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Label-free and sensitive strategy for microRNAs detection based on the formation of boronate ester bonds and the dual-amplification of gold nanoparticles

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ABSTRACT

MicroRNAs (miRNAs), regulating gene expression by translational repression or degradation of messenger RNAs, are believed to be important for cancer diagnosis and prognosis serving as reliable molecular biomarkers. Simple, sensitive, and cost-effective assays for miRNAs are therefore in urgent demand. The main difference in the structure of RNA versus DNA is the presence of a hydroxyl group at the 2′ position of the ribose sugar in RNA, which makes the RNA molecule contain cis-diol at the end of the chain. Hydrophilic boronic acids are well known to form covalent bonds with cis-diols. In this work, we reported a label-free and sensitive method for the detection of miRNAs based on the formation of boronate ester covalent bonds and the dual-amplification of gold nanoparticles (AuNPs). Specifically, miRNAs were captured by the pre-immobilized DNA probes at the gold electrode, and derivatized with 4-mercaptophenylboronic acid (MBA)-capped AuNPs (MBA-AuNPs) through the formation of tight covalent bonds between the boronic acids of MBA-AuNPs and diols of miRNAs. Electrochemically active dopamine (DA)-capped AuNPs (DA-AuNPs) were then attached by the anchored MBA-AuNPs via the interaction of boronic acids and DA tags, which facilities the amplified voltammetric detection of miRNAs. Analytical merits (e.g., sensitivity, reproducibility, storage stability, dynamic range, and selectivity) were addressed. We believe that the results will be valuable for the development of biosensor for the detection of miRNAs in a biological matrix.

1. Introduction

MicroRNAs (miRNAs) are small RNA molecules (typically 18–24 nucleotides long) that can regulate gene expression in plants and animals by translational repression or degradation of messenger RNAs (Ambros, 2004; Cissell et al., 2007; Zhang et al., 2009). The human genome may encode over 1000 miRNAs, which may target about 60% of mammalian genes and are abundant in many human cell types (Bentwich et al., 2005; Lewis et al., 2005). Recently, some tumor-related miRNAs have been detected in serum (Asangani et al., 2007; Calin and Croce, 2006; Chan et al., 2005; Tricoli and Jacobson, 2007). Therefore, the expression levels of individual miRNAs may serve as reliable molecular biomarkers for cancer diagnosis and prognosis (Bartel, 2004; Cissell et al., 2007). However, currently used methods for the routine detection of miRNAs, such as northern blotting assay, real-time fluorescence quantitative, microarray and surface plasmon resonance are usually time-consuming, requiring fluorescent- or radio-labeling, complicated instruments and/or lacking sensitivity (Fang et al., 2006; Kim et al., 2010; Liang et al., 2005; Yan et al., 2008). Simple, sensitive, cost-effective and rapid detection assays for miRNAs are therefore in urgent demand in view of the low intracellular content and instability of miRNAs (Cissell et al., 2007; de Planell-Saguer and Rodicio, 2011; Driskell et al., 2008; Zhang et al., 2011).

In recent years, there have been some attempts for miRNAs detection using electrochemical biosensor based on the signal amplification of nanoparticles in view of its high sensitivity, simplicity, rapid response, and compatibility with miniaturization (Dong et al., 2012; Fan et al., 2007; Gao, 2012; Gao and Yu, 2007; Gao and Yang, 2006; Gao and Yuan, 2007; Kilic et al., 2012; Lusi et al., 2009; Pöhlmann and Sprinzl, 2010; Peng and Gao, 2011; Peng et al., 2010; Wang et al., 2012; Yin et al., 2012a, 2012b). For example, Gao's group was the first to develop electrochemical biosensors for miRNAs detection with OsO_2 nanoparticles as tags for RNA labeling (Gao and Yang, 2006). Hereupon, they developed a series of sensitive electrochemical biosensors for miRNAs

