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Universal Ratiometric Photoelectrochemical Bioassay with Target-Nucleotide Transduction-Amplification and Electron-Transfer Tunneling Distance Regulation Strategies for Ultrasensitive Determination of microRNA in Cells

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ABSTRACT: A universal ratiometric photoelectrochemical (PEC) bioassay, which could be readily expanded for untrasensitive determination of various targets in complex biological matrix, was established by coupling a target-nucleotide transduction-amplification with DNA nanomachine mediated electron-transfer tunneling distance regulation strategies. With the help of target-nucleotide transduction-amplification strategy, the one input target signal could be transducted to corresponding multiple output DNA signals by nucleotide specific recognition technology, simultaneously leading to an efficient signal amplification for target. Then the output DNA could initiate the formation of four-way junction DNA nanomachine through binding-induced combination, by which the electron-transfer tunneling distance between photoactive materials and sensing interface could be regulated, simultaneously resulting an enhanced photocurrent signal from SiO_2@methylene blue (SiO_2@MB) as wavelength-selective photoactive material in close proximity to sensing interface and a reduced photocurrent signal from another wavelength-selective photoactive material CdS quantum dots (CdS QDs) away from sensing interface for photocurrent signal ratio calculation. Using microRNA-141 (miRNA-141) as target model, the constructed biosensor demonstrated favorable accuracy and excellent sensitivity down to femtomolar level. Impressively, the proposed assay overcame the heavily dependence of target on photoactive materials in current ratiometric PEC assay, and demonstrated admirably universal applicability for determination of various targets such as metal ions, miRNAs, DNAs and proteins by merely two different photoactive materials (SiO_2@MB and CdS QDs), paving the way to applicate universal ratiometric PEC assay in environment test, clinical diagnosis and other related subjects.

KEYWORDS: photoelectrochemical, ratiometric, universal, signal transduction, DNA nanomachine

INTRODUCTION

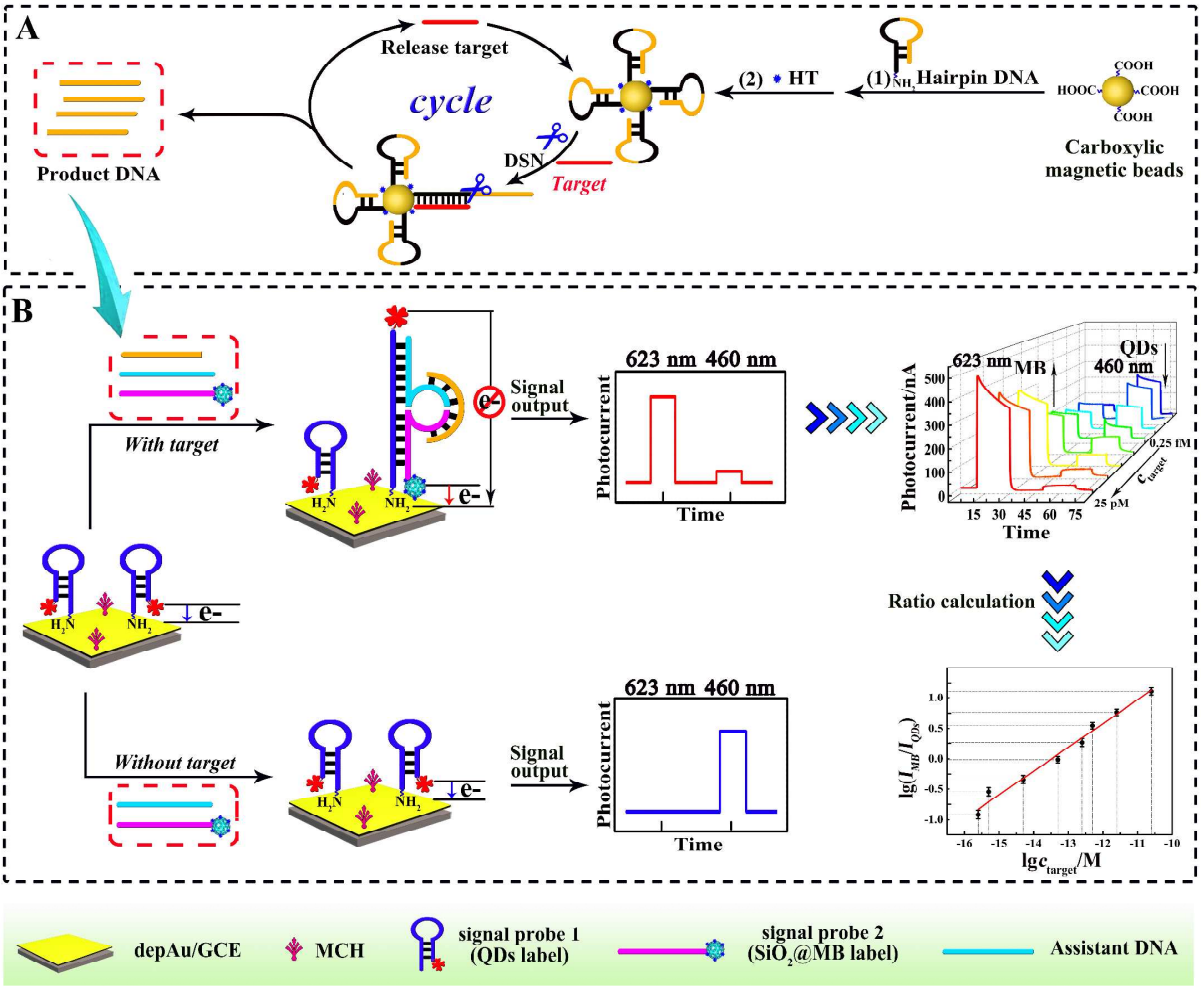
Ultrasensitive analysis in complex biological matrix with low background and good anti-interference ability benefits from ratiometric techniques, in which a dual-signal approach was used to reduce false-positive or false-negative signals.[1-2] Currently, numerous great efforts have been made to develop the ratiomatric technique in fluorescence, Raman, electrochemistry and electrochemiluminescence fields to construct highly precise and ultrasensitive analytical methods.[3-5] Regrettably, until now, the ratiometric techniques have been rarely reported in photoelectrochemical (PEC) assay, which as an actively developing technique has attracted extensively research interests due to its inherent advantages, such as low background, high sensitivity, high accuracy and low cost.[6-9] The main reason could be attributed to the difficulty in searching for a pair of wavelength-selective photoactive materials to serve as different signal labels, in which one signal of photoactive material could be quenched and that of another photoactive material must be simultaneously enhanced by target. Fortunately, recently, Ju's group has successfully searched out a couple of photoactive materials, CdS quantum dots (QDs) and methylene blue (MB), whose photocurrent signals could be respectively quenched and enhanced by Cu^{2+}, to construct ratiometric PEC assay for detection of Cu^{2+}.[10] However, for most of the targets in nature such as microRNAs (miRNAs), DNAs, proteins and other metal ions, it remains an enormous challenge to find corresponding a pair of photoactive materials to realize ratiometric PEC assay. Therefore, it is highly meaningful but full of challenge to conquer the

