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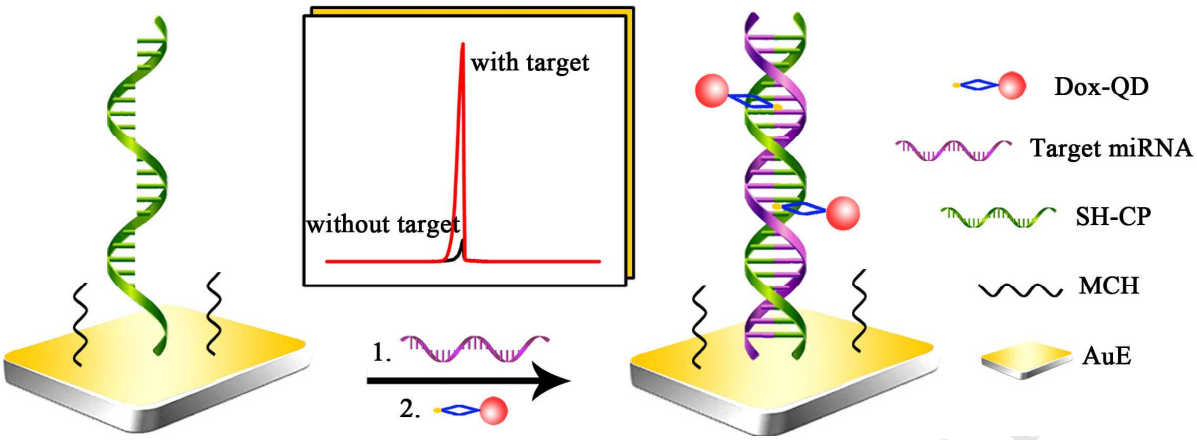

Intercalation of quantum dots as the new signal acquisition and amplification platform for sensitive electrochemiluminescent detection of microRNA

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Abstract: Herein, we report on the development of a simple and sensitive biosensor for electrochemiluminescent (ECL) detection of microRNAs (miRNA) based on the intercalation of doxorubicin-conjugated quantum dot nanoparticles (Dox-QDs) into the DNA/RNA hybrids as the new signal acquisition and amplification platform. The thiolated DNA capture probes are self-assembled onto gold electrodes *via* the formation of Au-S bonds. The sensing surface is then incubated in a target miRNA-containing buffer solution to form the double-stranded duplexes. In this case, massive Dox-QDs can intercalate into the base pairs of the hybrid duplexes, resulting in amplified ECL emissions due to their reactions with the coreactant $S_2O_8^{2-}$ and the dissolved oxygen in the detection buffer. The increase in ECL intensity proportional to the amount of target miRNA in the testing samples serves as the quantitative basis. Different from traditional QDs-based methods such as labeling and embedding, our sensor involves the employment of the intercalation of the Dox-QDs as the signal acquisition and amplification platform. The combination of the QDs intercalation

amplification with the high sensitivity of the ECL technique enables us to detect miRNA down to the low femtomolar level. Moreover, our method is also coupled with acceptable selectivity in discriminating the target miRNA and against its family members as well as other interference sequence, and can monitor miRNAs from human prostate carcinoma (22Rv1) cell lysates.

Keywords: doxorubicin; quantum dot; microRNA; electrochemiluminescence.

1. Introduction

MicroRNAs (miRNAs) are a class of small, endogenous and nonprotein coding RNAs composed of about 18-24 nucleotides [1-3]. It is more than two decades since the first miRNA lin-4 was discovered [4], and from that moment on a new era of genetic-related research has started. Derived from long primary transcripts, miRNA are considered to be responsible for the regulation of gene expression, which is achieved by binding to their complementary 3'-untranslated regions of target messenger RNAs (mRNAs), leading to the target degradation or suppression of the translation process [5-7]. Recently, increasing evidence reveals that aberrant expression of miRNAs is related to various diseases and disorders [8], especially cancers [9, 10], endowing them great potential in diagnostics and therapy. However, the intrinsic properties of miRNAs such as instability, short lengths, low abundance and sequence similarity among family members [11, 12] hinder the progress in the development of effective approaches for the detection of miRNA. Northern blotting [13], real-time PCR [14, 15] and microarray analysis [16] are conventional methods

for miRNA detection, but these methods suffer from the limitations of low sensitivity, harsh experimental conditions, time-consuming procedure and expensive equipment, which restrict their practical application. Consequently, the development of rapid, simple, sensitive and economical approaches for miRNA determination is urgently needed.