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detection based on miRNAs labeled with $Os(dmpy)_2(IN)Cl^+$ (Gao and Yu, 2007), $Ru(PD)_2Cl_2Os$ (Gao and Yuan, 2007), and RuO_2 nanoparticles (Peng and Gao, 2011; Peng et al., 2010). The detection limit of femtomolar was achieved (Peng and Gao, 2011; Peng et al., 2010), which is comparable to that of the more common PCR-based assays. Recently, Dong et al. reported a sensitive method for miRNAs detection using oligonucleotide encapsulated silver nanoclusters (Ag-NCs) as effective electrochemical detection probes (Dong et al., 2012). However, the engagement of chemical ligations between detection probe and Ag-NCs may be a technical hurdle in bringing them to the next stage due to the instability of Ag-NCs. Labeling of DNA or miRNAs with nanoparticles offers better selectivity and sensitivity, but there are some limitations in the extensive application of labeled DNA or miRNAs due to the operation complexity and the high cost. Label-free biosensors play an important role for their simplicity, convenience, and low cost. Schiavo's group developed a label-free miRNAs detection method based on guanine oxidation signal of guanine-containing miRNAs after hybridization with guanine-free probe at electrode (Lusi et al., 2009). However, the application of this method is limited in detection of guanine-free miRNAs.

For the sensitive, selective and label-free detection of biomolecules, sandwich-type affinity biosensor is one of the most attractive tools. In this format, the crucial step is the capture and identification of analytes. Recently, we have achieved the amplified detection of amyloid-β peptides, glycoproteins and dopamine in sandwich format (Liu et al., 2013; Xia et al., 2013, 2010). In this work, we attempted to develop a sandwich-type electrochemical biosensor for miRNAs detection. The main difference in the structure of RNA versus DNA is the presence of a hydroxyl group at the 2′ position of the ribose sugar in RNA, which makes the RNA molecule contain cis-diol at the end of the chain. Based on the difference, Gao and Yang developed a biosensor based on electrocatalytic OsO_2 nanoparticles (Gao and Yang, 2006). In this method, target miRNAs are first treated with periodate to produce 2′- and 3′-terminal dialdehydes at the 3′ end of the molecule. The periodate-treated target miRNAs are captured by the pre-immobilized complementary oligonucleotide probes. Isoniazid-modified OsO_2 nanoparticles are then attached onto the sensor surface through the interaction between isoniazid and dialdehydes of treated miRNAs. The amount of miRNAs can be determined according to the electrochemical current produced by the nanoparticle-catalyzed oxidation of hydrazine. It is well known that phenylboronic acids can form boronate ester covalent bonds with cis-diols (e.g. catechol derivatives, sugars, nucleosides and glycoproteins) on substrate surface, which has been characterized by electrochemistry, fluorescence, quartz crystal microbalance, surface plasmon resonance, Raman spectroscopy and UV–visible spectroscopy (Aytaç et al., 2011; Bull et al., 2013; de Guzman et al., 2010; Deore and Freund, 2005; Granot et al., 2008; Kanayama and Kitano, 2000; Kanekiyo et al., 2004; Li et al., 2011; Liu et al., 2005; Nishiyabu et al., 2011; Park et al., 2008; Rahman and Elaissari, 2012; Song and Yoon, 2009; Takahashi and Anzai, 2005; Wang et al., 2010). This binding property enables boronic acids-functionalized particles to be used for the fluorescent, electrochemical and colorimetric detection of diol derivatives (Jin et al., 2010; Kong et al., 2011; Li et al., 2012; Liu et al., 2012b), and the specific capture and purification of RNA and glycoproteins (Rahman and Elaissari, 2012; Zhang et al., 2012; Zhou et al., 2008). More intriguingly, the hybridization methods based on quantum dots and metal nanoparticles are highly sensitive and are viable alternatives to the routine detection of miRNAs (Alhasan et al., 2012; Gill et al., 2008). Because of the unique combination of chemical and physical properties, gold nanoparticles (AuNPs) as labels have been widely used in diagnostics and detection (Katz and Willner, 2004). Herein, we reported a dual-amplified sandwich-type electrochemical biosensor for the detection of miRNAs at low levels using 4-mercaptophenylboronic acid (MBA)-capped AuNPs (MBA-AuNPs) and electrochemically active dopamine (DA)-capped AuNPs (DA-AuNPs). The sandwich-type system was formed by specific recognition of anti-miRNAs probes on gold electrode to miRNAs, followed by the successive attachment of MBA-AuNPs and DA-AuNPs through the formation of boronate ester covalent bonds. This biosensor obviates the use of expensive bioreagents and labeled target/detection DNA or miRNAs, reducing the operation complexity and assay cost.