heavily dependence of the target to photoactive materials for construction of universal ratiometric PEC assay to detect various kinds of the targets.

To address the challenge for construction of universal ratiometric PEC assay, the first key factor is to search for a new signal response pattern to target by electron-transfer tunneling distance regulation strategy, in which two different photoactive materials merely acted as signal enhanced and reduced labels respectively rather than materials which signals were enhanced and quenched by target respectively. Recently developed electron-transfer tunneling distance regulation strategies with controllability for the signal intensity offer new possible solution to construct the aforementioned signal response pattern for analytical detection in both biomedical and environment researches.[11-15] Despite the continued interest and effort put forward constructing this type of regulation strategy in various related subjects, however, great progresses in improving the stability and accuracy of detection based on this strategy have been hindered by the poor rigidity of the formed double-stranded DNA nanostructures. Therefore, it is an urgent affair to find a structurally stable but not overcomplicated nanostructure to construct electron-transfer tunneling distance regulation strategy. Extensive research suggests that the four-way DNA nanostructure has striking enhancement of rigidity compared to the double-stranded DNA nanstructure.[16-19] Inspiring by these researches, we designed a new type of four-way junction DNA nanomachine mediated electron-transfer tunneling distance regulation strategy to construct universal ratiometric PEC assay, which demonstrated remarkable enhancement of rigidity over the currently general regulation strategy, leading to enhanced accuracy and stability for PEC assay.

Herein, a universal ratiometric PEC assay was proposed for the first time for ultrasensitive and highly accurate determination of analyte in complex biological matrix by coupling the target-nucleotide transduction-amplification with binding-induced four-way junction DNA nanomachine mediated electron-transfer tunneling distance regulation strategies. As shown in Scheme 1, miRNA-141 was employed as target model to investigate the performance of our design. For the purpose of target-nucleotide transduction-amplification strategy, a DSN enzyme-assisted target recycling was introduced by cleaving the hairpin DNA upon binding with target to reuse the target and release product DNA from hairpin DNA. The duplex specific nuclease (DSN) enzyme was a kind of enzyme with strong preference for cleaving double-stranded DNA and DNA in DNA-RNA hybrid duplexes, and show minor activity for single-stranded DNA or single or double-stranded RNA.[20] In absence of target, an initial photocurrent signal (I_{QDs}) at wavelength of 460 nm was produced by CdS QDs labeled hairpin DNA (signal probe 1) in close proximity to sensing interface. In the existence of target, the binding-induced four-way junction DNA nanomachine could be formed by opening the signal probe 1 with the help of SiO_2@MB labeled DNA (signal probe 2), assistant DNA and product DNA, which served as a bridge DNA for combination of the assistant DNA with signal probe 2, simultaneously resulting CdS QDs away from sensing interface with reduced CdS QDs photocurrent signal (I'_{QDs}) at wavelength of 460 nm and SiO_2@MB in close proximity to sensing interface with enhanced SiO_2@MB photocurrent signal (I_{MB}) at wavelength of 623 nm. The ratiometric PEC assay strategy could be realized by monitoring the ratios of these two different PEC signals (I_{MB}/I'_{QDs}). Although the concept of the universal ratiometric PEC assay has only been demonstrated for determination of miRNA, it could also be readily expanded for determination of other targets such as cells,

proteins and metal ions, paving the way to applicate ratiometric PEC assay in environment test, clinical diagnosis and other related subjects.