Up to now, with the great achievements of nanotechnology, nanomaterials are widely used in various miRNA detection schemes due to their fascinating properties in connection to colorimetric [17], photoelectrochemical (PEC) [18], electrochemical (EC) [19-22] and electrochemiluminescent (ECL) [23-25] techniques for signal transduction. Among the popular nanomaterials, semiconductive quantum dots (QDs) have emerged as excellent candidates in biosensor applications attributing to their specific optical and EC properties, which can act as signal tags to trace the recognition events through the target-induced fluorescent or ECL emission variation, or the current change of the metal ions released from the acid-dissolved QDs [24]. Generally speaking, the signal acquisition is accomplished by chemical modification of capture probes with QDs [27-29], or the formation of a QDs-containing film on the electrode surface [31-33]. However, a laborious and tedious labeling processes for covalent cross-linking of biomolecules and QDs are required in the former method, and easy leakage of QDs from its composited film makes the latter method inaccurate. A effecient way to get QDs involved into a biosensing platform is thus desired.

Intercalation of small molecules into the base pairs of double-stranded DNA is a strong noncovalent interaction depending on the planarity and aromaticity of their

structural features [34], which serves as an effective way to introduce signal molecules into a hybridization-based biological detection system. The surface groups of QDs provide support for further functionalization with intercalative molecules, which paves the way for the intercalation of QDs into the DNA duplex. As one of the many intercalators, doxorubicin (Dox) is a popular anthracycline commonly used in anticancer drug [35]. Previous study has demonstrated the preparation of Dox-conjugated QDs nanoparticles and their application in cell imaging [36]. However, to the best of our knowledge, such Dox-QDs nanoconjugates have not been used for ECL hybridization detection.

In the present work, a simple and yet sensitive miRNA biosensor was described based on the intercalation of Dox-QD nanoconjugates into the DNA/RNA hybrids as the new signal acquisition and amplification platform. With the presence of the target miRNA, hybridization events occur along with the successive intercalation of massive Dox-QDs into the base pairs of the formed DNA/RNA hybrids, leading to an amplified signal output. The combination of the QDs intercalation amplification with the high sensitivity of the ECL technique enables us to detect miRNA down to the low femtomolar level.

2. Experimental

2.1 Materials and reagents

Mercaptopropionic acid (MPA), N-(3-dimethylamminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), 6-mercapto-1-hexanol (MCH) and Tris-HCl were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Sodium tellurite

(Na_2TeO_3) and doxorubicin (Dox) were from J&K Scientific Ltd. (Guangzhou, China). $NaBH_4$, $CdCl_2$•$2.5H_2O$, trisodium citrate, dihydrate ethylenediaminetetra-acetic acid (EDTA), $K_2S_2O_8$, sodium phosphate monobasic, sodium phosphate dibasic, sodium chloride, potassium chloride, potassium phosphate dibasic, potassium phosphate monobasic were received from Kelong Chemical Company (Chengdu, China).

The synthetic DNA and miRNAs were ordered from Invitrogen Biotechnology Co., Ltd. (Shanghai, China) and the sequences of the oligonucleotides are listed below:

thiolated capture probe (SH-CP): 5'-SH-$(CH_2)_6$-CCATCTTTACCAGACAGTGTT A-3'

miR-141 (target): 5'-UAACACUGUCUGGUAAAGAUGG-3'

miR-200b: 5'-UAAUACUGCCUGGUAAUGAUGA-3'

miR-429: 5'-UAAUACUGUCUGGUAAAACCGU-3'

let-7d: 5'-AGAGGUAGUAGGUUGCAUAGUU-3'

All reagents were analytical grade and solutions were prepared using ultrapure water (specific resistance of 18 MΩ·cm).