2. Experimental

2.1. Chemicals and reagents

Dithiobis(succinimidyl propionate) (DSP), MBA, tris (carboxyethyl)phosphine (TCEP), 1-hexanethiol (HT), KH_2PO_4, K_2HPO_4, and tris-(hydroxymethyl)aminomethane hydrochloride (Tris–HCl) were obtained from Sigma-Aldrich. Dopamine hydrochloride, adenosine-5′-monophosphate (AMP) 2′-deoxyadenosine 5′-monophosphate (dAMP) and thiolated single-stranded DNA (ss-DNA) probe (5′-TCAACATCAGTCTGATAAGCTA-$(CH_2)_6$-SH-3′) as well as its target DAN (5′-TAGCTTATCAGACTGATGTTGA-3′) were purchased from Sangon Biotech. Co., Ltd. (Shanghai, China). The target miRNA-21 and its similar sequences were obtained from GenePharma Co., Ltd. (Shanghai, China), which have the following sequences: 5′-UAGCUUAUCAGACUGAUGUUGA-3′ (miRNA-21), 5′-UAGCUUAUCGGACUGAUGUUGA-3′ (single-based mismatch), 5′-UUGCUUAUCGGACUGAUCUUGA-3′ (three-based mismatch), and 5′-GUAAGGCAUCUGACCGAAGGCA-3′ (non-complementary). The DA-DSP complex was prepared by mixing two-equivalent DA (20 mM) and one-equivalent DSP (10 mM) in DMF containing 20 mM triethylamine for 12 h as our previous report (Xia et al., 2013). The resultant product was characterized by LCT Premier XE mass spectrometry (m/e=481.1390) to ensure that all DA molecules react completely with DSP, and then stored at 4 °C under O_2-free condition. The DNA solutions were prepared using TE buffer solution (10 mM Tris–HCl, 1 mM EDTA, pH 7.4), and kept at −18 °C. The miRNAs stock solutions were prepared daily with diethylpyrocarbonate-treated water in an RNase-free environment. All aqueous solutions were prepared with a Millipore system (Simplicity Plus, Millipore Corp.), and saturated with N_2. The hybridization solution was prepared with TNE buffer (TE +0.1 M NaCl). The supporting electrolyte was phosphate-buffered saline solution (PBS buffer, 10 mM, pH 7.4) containing 50 mM Na_2SO_4.

2.2. Preparation of MBA-AuNPs and DA-AuNPs

The preparation and characterization of DA-AuNPs and MBA-AuNPs have been reported by us previously (Xia et al., 2013). Brief, the stock solution of DA-DSP was diluted with deionized water to 500 μM. To avoid the oxidation of DA-DSP, 1 mM Na_2SO_3 was added into the DA-DSP solution. The mixed solution of DA-DSP (1.4 μM) and citrated-stabilized AuNPs (3.2 nM) was then stirred at room temperature for 2 h to produce the DA-AuNPs. The MBA-AuNPs were prepared by mixing 500 nM MBA and 3.2 nM AuNPs for 2 h. Note that trace Fe^{3+}, Al^{3+}, and Cu^{2+} can induce the aggregation of DA-AuNPs (Liu et al., 2012a). The modified AuNPs suspensions were stored in a clean environment at 4 °C.

