



Electrochemical detection of zeptomolar miRNA using an RNA-triggered Cu^{2+} reduction method

Hyo Yong Kim^{a,b,1}, Jayeon Song^{a,c,1}, Hyun Gyu Park^{a,*}, Taejoon Kang^{c,*}

^a Department of Chemical and Biomolecular Engineering (BK21+ program), Korea Advanced Institute of Science and Technology (KAIST), 291 Daehak-ro, Yuseong-gu, Daejeon, 34141, Republic of Korea

^b California NanoSystems Institute, Crump Institute for Molecular Imaging, Department of Molecular and Medical Pharmacology, David Geffen School of Medicine at UCLA, Los Angeles, CA, 90095, United States

^c Bionanotechnology Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), 125 Gwahak-ro, Yuseong-gu, Daejeon 34141, Republic of Korea

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ABSTRACT

The development of ultrasensitive, selective, simple, and rapid microRNA (miRNA) detection strategies has been crucial because of their use as probable biomarkers for diagnosing human diseases. We herein described a label- and wash-free electrochemical miRNA detection method with zeptomolar sensitivity, which relies on target miRNA-induced reduction of Cu^{2+} and consequential changes in electrochemical signals generated from the remaining Cu^{2+} . Target miRNA was successfully identified with a detection limit of 33.2 zM based on this simple principle. The synergistic combination of miRNA recycling and Cu^{2+} reduction reactions contributed to this ultrasensitivity. Moreover, the developed electrochemical sensing method exhibited label- and wash-free detection of miRNA, showing potential applicability as a point-of-care testing system. Furthermore, the practical application of the designed technique was demonstrated by reliably detecting the target miRNA in the total RNA samples extracted from various cancer cell lines. We also believe that the conceived approach could be widely used to detect not only miRNAs but also diverse biomolecules by simply replacing the detection probe.

1. Introduction

A microRNA (miRNA), which is a single-stranded non-coding RNA consisting of ~22 nucleotides, has a regulatory effect on gene expression and plays a pivotal role in various physiological processes, such as proliferation, differentiation, and apoptosis [1–5]. Notably, the miRNA expression level can be altered in cancer cells, leading to the utility of the miRNA as a promising biomarker for cancer diagnosis [6–10]. For example, high expressions of miR-141 and miR-21 can be indicative of prostate and breast cancers, respectively [11,12], and down-regulation of Let-7f has been observed in lung and ovarian cancer cells [13–15]. Besides these examples, there have been plenty of findings regarding the aberrant expression of miRNAs in different types of cancers (Table S1). As shown in this summary, a variety of miRNAs are closely associated

with diverse cancer types and thus of significant importance as promising biomarkers for cancer prognosis/diagnosis.

Conventionally, miRNAs have been detected using techniques such as Northern blotting, microarray technology, and reverse transcription-polymerase chain reaction (RT-PCR) [16–18]. Although these methods exhibit distinct merits for miRNA detection, such as high sensitivity and specificity, simple miRNA detection is still difficult, especially in facility-limited environments, owing to several drawbacks, including tedious procedure (Northern blotting), low reproducibility (microarray technology), and requirement for a bulky and expensive instrument (RT-PCR) [19–21]. With the aim of finding alternative approaches, there have been several reports on the development of sensitive, specific, and even facile methods for the determination of target miRNA by employing the combinatorial activities of two enzymes, duplex-specific

Abbreviations: miRNA, microRNA; RT-PCR, reverse transcription-polymerase chain reaction; DSN, duplex-specific nuclease; TdT, terminal deoxynucleotidyl transferase; poly T, poly thymine; Cu NP, copper nanoparticle; POC, point-of-care; DP, detection probe; dTTPs, deoxythymidine triphosphates; MOPS, 3-(N-morpholino)propanesulfonic acid; dTTP, deoxythymidine triphosphate; DMEM, Dulbecco's modified Eagle's medium; FBS, Fetalgo Bovine Growth Serum; DW, distilled water; PAGE, polyacrylamide gel electrophoresis; CV, cyclic voltammetry; LOD, limit of detection; RSD, relative standard deviation.