2.2 Apparatus

UV-Vis and photoluminescence (PL) spectra were obtained on a UV-2450 (Shimadzu, Japan) and a RF-5301PC spectrophotometer (Shimadzu, Japan), respectively. Electrochemical measurements were performed on a CHI 660D electrochemistry workstation (CH Instruments Inc., Shanghai, China). A conventional three-electrode configuration was used, with a modified gold electrode (AuE, 3 mm in

diameter), a Ag/AgCl (3 M KCl) reference electrode and a platinum wire counter electrode. The ECL emission was monitored by a MPI-A electrochemiluminescence analyzer (Xi'an Remax Electronic Science & Technology Co. Ltd., Xi'an, China) in 0.1 M pH 7.4 phosphate buffered saline (PBS) containing 0.1 M KCl and 0.1 M $K_2S_2O_8$ with the voltage of the photomultiplier tube (PMT) at 900 V.

2.3 Preparation of the Dox-QD nanoconjugates

Water soluble MPA-capped CdTe QDs (MPA-CdTe) were prepared according to our previously reported protocol [37]. The mean size of the QDs is estimated from the corresponding UV-Vis adsorption peak to be 3.1 nm and thus the concentration of the QDs solution is calculated to be 140 μM on the basis of the Peng's empirical equation [36]. Formation of the Dox-QD nanoconjugates was accomplished by mixing 7 μL of CdTe QDs (140 μM) and 2 mg EDC with 187 μL of 1×PBS (pH 7.4) under gentle stirring in the ice-bath for 1 h. Then, 6 μL of Dox (1 mg mL^{-1}) was added to the above mixture and incubated for another 2 h. Finally, the obtained reddish solution was centrifuged at 8000 rpm for 10 min followed by removal of the supernatant. After washing twice with water, the pellets (Dox-QD nanoconjugates) were collected by centrifugation and resuspended in water for further use.

2.4 Cell Culture and Total RNA Extraction

All appliances were pretreated with 0.01% DEPC solution, and solutions used throughout the experiments were prepared with DEPC-treated water. The RPMI 1640 medium (Thermo Scientific Hyclone) supplemented with 10% fetal bovine serum, 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin were employed for the culture

of human cancerous cell lines (22Rv1 and Hela) at 37 °C in a 5% CO_2 incubator with humidity. Total RNA samples were extracted from each cell line by using Trizol Reagent (Invitrogen Biotechnology Co., Ltd.) according to the manufacturer's protocol. Briefly, the appropriate amount of Trizol Reagent was added to the cell pellet for cell disruption, followed by the incubation at room temperature for 5 min. Subsequently, the RNA extraction was precipitated by 2-propanol. Finally, the extracted RNA solution was diluted to 10^6 cells per 100 μL water and stored at −20 °C for further use

2.5 Fabrication of the sensors

Firstly, the pretreatment of the AuEs was accomplished according to the previous method [39]. The freshly pretreated AuE was immediately soaked with SH-CP (1 μM) in immobilization buffer (IB, 10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, 10 mM TCEP, pH 7.4) and incubated overnight at room temperature in humidity. After being rinsed thoroughly with 1×PBS and dried with nitrogen, the CP-assembled electrode was blocked with 1 mM MCH for 2 h to fabricate the sensing surface. Next, the surface was immersed in hybridization buffer (HB, 10 mM Tris-HCl, 1 mM EDTA, 300 mM NaCl, 1 mM $MgCl_2$, pH 7.4) containing different concentrations of the target miRNA at room temperature for 2 h, followed by rinsing with 1×PBS. After that, a droplet of 10 μL Dox-QD nanoconjugates (10 μM) in HB was cast onto the modified electrode and incubated for 1 h. Finally, after washing by 1×PBS, the resulting functionalized electrode was placed in detection buffer (0.1 M PBS containing 0.1 M $K_2S_2O_8$ and 0.1 M KCl, pH 7.4) for ECL measurement by scanning the potential

from 0 to -1.5 V (versus Ag/AgCl) at the scan rate of 50 mV s^{-1} with the voltage of photomultiplier tube (PMT) of 900 V.