Scheme 1. Schematic Diagram of (A) Signal Transduction-Amplification Strategy; (B) Fabrication Procedure for the Ratiometric PEC assay.

EXPERIMENTAL METHODS

Chemicals and Materials. Polyamidoamine dendrimer with arboxyl terminated (PAMAM, fifth generation), magnetic beads (MBD), 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC), n-hydroxysulfosuccinimide (NHS), 1-hexanol, ethylsilicate (TEOS), triton

X-100, cyclohexane, 3-aminopropyltriethoxysilane (APTMS), cadmium chloride ($CdCl_2$), sodium sulfide (Na_2S), 3-mercaptopropionic acid (MPA), methylene blue (MB), gold chloride ($HAuCl_4$), 6-mercaptohexanol (MCH), ascorbic acid (AA) and ethidium bromide were obtained from Sigma Chemical Co. (St. Louis, MO., USA). DSN enzyme and 10×DSN master buffer were obtained from Axxora, LLC (San Diego, CA., USA). The phosphate buffer solution (PBS, pH 7.0, 0.1 M) and tris-EDTA buffer (TE buffer, pH 8.0) were purchased from Beijing Dingguo Changsheng Biotechnology Co. Ltd. (Beijing, China). Ferricyanide/ferrocyanide mixed solution ($[Fe(CN)_6]^{3-/4-}$, 5.0 mM) was prepared by dissolving potassium ferricyanide and potassium ferrocyanide by PBS solution.

The miRNA and DNA oligonucleotides in the experiment were synthesized by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China). The corresponding sequences were listed in the following table:

Table 1. The sequences used in the experiment.

Name	Sequence (5'→3')
miRNA-141	UAA CAC UGU CUG GUA AAG AUG G
miRNA-155	UUA AUG CUA AUC GUG AUA GGG GU
miRNA-21	UAG CUU AUC AGA CUG AUG UUG A
miRNA-199a	ACA GUA GUC UGC ACA UUG GUU A
Let-7a	UGA GGU AGU AGG UUG UAU AGU U
Hairpin DNA	TAT TAT GCC TAT GAA AAT GCG GCG CCA TCT TTA CCA GAC AGT GTT A-NH_2
Assistant DNA	TTT AAA GTA TGG TTA GAT GAC GCC GCA TTT TC
DNA for signal probe 1 (S1)	SH-TTT TTT TCT AGA GCC ATA CTT TTT TTT TTT CTA GTT TTT-NH_2
DNA for signal probe 2 (S2)	ATA GGC ATA ATA TAT CAT CAG GGC TCT AGA AAT TT-NH_2

Apparatus. The PEC measurement was recorded on an Iviumstat PEC workstation from Ivium (Netherlands). A three-electrode system was used through the experiment according to our reported work.[21] Cyclic voltammetry (CV), electrochemical impedance spectroscopy (EIS) and electrochemical deposition were performed on a CHI 660e electrochemical workstation (Shanghai Chenhua Instrument, China). The morphologies of the synthesized nanomaterials were characterized using a scanning electron microscopy (SEM, S-4800, Hitachi, Japan) and a high-resolution transmission electron microscopy (HRTEM, JEM-2100, JEOL, Japan). The fluorescence characterizations were obtained from a fluorescence spectrophotometer (F-7000, Hitachi, Japan). The laser confocal microscopy measurement was performed on a laser confocal microscope (F3000, Olympus, Tokyo, Japan).

Gel Electrophoresis. The polyacrylamide gel electrophoresis (PAGE) was used for characterizing the enzymes-assisted target quadratic recycling strategy in our experiment. Briefly, 10 μL different DNA samples were mixed with 2 μL loading buffer solution, followed by injecting into the grooves of freshly prepared polyacrylamide gel (16%) system or agarose gel electrophoresis system (10%). The electrophoresis was performed in 1×TBE buffer at 120 V constant voltage. Then the gels were carefully washed with ultrapure water and stained by ethidium bromide for 30 min. Finally, the image of the gel was taken using a digital camera (EOS 550D, Canon, Japan) under UV light illumination.

Preparation for the CdS QDs and Signal Probe 1. The CdS QDs was prepared according to the literature firstly.[10] For preparation of the signal probe 1, briefly, 200 μL CdS QDs was dissolved in 900 μL PBS solution. Then, 20 μL EDC solution and 20 μL NHS solution were dropped to the mixture and stirred for 20 min to activate the carboxyl group on the CdS QDs.

Subsequently, 20 μL S1 (100 μM) was added to the mixture and stirred for 3 h. The obtained solution was centrifuged, washed and redispersed in 1 mL PBS (pH 7.4) solution for further use.