* Corresponding authors.

E-mail addresses: hgpark@kaist.ac.kr (H.G. Park), kangtaejoon@kribb.re.kr (T. Kang).

¹ These authors contributed equally to this work.

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nuclease (DSN) and terminal deoxynucleotidyl transferase (TdT) [22–24]. Commonly, they used the target miRNA-specific detection probe modified with a phosphate group at its 3' end to restrict the non-specific extension reaction by TdT regardless of the target miRNA. As the designed detection probe forms a complex with the target miRNA, the DSN specifically cleaves the DNA strand, i.e., the detection probe, producing DNA fragments with 3'-hydroxyl groups and, at the same time, recycling the target miRNA for the cleavage of multiple phosphate-modified detection probes. TdT is then able to promote the extension reaction from the 3' ends of the DNA fragments; the elongated DNA product was identified by simply observing the fluorescent signal generated by either a fluorophore/quencher-labeled molecular beacon or poly thymine (poly T)-templated copper nanoparticle (Cu NP). Even though these methods presented a high sensing performance level even compared to the aforementioned conventional miRNA detection methods, their innate critical disadvantage, the requirement for a bulky and costly spectrophotometer, still limits their practical utility in facility-limited settings.

Compared to the fluorescence-based biosensor, electrochemical sensing strategies have shown tremendous promise owing to their advantageous characteristics, including high sensitivity, cost-effectiveness, and ease of instrument miniaturization [25,26]. Due to these obvious advantages of electrochemical sensors compared to fluorescence-based ones, several electrochemical miRNA detection methods have been intensively developed in recent years [27–41]. The existing electrochemical miRNA detection approaches have typically relied on target-induced electrochemical signal generation from either electroactive redox materials or biological enzymes that can promote electrochemical catalytic reactions on electrode surfaces. However, despite their noteworthy sensitivities and specificities, their practical uses as point-of-care (POC) testing systems have been hampered by the requirement of DNA capture probe immobilization on the electrode surface. This step makes the entire assay process time-consuming, labor-intensive, unstable, and expensive. Therefore, the development of an electrochemical miRNA detection method that does not require capture probe immobilization on the electrode could pave a new way for electrochemical miRNA sensors that overcome the limitations of previous approaches.

This paper describes the label- and wash-free electrochemical detection of miRNA based on target-triggered Cu^{2+} reduction. The high affinity of DNA toward metal cations can produce DNA-templated nanoparticles (NPs) [42]. In particular, Cu^{2+} is easily reduced in the presence of poly-T DNA templates and consequently behaves as an efficient electrochemical signal transducer [43]. Briefly, a target-specific 3'-phosphorylated detection probe (DP) is cleaved by DSN when it forms a DNA/RNA hybrid by binding with the target miRNA, producing short DNA fragments with 3'-hydroxyl groups. Subsequently, TdT catalyzes the extension of these DNA fragments using deoxythymidine triphosphates (dTTPs) as monomers, generating long poly T strands that serve as templates for Cu^{2+} reduction, which consequently induces target-dependent changes in the electrochemical signal from Cu^{2+} remaining in the reaction solution. With this strategy, the target miRNA, miR-141, was detected at low concentrations down to 33.2 zM and with high specificity against different types of miRNAs containing high sequence homology levels with the target miRNA. In addition, we successfully verified the ability of the developed method to carry out reliable identification of the target miRNA even in the total RNA extracted from diverse cancer cell lines by comparing the electrochemical signal results with those of RT-PCR. We firmly believe that the designed method can facilitate POC testing of miRNA because of its zeptomolar sensitivity, wide dynamic range, label- and wash-free detectability, simplicity resulting from the elimination of electrode modification, rapidity, and stability. Furthermore, this method may enable the detection of various nucleic acid biomarkers and possibly other biomolecules by employing the appropriate DP that can bind to and form complexes with the analyte of interest.