3. Results and discussion

Herein, we prepared a new class of signal amplification label (Dox-QD nanoconjugates), which can intercalate into the base pairs of the hybrid duplex structures with its functional Dox groups and act as ECL emitters. For proof-of-concept demonstration, miR-141 was selected as the target model to fabricate a simple sensitive ECL biosensor. As shown in Scheme 1, the sensing interface is easily obtained by immobilizing a mixed self-assembled monolayer of the single-stranded DNA CPs and MCH on the pretreated AuE surface. When the target miRNA is present, it is captured by hybridizing with the immobilized CPs to form double-stranded helix structures. Upon the addition of the Dox-QD nanoconjugates and incubation with the electrode, these nanoconjugates are capturedby intercalating into the base pairs of the hybrid duplexes, resulting in an amplified ECL emission in the coreactant ($S_2O_8^{2-}$) containing detection buffer. The increase in ECL intensity is related to the amount of target miRNA in the testing samples, which serves as quantitative basis. Because several signal tags are introduced in each target recognition event due to the formation of many bases pairs, our proposed method is expected to achieve amplified signal output compared with the conventional single QD-labeled detection.

Nowadays, there are various papers being reported with the use of CdTe labels for ECL detection of biomolecules [40-44]. According to these papers, the ECL

mechanism is mainly attributed to the emission of the excited QDs (QDs*). In typical QDs-$S_2O_8^{2-}$ system, when a cathodic potential scan is applied to the modified working electrode, the QDs* are formed through the reaction between the electrochemically reduced QDs ($CdTe^{-\bullet}$) and the $SO_4^{-\bullet}$ produced by the reduction of the co-reactant $S_2O_8^{2-}$. In addition, the OOH^- generated from the dissolved oxygen can also participate in assisting the formation of QDs*. The detailed ECL route is expressed in the equations as follows:

$CdTe + e^- \rightarrow CdTe^{-\bullet}$

$S_2O_8^{2-} + e^- \rightarrow SO_4^{-\bullet} + SO_4^{2-}$

$CdTe^{-\bullet} + SO_4^{-\bullet} \rightarrow CdTe^* + SO_4^{2-}$

$O_2 + H_2O + 2e^- \rightarrow OOH^- + OH^-$

$2\ CdTe^{-\bullet} + OOH^- + H_2O \rightarrow 2CdTe^* + 3OH^-$

$CdTe^* \rightarrow CdTe + h\upsilon$

The successful conjugation of MPA-QDs with Dox *via* amido bonds was confirmed by optical methods. The absorption and PL spectra of the MPA-QDs and Dox-QD nanoconjugates are respectively exhibited in Fig. 1. Previous studies have shown that quantum-confined size effect leads to the size-dependent optical properties, and the absorption and emission wavelength usually increases with the increasing particle diameter [36, 45, 46]. It is obvious that both longer wavelength-shift of absorption (curve b *vs.* a) and emission (curve d *vs.* c) spectra were observed, indicating the formation of larger size nanoparticles after modifying the QDs with Dox and the successful preparation of the Dox-QD nanocojugates.

During the fabrication process of the biosensor, electrodes with different modifications were characterized by cyclic voltammetry (CV) in 0.1 M KCl solution containing 1 mM $[Fe(CN)_6]^{3-/4-}$, which is a widely used indicator for surface chemistry. Curve a with a pair of quasi-reversible peaks in Fig. 1A shows the CV wave of the pretreated bare AuE. After self-assembling a mixed monolayer of the DNA CPs and MCH, obvious decreases in current response (curve b) are observed. Because the abundant negative charges of the DNA phosphate backbones repel $[Fe(CN)_6]^{3-/4-}$ from approaching the electrode surface, which confirms the successful formation of the sensing surface. Upon the addition of the target miRNA (400 fM), further decrease in current intensity can be seen from curve c, suggesting the successful hybridization between miRNA and CPs. The feasibility of this biosensor using the Dox-QDs intercalated into base pairs as ECL probes for miRNA analysis was evaluated by ECL measurements. Curve a in Fig. 2B shows the background emission peak on the CPs/MCH modified AuE in the absence of the target miRNA, which is caused by the reaction of $S_2O_8^{2-}$ and the dissolved oxygen in detection buffer. After incubating the sensing surface with the target miRNA (400 fM), a neglect variation in the ECL intensity is observed (curve b *vs.* a). However, subsequent incubation of the modified AuE with Dox-QD nanoconjugates leads to an obvious increase in ECL intensity (curve c *vs.* b) due to the reactions between QDs and coreactant $S_2O_8^{2-}$, suggesting the successful intercalation of the Dox-QDs into the double helix. Such increase in ECL intensity provides evidence for the target miRNA quantitation, and thus confirms the feasibility of our proposed biosensor.