Preparation for the SiO_2@MB Nanoparticles, Signal Probe 2, and MBD-Hairpin DNA Bionanoconjugates. It has been found that the photocurrents from single or several MB were quite small that were not suitable for a PEC analytical experiment. Therefore, it was highly needed to search for a method to immobilize large amount of MB with high stability and efficiency for the PEC assay. Besides, the immobilization method must have no influence for the wavelength response of MB. Researches suggests that silica nanoparticle was an excellent nanocarrier for immobilization of a large amount of small molecular materials (e.g. $Ru(dcbpy)_3^{2+}$) by doping the materials in the silica formed nanoparticles.[22-25] Besides, the operations were quite simple and convenient. Therefore, on the basis of these significant studies, in this work we adopted SiO_2 as nanocarries for photoactive materials MB to largely improve the loading amount of MB for a significantly enhanced photocurrent response. For preparation of the SiO_2@MB nanoparticles, 7.5 mL cyolohexane, 1.8 mL 1-hexanol, 1.7 mL triton X-100, 370 μL ultrapure water and 500 μL MB solution were mixed with stirring for 30 min. Then, 50 μL TEOS and 10 μL APTMS were added to the mixture followed with gentle stirring for 30 min. Subsequently, 100 μL 25 % ammonium hydroxide was dropped to the mixture solution and stirred for 48 h to obtain a homogeneous blue solution. The obtained solution was centrifuged and washed by ethanol and water alternately for several times to remove the redundant reagents. Finally, the product was redispersed in 5 mL ultrapure water and stored in 4 °C.

For preparation of the signal probe 2, firstly, 300 μL SiO_2@MB solution and 20 μL S2 (100 μM) were added to 900 μL PBS (pH 7.4) solution. Then, 20 μL glutaraldehyde (5%) was added

to the mixture solution and gently stirred for 1 h at 4 °C. The obtained solution was centrifuged and washed to remove the redundant reagents. Finally, the product was redispersed in 1 mL PBS (pH 7.4) solution and stored in 4 °C for further use.

For preparation of the MBD-hairpin DNA bionanoconjugates, firstly, 40 μL MBD was dissolved in 900 μL PBS solution. Next, 20 μL EDC solution and 20 μL NHS solution were dropped to the mixture and stirred for 20 min to activate the carboxyl group on the MBD. Then, 20 μL hairpin DNA (100 μM) was added to the mixture followed with gentle stirring for 3 h. Under magnetic separation, the obtained product was washed and redispersed in 1 mL PBS (pH 7.4) solution.

Signal Transduction and Amplification for miRNA Detection. The DSN enzyme-assisted target recycling strategy was performed by mixing the MBD-hairpin DNA solution with target, 1 U DSN enzyme and 1×DSN master buffer, followed by incubating at 65 °C for 40 min. Then the DSN enzyme in the mixture solution was denatured by adding 5 μL DSN stop solution, followed by incubating for 10 min. After that, the mixture solution was separated by magnet to obtain the clean liquid for further immobilization on the modified electrode surface.

Fabrication Procedure for the PEC Biosensing Interface. Before modification, the glassy carbon electrode (GCE, Φ = 4 mm) was pretreated according to our previously reported literature to obtain a mirror-like surface.[21] Subsequently, the electrochemical deposition was performed by immersing the electrode in 1% $HAuCl_4$ solution under -0.2 V constant potential for 30 s to modify gold nanoparticles (depAu) on the bare electrode surface. Afterwards, the obtained electrodes were washed carefully with double distilled water to remove the abundant regents. Then the modified electrodes were dried in air. Next, 20 μL signal probe 1 was incubated on the

electrode surface at 4 °C for 12 h by the Au-S covalent bond. Finally, 20 μL MCH solution was dropped on the modified electrode surface for 40 min to eliminate the non-specific binding sites on the sensing interface. The redundant reagents was removed by washing the electrode with ultrapure water after each step.

PEC Measurement. Before measurement, the modified electrode was immersed in the solution containing signal probe 2, assistant DNA 2 and the obtained solution containing product DNA from DSN enzyme-assisted target recycling strategy, at 37 °C for 1 h to form the binding-induced four-way junction DNA nanomachine on the sensing interface. Then the PEC measurement was performed by immersing the electrode in 6 mL PBS solution (0.1 M, pH 7.0) containing 0.1 M AA with a light-emitting diode (LED) light source switched from 623 nm (off-on-off) to 460 nm (off-on-off).

RESULTS AND DISCUSSION

Morphology and Photophysical Characterizations of the Synthesized Nanomaterials. The morphologies of the synthesized SiO_2@MB and CdS QDs were characterized by SEM and HRTEM, respectively. The SiO_2@MB showed a well-defined globular structures at around 50 nm (Figure 1A), indicating the successful synthesis of SiO_2@MB. Figure 1B showed the HRTEM image of CdS QDs, which displayed a homogeneous distribution of around 5 nm in diameter. Furthermore, the water solubility and photophysical properties of these nanomaterials were characterized under an ambient daylight (Figure 1C) and an ultraviolet light (D), respectively. As shown in Figure 1C and Figure 1D, the SiO_2@MB solution (bottle 1) showed a pale blue color under ambient daylight, while a pale green color under ultraviolet light. The CdS QDs solution (bottle 2) showed a golden yellow color under ambient daylight, while an orange

color under ultraviolet light. Importantly, the results in Figure 1C and Figure 1D indicated the synthesized nanomaterials possessed a well dispersity and fluorescence property.