2.3. Procedure

The gold disk electrode with a diameter of 2 mm was polished with diamond pastes down to 3 μm and alumina pastes down to 0.3 μm, and then sonicated in ethanol and water. The DNA probe-covered electrode was prepared by immersing the cleaned gold

electrodes in a solution of 1.0 μM thiolated ssDNA containing 5 μM TCEP in the darkness for 12 h. The amount of probe immobilized on gold electrode was chosen according to the well-established protocol (Steel et al., 1998; Wang et al., 2012). This step was followed by washing the electrode thoroughly with water and soaking the electrode in a 0.1 mM HT for 5 min to block the unreacted gold surface. Then, 10 μL of TNE comprising a given concentration of miRNAs was cast onto the electrode surface for the hybridization between miRNAs and pre-immobilized DNA probe for 30 min (Dong et al., 2012). Again, the electrode was rinsed with water to rid any non-specifically adsorbed substance. To attach DA-AuNPs, the electrode was first allowed to react with 20 μL of MBA-AuNPs suspension (pH 7.4) for 30 min, and then exposed to 20 μL of DA-AuNPs suspension (pH 7.4) for 10 min. After the electrode had been rinsed with water, voltammetric determination in supporting electrolyte was performed on a DY2013 electrochemical workstation (Digi-Ivy, Inc., Austin, TX) using a homemade plastic electrochemical cell. A platinum wire and a Ag/AgCl electrode were used as the auxiliary and the reference electrodes, respectively.

After each assay, the electrode surface was regenerated with 10 mM HCl for 30 s (desorbing the target miRNAs, MBA-AuNPs, and DA-AuNPs). To test the stability, the probe DNA modified electrode was stored in the dark, at room temperature in TE buffer in a sealed container. This allowed long-term stability testing for up to 10 days.

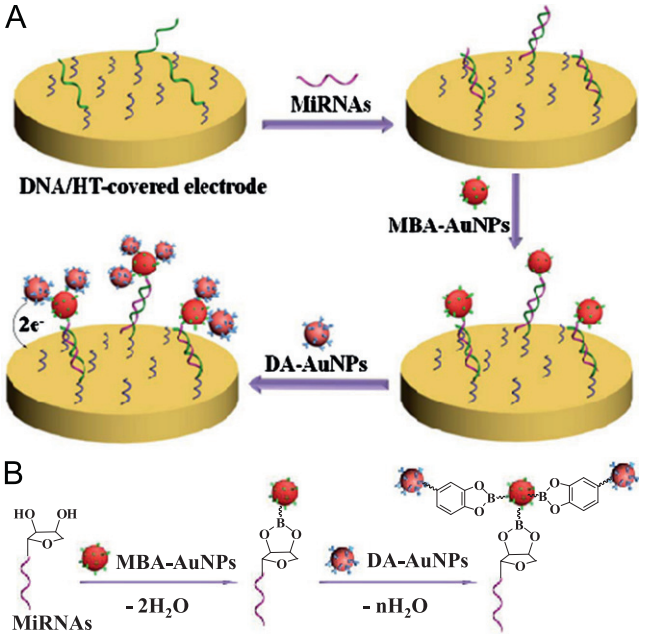

Fig. 1. Schematic representation showing the capture of miRNAs by the immobilized DNA probes and the detection of miRNAs by the attachment of MBA-AuNPs and DA-AuNPs (A) and the formation of boronate ester bonds between MBA-AuNPs and miRNAs as well as DA-AuNPs (B).

3. Results and discussion

Although the specific binding of boronic acid to ribose sugar in ribonucleotide and dopamine has been demonstrated previously (Deore and Freund, 2005; Kanekiyo et al., 2004; Kim et al., 2004; Kong et al., 2011; Shimomura et al., 2003; Strawbridge et al., 2000), we first investigated the formation of the phenylboronic acid–nucleotide complex with mass spectrometry to confirm the binding of phenylboronic acid and nucleotide (Fig. S1 in Supplementary material). The result of mass spectrometry also demonstrated that phenylboronic acid did not interact with deoxyribonucleotide (data not shown).