Quantitative performance of our proposed sensor with Dox-QDs as hybridization indicators for miRNA analysis was examined by incubating the sensing surface with different target concentration and recording the corresponding ECL responses. Fig. 3 shows the typical target concentration-dependent ECL curves. It can be clearly seen that the ECL intensity increases accordingly when the concentrations of the target miRNA increases. Inset of Fig. 3 exhibits the calibration plot for the quantitative determination of miRNA. The corresponding ECL response is proportional to the target miRNA concentration ranging from 10 fM to 1.2 pM with an estimated detection limit of 5.5 fM according to three times the average standard deviation of the blank response. Compared with some other common amplified miRNA detection schemes listed in Table 1, this proposed strategy achieves comparable or even better sensitivity. Moreover, our method is also coupled with good reproducibility, which was proved by performing six duplicate measurements of 400 fM target miRNA with a relative standard deviation of 4.4%.

Distinguishing miRNA from its family members remains a great challenge, which plays a significant role in better understanding the biological functions of each miRNA. Herein, we chose two members of the target miR-141-belonged miR-200 family (miR-200b and miR-429) and one irrelevant let-7d as interferences to test the selectivity of this hybridization indicator-based method. As revealed in Fig. 4A, the variation in ECL intensity corresponding to the irrelevant let-7d (700 fM) compared with the blank control experiment is nearly negligible. The ECL responses with the presence of 700 fM miR-429 and miR-200b respectively shows slight increase

compared with the blank test. Only the perfect complementary miR-141 (700 fM) causes remarkable signal increment. The above results suggest the high fidelity of our method in discriminating between the miR-200 family members as well as other interference sequence. Moreover, a series of intensity-constant ECL peaks is observed by performing 7 cycles of CV scans in Fig. 4B, which indicates our method has good stability.

We further investigated the feasibility of this sensor for real samples by testing the total small RNAs extracted from two human cancerous cell lines, 22Rv1 (human prostate carcinoma cells) and Hela (cervical cancer cells). As shown in Fig. 5, the incubation of the sensor with the lysate from Hela cells (10000 cells) caused negligible variations in ECL intensity compared to the blank buffer test (column b *vs.* a), suggesting the inexistence of miR-141 overexpression in Hela cells, which is in accordance with the previous report [52]. However, the incubation of the sensor with the lysates from 22Rv1 cells made the ECL response of the sensor change obviously (column c and d). To be specific, the emission intensity increased with increasing number of the cells. These results shows the overexpression of miR-141 in 22Rv1 cells, and the amount of miRNA-141 found in 22Rv1 cells was calculated to be about 2500 copies per cell according to the ECL intensity values, which is in agreement with reported research findings [53]. Therefore, the good feasibility in real samples analysis provides our proposed strategy great potential for early diagnosis of cancers.

4. Conclusion

In summary, we have successfully synthesized doxorubicin-conjugated QDs nanoparticles as labels in the ECL determination of the sequence-specific miRNA using thiolated DNA CPs modified gold electrode. The ECL emission is generated from the Dox-QDs intercalated into the hybrid duplex structures reacting with $S_2O_8^{2-}$ and the dissolved oxygen in the detection buffer. Compared with the common QDs-based detection, our method avoids chemical labeling of CPs with QDs or the easy leakage of the embedded QDs from the composite film at electrode surface. The simplicity of this two-step (hybridization and intercalation) strategy provides it with a distinct advantage. Moreover, the reported approach herein toward the target miRNA detection is not only coupled with good sensitivity and selectivity, but also can monitor miRNAs from real samples. Thus our proposed method seems to be a promising analysis platform to be applied for a wide range of miRNA or DNA sequences detection.

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Figure captions

Scheme 1. Illustration for the principle of the developed ECL sensor for detecting miRNA based on the Dox-QDs nanoconjugates as the new signal acquisition and amplification platform.