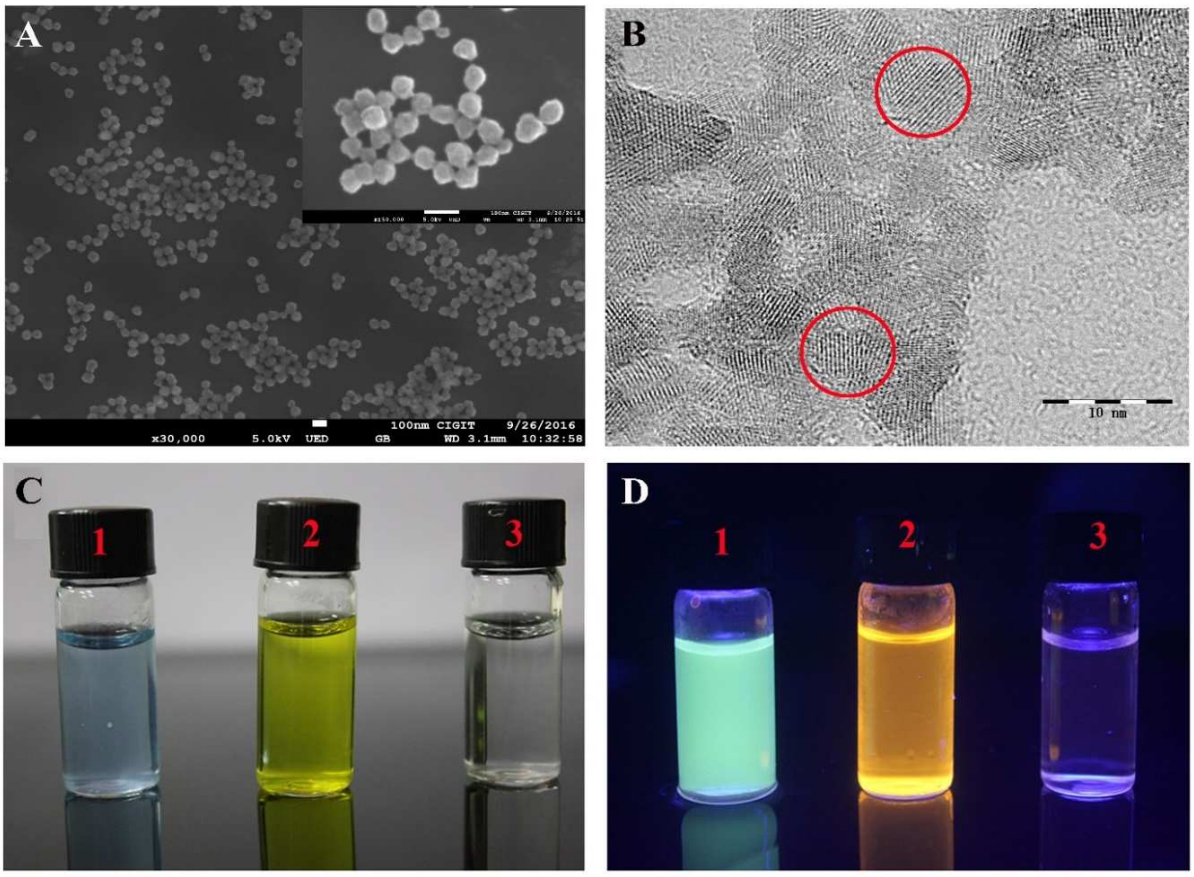


Figure 1. The SEM images of SiO_2@MB (A) and HRTEM image of CdS QDs (B). Aqueous solution of SiO_2@MB (bottle 1), CdS QDs (bottle 2) and ultrapure water (bottle 3) under an ambient daylight (C) and ultraviolet light (D). The insert image of A shows a partically enlarged SEM image of SiO_2@MB.

Analytical Performance of the Ratiometric PEC Assay. To investigate the analytical performance of the binding-induced universal ratiometric PEC assay, the established biosensor was explored to monitor miRNA-141 with different concentrations. In the presence of target miRNA-141, the signal probe 1 could be opened and then formed a four-way junction DNA

nanomachine with the help of signal probe 2, product DNA and assistant DNA 2. Then a decreased photocurrent signal of signal probe 1 (I_{QDs}) at wavelength of 460 nm and an increased photocurrent signal of signal probe 2 (I_{MB}) at wavelength of 623 nm could be obtained due to the formation of four-way junction DNA nanomachine on sensing interface. The photocurrent ratio of I_{MB}/I_{QDs} was used to detect miRNA-141. As shown in Figure 2(A), the photocurrent signal of signal probe 1 at 623 nm decreased and the photocurrent signal of signal probe 2 at 460 nm increased with increasing the concentration of miRNA-141, respectively. Figure 2(B) showed the corresponding linear relationship between photocurrents of signal probe 1, signal probe 2 and the concentration of miRNA-141 (from 25 pM to 0.25 fM). In Figure 2(C), a good linear relationship was presented between the photocurrent ratio of I_{MB}/I_{QDs} and the concentration of miRNA-141 with detection limit of 83.3 aM. As shown in Table 2, the proposed ratiometric PEC assay showed excellent performance compared with other reported methods, which could be contributed to the target-nucleotide transduction-amplification strategy and DNA nanomachine mediated electron-transfer tunneling distance regulation strategy.