3.1. Principle of the strategy for miRNAs detection

To prove the feasibility of our strategy, miRNA-21 (5′-UAGCUUAUCGGACUGAUGUUGA-3′) over-expressed in 11 types of solid tumors was tested as model analyte. The analysis principle of this method is shown in Fig. 1. Thiolated ss-DNA probes (5-TCAACATCAGTCTGATAAGCTA-$(CH_2)_6$-SH-3′) were immobilized on the gold electrode surface to capture miRNA-21. The use of the mixed self-assembled monolayers (SAMs) of thiolated DNA and HT eliminates nonspecific adsorption of ss-DNA on gold surface and orients the immobilized DNA molecules for more efficient hybridization (Dong et al., 2012; Levicky et al., 1998). MiRNA-21 molecules were captured by DNA-modified electrode via the hybridization reaction. The cis-diol groups in ribose sugar of miRNA-21 molecules were derivatized with MBA-AuNPs through the formation of boronate ester covalent bonds. This step was followed by the attachment of DA-AuNPs. MAB-AuNPs and DA-AuNPs were attached step by step since DA-AuNPs could induce the aggregation of MBA-AuNPs in solution through the DA-MBA interaction (Kong et al., 2011). As a result, facile electron transfer reactions between the DA tags and the electrode would take place. Note that a MBA-AuNP can attach more than one DA-AuNP and each DA-AuNP contains a large number of electrochemically active DA molecules (Kong et al., 2011; Xia et al., 2013). Therefore, the electrochemical signals will be greatly amplified, and the peak currents are proportional to the amounts of captured miRNA-21 molecules.

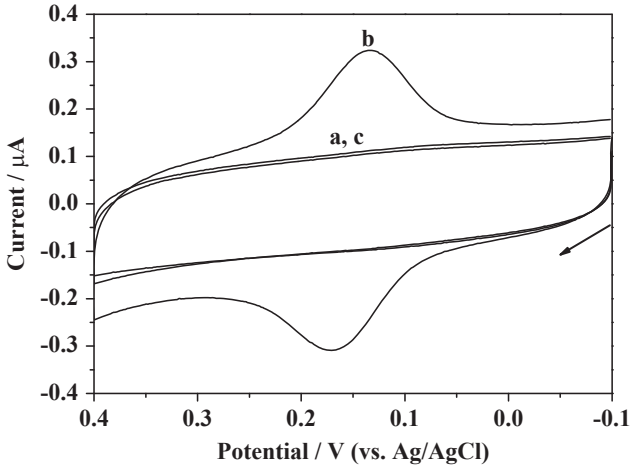

Fig. 2. Cyclic voltammograms (CVs) acquired at mixed SAMs of DNA probe/HT before (a) and after (b) hybridization with 10 pM miRNA-21, followed by the attachment of MBA-AuNPs and DA-AuNPs. Curve c corresponds to that after exposure to 10 pM target DNA instead of miRNA-21. The scan rate was 0.1 V/s, and the arrow indicates the scan direction.

3.2. Feasibility and regeneration

As shown in Fig. 2, no redox peak was observed at the DNA modified electrode coated successively with MBA-AuNPs and DA-AuNPs (curve a), indicating that MBA-AuNPs and DA-AuNPs did not interact with the DNA probes. The result is understandable since deoxyribonucleotide did not form a complex with phenylboronic acid due to the lack of hydroxyl group at the 2′ position of the ribose sugar, which was confirmed by mass spectrometry. Curve b is a representative CV collected at an electrode modified with probe DNA for miRNA-21 capture and the subsequent attachment of MBA-AuNPs and DA-AuNPs. The redox peaks with