Fig. 1. UV-Vis spectra of the MPA-QDs (a) and Dox-QDs (b), and PL (λ_{ex} = 450 nm) spectra of the MPA-QDs (c) and Dox-QDs (d).

Fig. 2. (A) Typical cyclic voltammograms of (a) bare AuE, (b) MCH/CPs/AuE and (c) miRNA/MCH/CPs/AuE. CV measurements were performed in 0.1 M KCl containing 1 mM $[Fe(CN)_6]^{3-/4-}$ by scanning the potential from -0.1 V to 0.6 V at a scan rate of 50 mV s^{-1}. (B) ECL curves of (a) MCH/CPs/AuE, (b) miRNA/MCH/CPs/AuE, (c) b incubated with Dox-QDs. ECL curves were recorded in 0.1 M PBS containing 0.1 M $K_2S_2O_8$ and 0.1 M KCl (pH 7.4) from 0 to -1.5 V.

Fig. 3. ECL responses of the sensor incubating with different concentration of target miRNA (a) 0 fM, (b) 10 fM, (c) 80 fM, (d) 200 fM, (e) 400 fM, (f) 700 fM, (g) 900 fM, (h) 1.2 pM. Inset:The resulting calibration plot of c_{miRNA}*vs.* ECL intensity (error bars = SD, n = 3). ECL measurement conditions, as in Fig. 2B.

Fig. 4. (A) Selectivity investigation of the Dox-QDs-based sensor for the target miR-141 against other interference sequences (let-7d, miR-429, miR-200b) with the same concentration 700 fM. (B) ECL emission-time curves corresponding to 400 fM target. ECL measurement conditions are as in Fig. 2B.

Fig. 5. Detection of miR-141 from different cancer cell lysates: (a) blank, (b) Hela (10000 cells), (c) 22Rv1 (100 cells) (d) 22Rv1 (1000 cells).

Table 1. Comparison of the proposed assay with some reported biosensors for miRNA detection