2. Methods/experimental

2.1. Materials

The oligonucleotides used in this study were synthesized by Bio-ner® (Daejeon, Korea) and the corresponding sequences are listed in Table S2. Copper sulfate, sodium ascorbate, and 3-(N-morpholino)propanesulfonic acid (MOPS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Deoxythymidine triphosphate (dTTP) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). DSN and TdT were purchased from Evrogen (Konkov, Moscow, Russia) and Enzynomics (Daejeon, Korea), respectively. A screen-printed electrode (SPE; DRP-C220AT) and a potentiostat (Reference 600) were purchased from DropSens (Oviedo-Asturias, Spain) and Gamry Instruments (Warminster, PA, USA), respectively. MCF-7 (human breast cancer cell line), HeLa and HeLa-141 (human cervical cancer cell lines), LNCaP-LN3 (human prostate cancer cell line), RAG (mouse renal adenocarcinoma cell line), and TCMK-1 (mouse kidney epithelial cell line) were obtained from Korean Cell Line Bank (KCLB; Seoul, Korea). Dulbecco's modified Eagle's medium (DMEM) and Fetalgro Bovine Growth Serum (FBS) were purchased from Welgene Inc. (Gyeongsan-si, Korea) and RMBIO® (Missoula, MT, USA), respectively. Easy-Blue™ Total RNA Extraction Kit and Luna® Universal Probe One-Step RT-qPCR Kit were purchased from Intron Biotechnology Inc. (Daejeon, Korea) and New England Biolabs Inc. (Beverly, MA, USA), respectively. Ultrapure DNase/RNase-free distilled water (DW) purchased from Bioneer® was used in all experiments.

2.2. Electrochemical detection of miRNA

The DP cleavage reaction was conducted in a 10 μL solution containing DW (2 μL), DSN reaction buffer (5 μL ; 500 mM Tris-hydrochloride (pH 8), 50 mM magnesium chloride, and 10 mM dithiothreitol), DP (1 μL , 500 nM), DSN (1 μL , 1 U/ μL), and miRNA (1 μL , at varying concentrations). The solution was incubated at 50 °C for 30 min using an S1000™ thermal cycler (Bio-Rad, CA, USA). Subsequently, TdT reaction buffer (5 μL ; 200 mM Tris-acetate (pH 7), 500 mM potassium acetate, and 100 mM magnesium acetate), CoCl_2 (5 μL , 0.25 mM), dTTP (2.5 μL , 10 mM), and TdT (1 μL , 1 U/ μL) were added to the solution, which was subsequently incubated at 37 °C for 30 min. Finally, DW (9 μL), MOPS buffer (5 μL ; 100 mM MOPS (pH 7.6) and 1.5 M sodium chloride), sodium ascorbate (10 μL , 10 mM), and copper sulfate (2.5 μL , 10 mM) were added to the as-prepared solution and the mixture was incubated at 25 °C for 10 min. For the electrochemical detection, a 50 μL reaction sample was loaded onto the SPE and the resulting electrochemical signal was measured using the potentiostat.

For the detection of miRNAs in a total RNA sample, different types of cell lines (MCF-7, HeLa, HeLa-141, LNCaP-LN3, RAG, and TCMK-1) were cultured in DMEM supplemented with 10% FBS under a humidified atmosphere containing 5% CO_2 at 37 °C, and 1×10^6 cells in the exponential growth phase were collected. The total RNA in the cultured cells was extracted thereafter using the Easy-Blue™ Total RNA Extraction Kit by following the manufacturer's instructions. The concentrations of the extracted total RNA were measured using the NanoDrop® ND-1000 spectrophotometer (Wilmington, DE, USA). Finally, the extracted total RNA (1 μL , 5 $\mu\text{g}/\mu\text{L}$) was added to the reaction sample instead of the target miRNA and subjected to the aforementioned detection procedure.

