₂S₃/ITO electrode was compared, where J_2 was the photocurrent density of Bi₂S₃/ITO in 0.1 M Tris–HCl (pH 9.8) containing 10 mM AAP after incubating with ALP/SA/DNA2/microRNA–DNA1/AuNPs/Bi₂S₃/ITO for 30 min, J_1 was the photocurrent density of Bi₂S₃/ITO in 0.1 M Tris–HCl (pH 9.8) containing 10 mM AAP. For achieving the investigation of detection specificity, the probe DNA S1 in ALP/SA/DNA2/microRNA–DNA1/AuNPs/Bi₂S₃/ITO was hybridized with 0.5 pM complementary (column a), single-base mismatched (column b), three-base mismatched (column c), and non-complementary (column d) microRNA, respectively. As seen in Fig. 3C, the ΔJ was 40.21 $\mu\text{A}/\text{cm}^2$ for probe DNA S1 hybridizing with complementary microRNA-21, which was 3.85 times to single-base mismatched microRNA, 4.95 times to three-base mismatched microRNA and 7.52 times to non-complementary microRNA. These results demonstrated that the developed method had good detection specificity.

The stability of the photocurrent responses of the fabricated biosensor (ALP/SA/DNA2/microRNA–DNA1/AuNPs/Bi₂S₃/ITO) was also studied (Fig. 3D). No significant change of photocurrent response was observed with light on and off for 15 times over 300 s (relative standard deviation (RSD) = 3.28%), indicating a good stability.

The reproducibility of the photoelectrochemical biosensor was evaluated by measuring the photocurrent of Bi₂S₃/ITO in 0.1 M Tris–HCl (pH 9.8) containing 10 mM AAP after the solution was incubated with ALP/SA/DNA2/microRNA–DNA1/AuNPs/Bi₂S₃/ITO for 30 min, where the probe was hybridized with 0.5 pM microRNA-21. Six Bi₂S₃/ITO and ALP/SA/DNA2/microRNA–DNA1/AuNPs/Bi₂S₃/ITO were prepared as the process described in Section 2.3. The RSD for six measurements was 6.87%, suggesting an acceptable reproducibility.

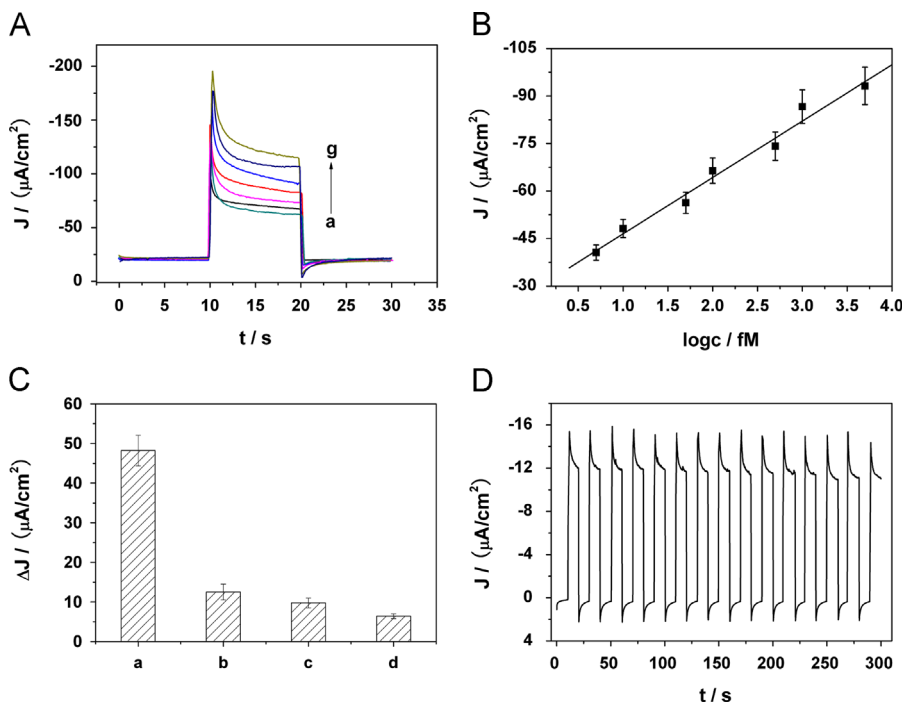


Fig. 3. (A) Photocurrent response of the biosensor with different hybridizations of microRNA-21 in 0.1 M Tris–HCl (pH 9.8) containing 10 mM AAP. MicroRNA-21 concentrations (from a to g): 5, 10, 50, 100, 500, 1000 and 5000 fM. (B) The calibration curve of the photocurrent versus logarithm value of microRNA-21 concentration. (C) Comparison of photocurrent change after the probe was hybridized with complementary (a), single-base mismatched (b), three-base mismatched (c), and non-complementary (d) microRNA ($c=0.5$ pM). (D) Time-based photocurrent responses of the biosensor in 0.1 M Tris–HCl (pH 9.8) containing 10 mM AAP with light on and off.

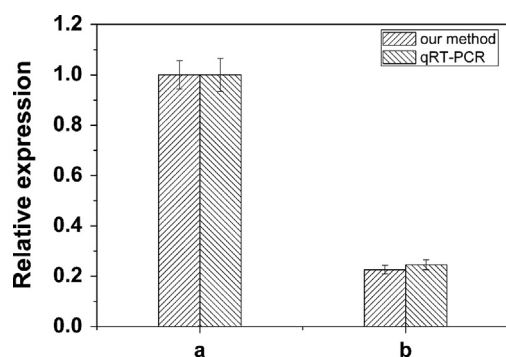


Fig. 4. The relative expression level of microRNA-21 in DF-1 chicken fibroblast cells infected with (b) and without (a) infected subgroup J ALVs.

3.7. MicroRNA-21 expression in DF-1 chicken fibroblast cells infected with subgroup J ALVs

In order to evaluate the applicability of the developed method, the fabricated biosensor was used to detect the expression level of microRNA-21 in DF-1 chicken fibroblast cells infected with subgroup J ALVs. As control, the expression level of microRNA-21 in normal DF-1 chicken fibroblast cells was also investigated. The results are shown in Fig. 4, which indicated that the expression level of microRNA-21 decreased after the DF-1 chicken fibroblast cells was infected with subgroup J ALVs when compared with control result. For proving the accuracy of this method, the expression level of microRNA-21 was also investigated by qRT-PCR, and the results were in agreement with them obtained by the method developed in this work, indicating that the fabricated photoelectrochemical biosensor had good accuracy. Moreover, according to the results that the over-expression of microRNA-21 in DF-1 chicken fibroblast cells infected with subgroup J ALVs, we think that microRNA might be an alternative biomarker for diagnosis of avian leukosis.

4. Conclusion

In summary, Bi_2S_3 nanorods with high photo-to-electronic conversion efficiency were applied in fabrication of photoelectrochemical bioelectrode. Based on it, we have developed a highly selective and sensitive photoelectrochemical biosensor for microRNA-21 assay on the basis of *in situ* production of electron donor under the catalytic chemistry of ALP. Additionally, the detection limit of the biosensor for microRNA-21 detection was lower than some of previous reports. Compared with other bioanalytical methods, our method has the advantages of time-saving, low cost, instrument miniaturization and operation simplicity. Accordingly, the proposed photoelectrochemical biosensor was not only suitable for screening biomarker of ALVs as shown above but also other diseases.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bios.2013.09.069>.

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