Scheme 1

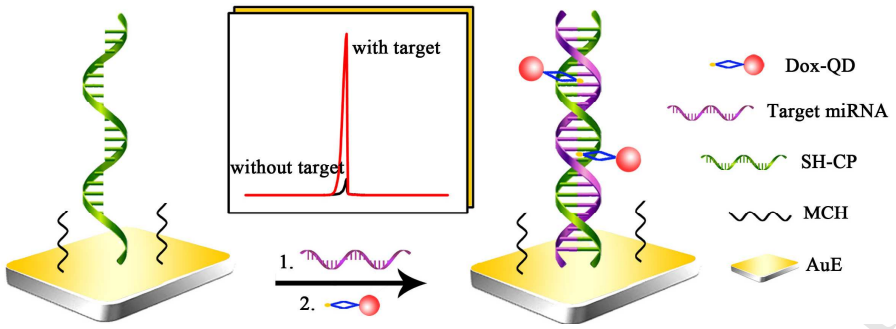

Fig. 1

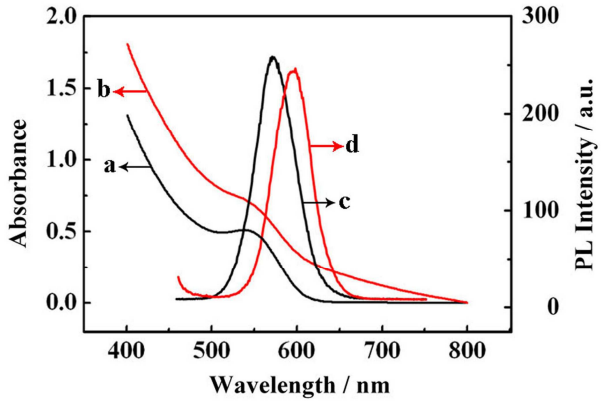

Fig. 2

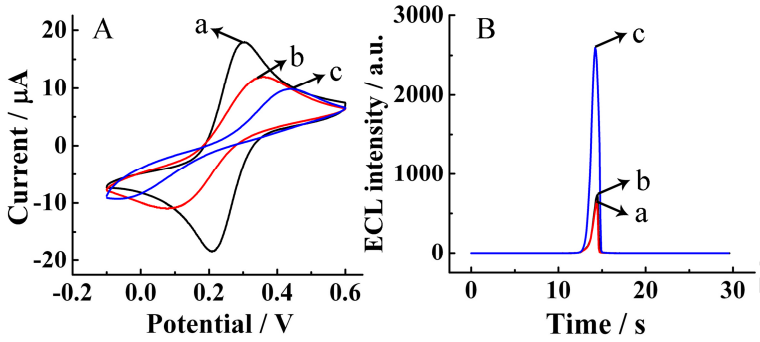

Fig. 3

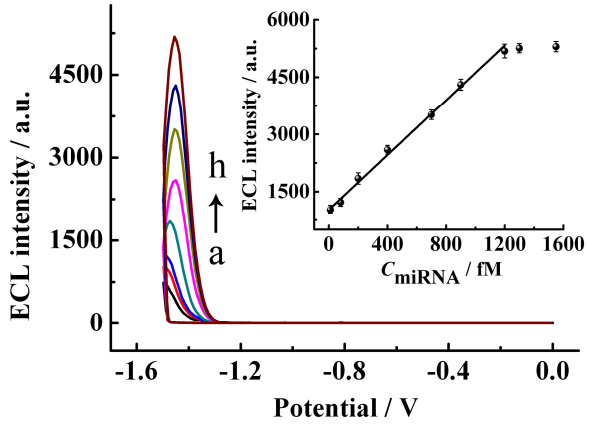

Fig. 4

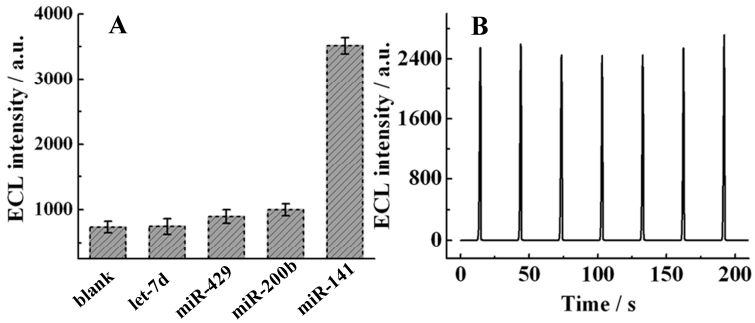

Fig. 5

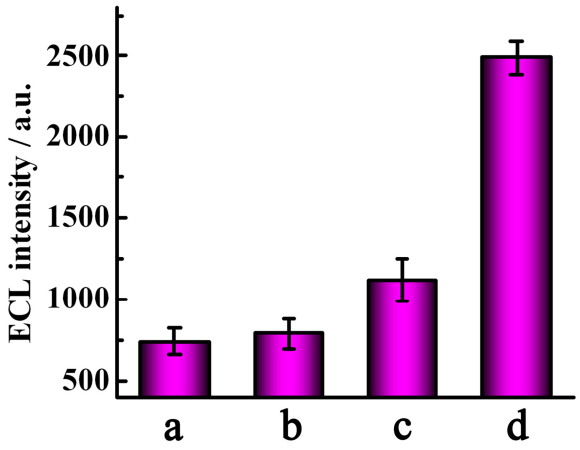

Table 1

ref	Detection method	DL	Signal amplification strategy
47	DPV	5.36 fM	isothermal amplification reaction
48	Amperometric measurements	80 fM	electrocatalytic nanoparticle tags
49	EIS	3 fM	RuO_2 NPs-initiated deposition of an insulating film
50	Colorimetric detection	10 nM	conformational changes of cationic polythiophene derivative
51	Fluorescence	180 fM	bifunctional strand displacement amplification-mediated hyperbranched rolling circle amplification
this work	ECL	5.5 fM	Dox-QDs intercalated into DNA/RNA hybrids

Highlights:

1. A new class of signal amplification tag (doxorubicin-QDs nanoparticles) is employed to simple detect MicroRNA.

2. The signal acquisition way by QDs intercalation into the RNA/DNA hybrids can overcome the drawbacks of labeling and embedding.

3. The method shows high selectivity toward the target microRNA and can be applied for real sample analysis.