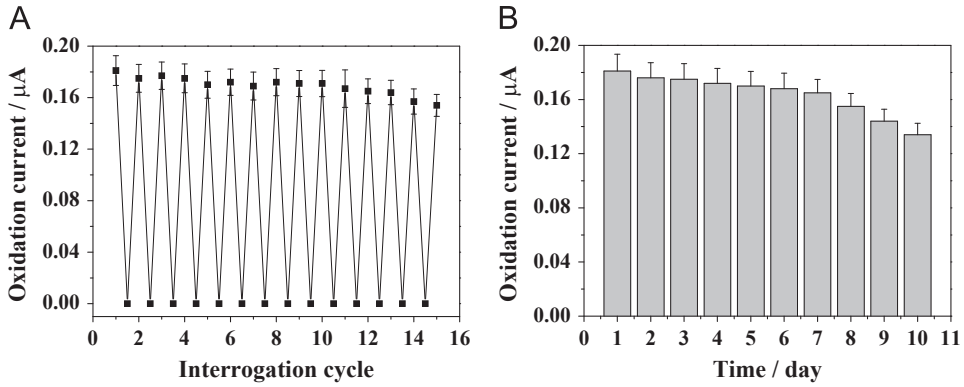

Fig. 3. (A) The oxidation currents for 15 regeneration/assay cycles and (B) the storage stability of the DNA-modified electrodes. The experimental conditions are the same as those in Fig. 2. Each point was averaged from at least three replicates, and the relative standard deviations (RSDs) are shown as the error bars.

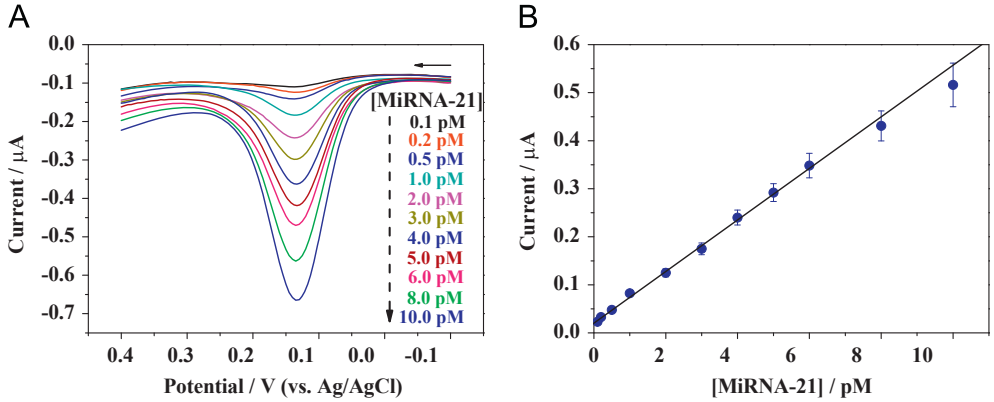

Fig. 4. (A) Representative differential pulse voltammograms (DPVs) acquired at mixed SAMs of DNA probe/HT after hybridization with different concentrations of miRNA-21 followed by the attachment of MBA-AuNPs and DA-AuNPs. (B) Dependence of the anodic peak currents on the miRNA-21 concentration (0.1–10 pM).

Epa=0.174 and Epc=0.133 were attributed to the oxidation/reduction of DA tags (Ji et al., 2012; Liu et al., 2013; Medintz et al., 2010). The well-defined redox waves demonstrate that this method is amenable to the detection of miRNA-21 in a highly sensitive manner. Note that no redox peak was observed in the absence of MBA-AuNPs (data not shown), indicating that the attachment of DA-AuNPs is dependent on the anchored MBA-AuNPs. For comparison, the same procedure was implemented with target DNA instead of miRNA-21 (curve c). The absence of any discernible peak in curve c indicates that binding of MBA-AuNPs is greatly dependent on the cis-diol groups in ribose sugar of captured miRNAs.