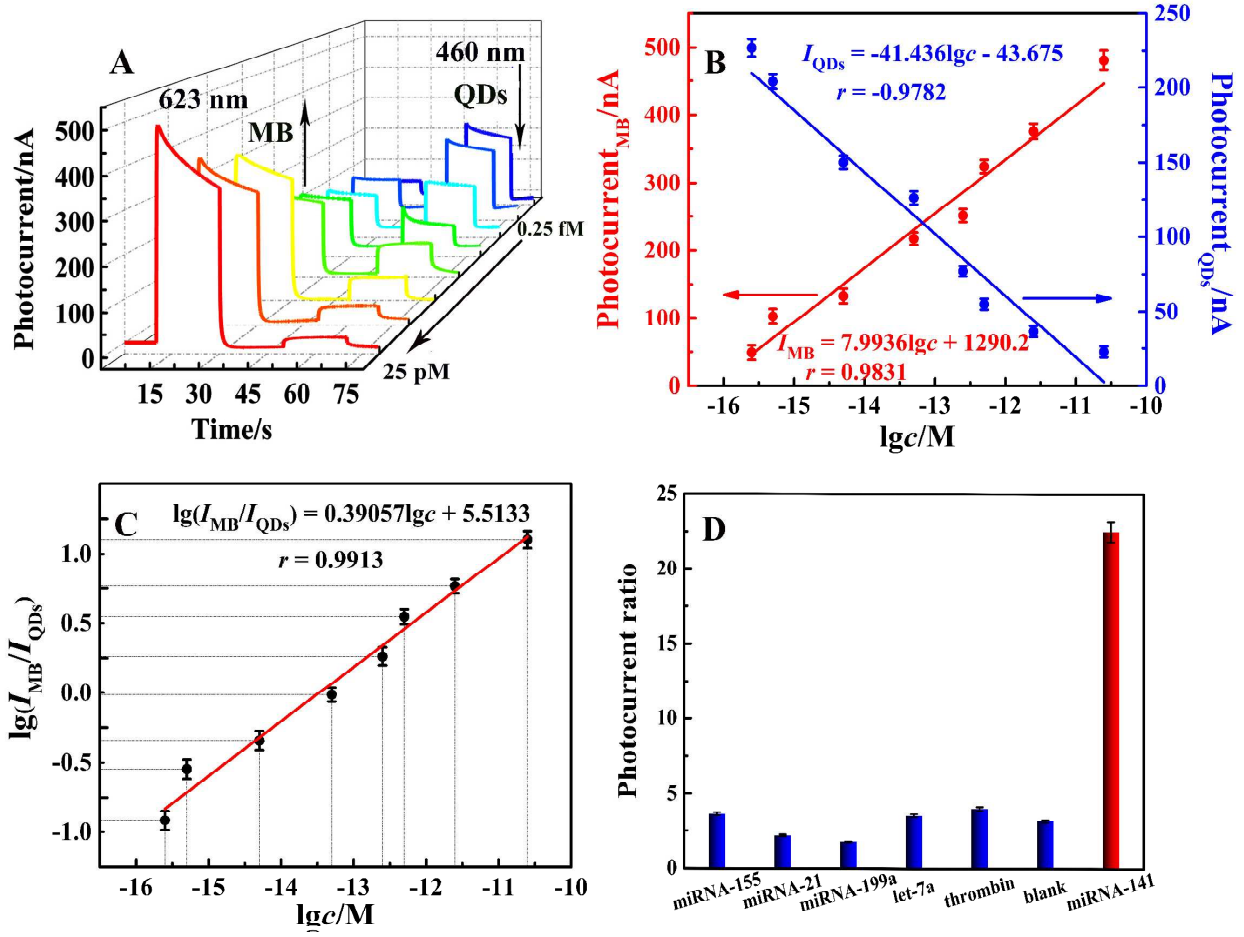


Figure 2. (A) Photocurrent response of the biosensor with different concentrations of miRNA-141 (0.25 fM, 0.50 fM, 5 fM, 50 fM, 0.25 pM, 0.50 pM, 2.5 pM, 25 pM) in 0.1 M PBS (pH 7.0) containing 0.1 M AA. (B) The linear relationship between photocurrent responses of signal probe 1 (QDs), signal probe 2 (MB) and the concentration of miRNA-141. (C) The linear relationship between photocurrent responses ratio and the concentration of miRNA-141. (D) The selectivity of the proposed PEC bioassay: miRNA-155 (2 nM), miRNA-21 (2 nM), miRNA-199a (2 nM), let-7a (2 nM), thrombin (2 nM), blank and miRNA-141 (25 pM).

Table 2. Comparison of Our Ratiometric PEC Bioassay with Other Detection Methods.

Analytical method	Target	Detection strategy	Linear range	Detection limit	Ref.
ECL	DNA	ratiometric	5.0 fM~1.0 pM	1.7 fM	26
ECL	miRNA	ratiometric	1.0 fM~1.0 nM	0.5 fM	27
DPV	DNA	ratiometric	0.01 pM~10 nM	32 fM	28
Fluorescence	miRNA	---	100 fM~100 nM	100 fM	29
EIS	miRNA	---	2.0 fM~2.0 pM	1.0 fM	30
PEC	miRNA	---	350 fM~5 nM	153 fM	31
PEC	miRNA	ratiometric	0.25 fM ~25 pM	83.3 aM	***Our work***

Abbreviation: electrochemiluminescence (ECL); differential-pulse voltammetry electrochemical (DPV); electrochemical impedance spectroscopic (EIS).

Selectivity of the PEC Bioassay. To investigate the selectivity of the proposed PEC assay, a contrast experiment was performed by incubating the biosensor with interferences including 2 nM miRNA-155, miRNA-21, miRNA-199a, let-7a and thrombin, respectively. As shown in Figure 2D, compared to the PEC signal ratio obtained from the miRNA-141 (25 pM), there was no obvious PEC signal ratio for the interferences, indicating good selectivity of the proposed strategy.

The Applicability of the Bioassay for miRNA Determination in Cancer Cells. Since miRNA performed close relationship to the occurrence of various cancers, monitoring special miRNA in cancer cells has become an effective tool for diagnosis, classification, progression and treatment of cancers. To investigate the feasibility of the proposed ratiometric PEC bioassay for ultrasensitive and accuracy determination of miRNA in complex biological matrix, five different cell lysate samples from cancer cell lines including MB231, 22Rv1, HeLa, A549 and MCF-7 has been tested using the proposed strategy. The light field microscopy (LF), fluorescence

microscopy (FL) and their merge images of the 22Rv1 cancer cells and MB231 cancer cells with high concentrations and low concentrations has been demonstrated in Figure 3A. Total RNA extraction samples derived from different numbers of 22Rv1 (Figure 3B) and MB231 cells (Figure 3C) (from 10 to 5000) has led an increased photocurrent ratio of the proposed method. The linear relationships can be obtained with good linear equation of lg (I_{MB}/I_{QDs}) = 0.45331 + 0.17164 lg N_{cell} (Figure 3B) and lg (I_{MB}/I_{QDs}) = 0.43471 + 0.24089lg N_{cell} (Figure 3C), where N_{cell} was the cell numbers. In Figure 3D, since the miRNA-141 was high expressed in MB231 and 22Rv1 cells and low expressed in A549, MCF-7 and HeLa cells, an unapparent photocurrent ratio response of the A549, MCF-7 and HeLa cells could be observed, which was fitted well with reported literatures.[32-34] The results indicated that the proposed ratiometric PEC bioassay possessed great potential for practical determination of miRNA in cancer cells with ultrasensitive and accuracy.