DNA hybridization and stability of boronate ester are pH-dependent (Springsteen and Wang, 2002; Wang et al., 2012). We further found that the electrode can be conveniently regenerated by rinsing the surface with a 10 mM HCl solution (desorbing the target miRNAs, MBA-AuNPs, and DA-AuNPs). As shown in Fig. 3A, no obvious change in the current was observed after 15 regeneration/assay cycles. Thus, multiple samples can be determined using one electrode, dramatically increasing the sample throughput and reducing the sample analysis time. We also found that the relative standard deviations (RSDs) are all below 13% by performing these regeneration/assay cycles at three different electrodes in parallel, indicating that multiple electrodes can be prepared concurrently for the assays of many different samples. Furthermore, we investigated the stability of the sensor and found that the electrode remains stable for almost one week and only loses 26% of its capture efficiency after 10 days (Fig. 3B). A point worth to mention is that redox tags (e.g., ferrocene or Fc) labeled DNA sensor exhibits more than 50% signal loss after 15 regeneration/assay cycles and 91% signal loss after 180 h storage due to the degeneration of Fc tags (Ge and Levicky, 2010; Kang et al., 2009). Evidently, label-free sensor presented here dramatically outperforms the redox tags labeled counterparts in terms of their regeneration and long-term storage stability.

Table 1
Comparison of the sensitivity obtained by employing different types of nanoparticles for signal amplification.

Type of nanoparticles	Linearity (pM)	LOD (fM)	Reference
Isoniazid-capped OsO_2	0.3–200	80	Gao and Yang, 2006
AgNPs-labeled DNA	0.1–1000	67	Dong et al., 2012
Ferrocene/streptavidin-capped AuNPs	0.01–2	10	Wang et al., 2012
RuO_2-labeled miRNAs	0.006–2	6	Peng and Gao, 2011
AuNPs-labeled DNA	0.1–70	60	Yin et al., 2012
MBA-AuNPs/DA-AuNPs	0.1–10	45	This work

3.3. Sensitivity

In view of the low level of miRNAs in biological samples (femtomolar or even lower level), high sensitive methods that are capable of performing assays of single nucleotide polymorphoism are in urgent demand for identifying and quantifying miRNAs (Cissell et al., 2007). With the regeneration of the method established, we assessed the sensitivity and reproducibility. Differential pulse voltammetry can decrease the background charging

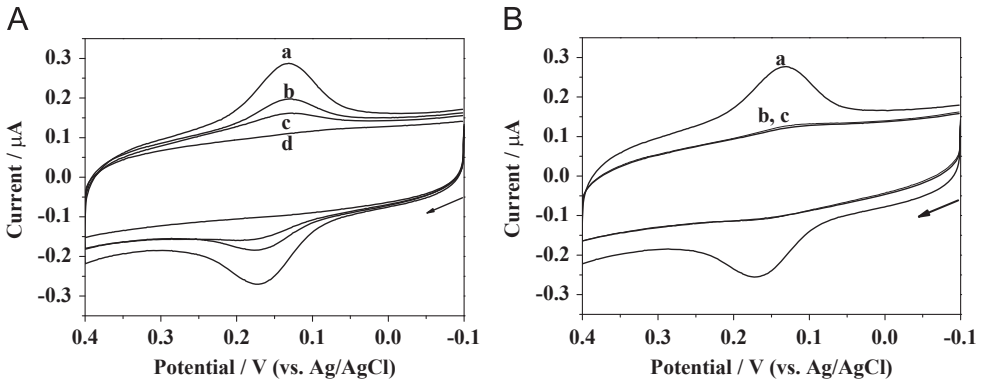

Fig. 5. CVs acquired at mixed SAMs of DNA probe/HT after hybridization with different miRNAs at 25°C (A) and 65°C (B), respectively, followed by the attachment of MBA-AuNPs and DA-AuNPs. Curve a: miRNA-21, curve b: single-base mismatch, curve c: three-base mismatch, and curve d: non-complementary. All the miRNAs concentrations are 8 pM. The other experimental conditions are the same as those in Fig. 2.