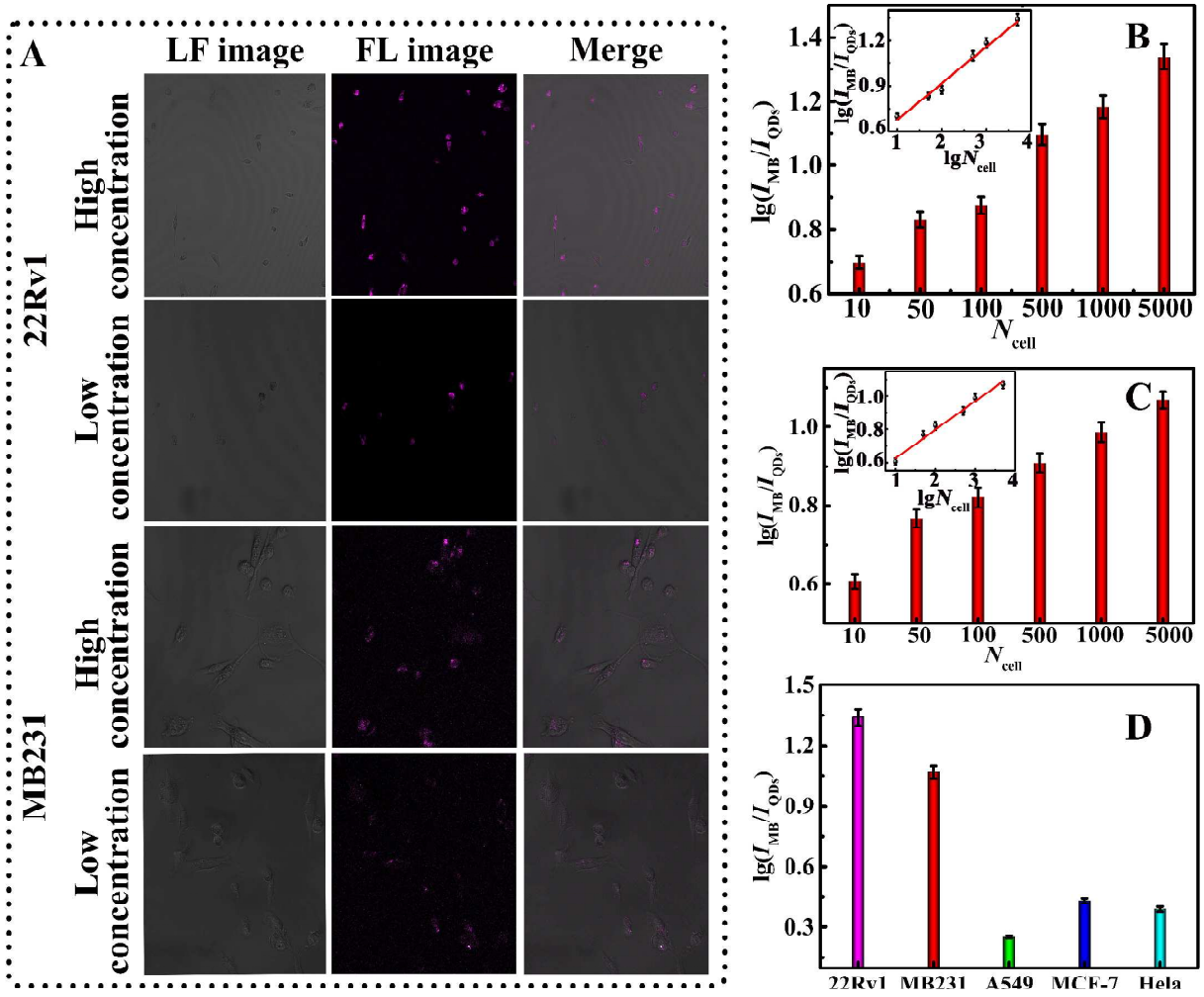


Figure 3. (A) The LF, FL and merge images of the 22RV1 cancer cells and MB231 cancer cells with different concentrations. Photocurrent responses and linear relationship (the inset images) of the PEC biosensor incubated with total RNA solutions derived from 22Rv1 cancer cells (B) and MB231 cancer cells (C) with different numbers. (D) Photocurrent responses of the PEC bioassay incubated with RNA sollutions from different cancer cell lysate samples including 22Rvl, MB231, A549, MCF-7 and HeLa cancer cells.

CONCLUSION

In summary, a universal ratiometric PEC bioassay was established based on target-nucleotide transduction-amplification and electron-transfer tunneling distance regulation strategies for untrasensitive determination of target in cells. Our proposed assay overcame the high dependence of target on photoactive materials in current ratiometric PEC bioassay and showed admirable universal applicability, by which various targets such as metal ions, miRNAs, DNAs and proteins could be easily detected by merely two different photoactive materials. As a result, the proposed ratiometric PEC estimation technique demonstrated significantly enhanced selectivity, ultrasensitivity and high accuracy for determination of target in complicated biological matrix, especially in cancer cells. The success in the establishment of the proposed universal ratiometric PEC bioassay provided an efficient strategy for determination of vast majority of targets with ultratrace level and high accuracy, paving the way to applicate ratiometric PEC assay in environment test, clinical diagnosis and other related subjects.

ASSOCIATED CONTENT

Supporting Information.

Experimental details for the preparation of DNA bioconjugates and related nanomaterials, characterization of the synthesized materials, nucleotide amplification strategy and fabrication of biosensor, supplementary figures and tables.

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Notes

The authors declare no competing financial interest.

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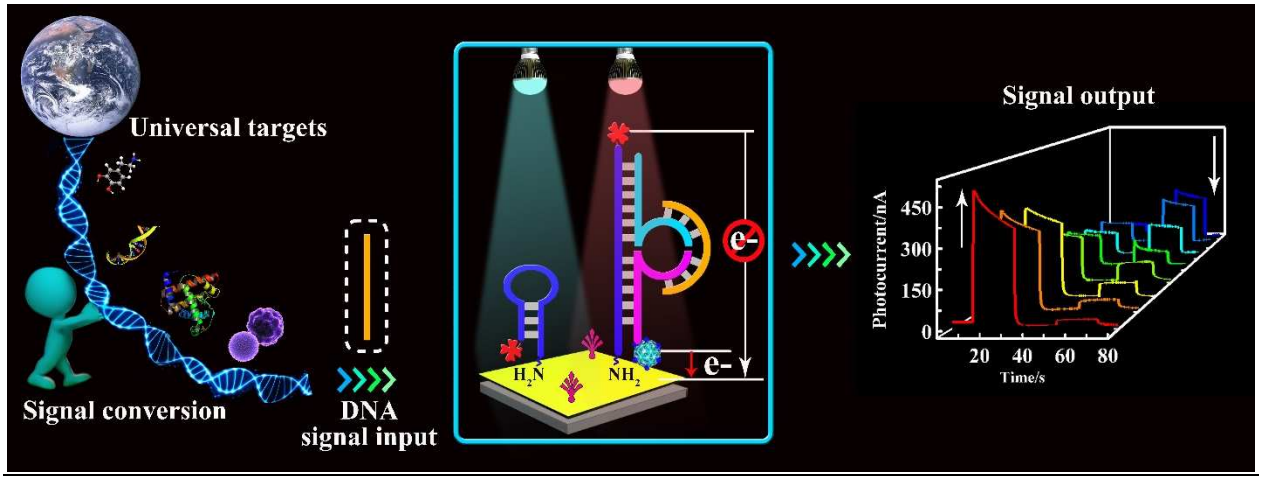
Universal targets
Signal conversion
DNA
signal input
e-
e-
H_2N
NH_2
Signal output
Photocurrent/nA
450
300
150
0
20
40
60
80
Time/s