currents and in turn increase the detection sensitivity. Herein, the dependence of the current on the concentration of miRNAs was investigated by differential pulse voltammetry. Fig. 4A shows the representative differential pulse voltammograms (DPVs). The aforementioned regeneration of the sensor surface contributes to good reproducibility of the method, as the RSDs shown as the error bars are all less than 8.7% (Fig. 4B). The peak currents (I_p) increased linearly with the concentrations of miRNA-21 ranging from 0.1 to 10 pM and began to level off when the concentration was beyond 10 pM. The linear regression equation is expressed as I_p (μA)=0.019+0.053 [miRNA-21] (pM) (R^2=0.99). The limit of detection (LOD) was estimated to be 45 fM (n=11), which is comparable to (or even lower than) those achievable with other types of amplified electrochemical strategies (Table 1). The lower detection limit is attributed to the dual-amplification of MBA-AuNPs and DA-AuNPs. Moreover, our method obviates the use of expensive bioreagents and labeled target/detection DNA or miRNAs, reducing the operation complexity and assay cost. The technical simplicity, high sensitivity and selectivity also demonstrated that electrochemical biosensor can serve as a viable alternative to conventional or modified biochemical techniques, such as fluorescent correlation spectroscopy, quantum dot-based miRNAs profiling microarray assay, and Northern blotting assay (Fang et al., 2006; Kim et al., 2010; Liang et al., 2005; Yan et al., 2008).

3.4. Selectivity

Due to the short lengths of miRNAs and the numerous miRNAs species in human genome, sequence similarity is frequently encountered in the miRNAs family. The selectivity of our method was also determined. As shown in Fig. 5A, the current of complementary target miRNA-21 (curve a) was much higher than those of the mismatch sequences at the same concentration. No redox peak was observed in the case of non-complementary (curve d), indicating that non-complementary miRNAs were not captured by the probes. However, the currents in the case of single-base mismatch (curve b) and three-base mismatch (curve c) miRNAs were 39 and 20% of that of complementary target miRNA-21, respectively, indicating that base mismatch miRNAs could influence the accuracy of the determination in real sample. It is well known that DNA hybridization depends on the temperature (Wang et al., 2012). We further investigated the effect of temperature on the hybridization of probe DNA and miRNA-21, and found that no obvious change in the current was observed at the temperature in the range of 25–65 °C (Fig. S2 in Supplementary material). When the temperature is higher than 65 °C, the currents began to decrease, indicating that less amount of miRNA-21 were captured by the pre-immobilized DNA probes. This result is understandable since double-stranded DNA/RNA is susceptible to denaturation at higher temperature. We also investigated the effect of temperature on the hybridization between DNA probes and base mismatch miRNAs. As a result, the currents in the case of the single-base mismatch and three-base mismatch miRNAs almost dropped to background level at 65 °C (Fig. 5B), demonstrating that these two miRNAs were not captured by the probes at such temperature. Therefore, the interference of base mismatch miRNAs could be avoided by elevating the hybridization temperature.

4. Conclusion

In this work, we reported a label-free and sensitive strategy for the detection of miRNAs based on the difference in the structure of RNA versus DNA and the dual-amplification of MBA-AuNPs and DA-AuNPs. Cis-diols at the 3′ end of miRNAs captured by the pre-immobilized DNA probes were derivatized with MBA-AuNPs for the attachment of electrochemically active DA-AuNPs. With miRNA-21 as model analyte, we demonstrated the feasibility and sensitivity of the proposed strategy. The detection limit is comparable to (or even lower than) those achievable with other amplified electrochemical strategies. Moreover, this biosensor outperforms those with the redox- or nanoparticles-labeled oligonucleotide probes in terms of its regeneration, reproducibility, long-term storage stability and/or sensitivity. We believe that the results will be valuable for the design of new types of biosensor for the label-free and sensitive detection of miRNAs in a biological matrix.

Acknowledgments

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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.2013.03.074.

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