2.3. Detection of target miRNA spiked into the total RNA extracted from the HeLa

Two μL solution prepared by mixing 1 μL HeLa total RNA solution (5 $\mu\text{g}/\mu\text{L}$) and 1 μL .

miRNA solution at varying concentrations was added into 8 μL reaction solution containing DW (1 μL), DSN reaction buffer (5 μL ; 500

mM Tris-hydrochloride (pH 8), 50 mM magnesium chloride, and 10 mM dithiothreitol), DP (1 μ L, 500 nM), DSN (1 μ L, 1 U/ μ L). The sample containing target miRNA spiked into the total RNA was then treated according to the same procedure described in '2.2. Electrochemical detection of miRNA'.

2.4. RT-PCR

RT-PCR was conducted using the Luna® Universal Probe One-Step RT-qPCR Kit following the manufacturer's instructions. The reaction was conducted in a 10 μ L solution containing DW (1.5 μ L), RT-PCR buffer (5 μ L, 2 \times), forward primer (0.4 μ L, 10 μ M), reverse primer (0.4 μ L, 10 μ M), TaqMan probe (0.2 μ L, 10 μ M), extracted total RNA (2 μ L, 0.5 μ g/ μ L), and an enzyme mix (0.5 μ L, 20 \times) comprised of reverse transcriptase and Taq DNA polymerase. The RT-PCR solution was incubated at 55 $^{\circ}$ C for 10 min, heated at 95 $^{\circ}$ C for 1 min, and subjected to 24 cycles involving denaturation at 95 $^{\circ}$ C for 10 s and annealing/extension at 60 $^{\circ}$ C for 30 s. The fluorescent signal from the TaqMan probe was measured at the end of every temperature cycle using CFX Connect™ Real-Time PCR Detection System (Bio-Rad, CA, USA).

2.5. Polyacrylamide gel electrophoresis

For polyacrylamide gel electrophoresis (PAGE) analysis, a mixture of the reaction solution (10 μ L) and loading buffer (2 μ L, 6 \times) purchased from Bioneer® was loaded on a 15% polyacrylamide gel using 1 \times Tris-borate ethylene-diamine-tetraacetic acid as a running buffer at a constant voltage of 120 V for 2 h. After SYBR Gold staining, the gel was scanned using a Gel Doc EZ Imager (Bio-Rad, CA, USA).

2.6. Transmission electron microscopy (TEM)

The sample for TEM analysis was prepared by casting a 15 μ L specimen onto a 300-mesh copper grid with a lacey carbon film (LC300-CU) purchased from Electron Microscopy Sciences (Hatfield, PA, USA). The sample was subsequently dried at room temperature overnight and analyzed by Cs-corrected scanning TEM (JEM-ARM200F, JEOL, Tokyo, Japan) at the National NanoFab Center (Daejeon, Korea).

3. Results and discussion

3.1. Electrochemical miRNA detection via miRNA-triggered Cu^{2+} reduction

The overall procedure of the proposed electrochemical miRNA detection platform based on miRNA-triggered Cu^{2+} reduction is illustrated in Fig. 1. The entire reaction is comprised of two parts: (A) the target miRNA-induced cleavage of the DP and (B) the extension of the poly T sequence with concomitant reduction of Cu^{2+} . The DP employed in this study was modified with a phosphate group at its 3'-end to prevent the extension reaction promoted by TdT [44]. The target miRNA present in a sample binds to the DP and forms a DNA/RNA hybrid. Then, DSN specifically degrades the DNA strand (DP) in this DNA/RNA hybrid, recycling the target miRNA to promote another DP cleavage reaction. Short DNA fragments containing hydroxyl groups at their 3'-ends are concomitantly produced during the DP cleavage reaction. Thereafter, the poly T sequence is extended from the 3'-hydroxyl groups of the produced short DNA fragments by TdT using dTTP as a monomer. Finally, the Cu^{2+} concentration in the reaction solution is reduced as the poly T-templated Cu NPs are produced in the presence of the reducing agent, i.e., ascorbate, leading to a decrease in the electrochemical signal [45]. Overall, the proposed reaction enables label- and wash-free electrochemical detection of miRNA.

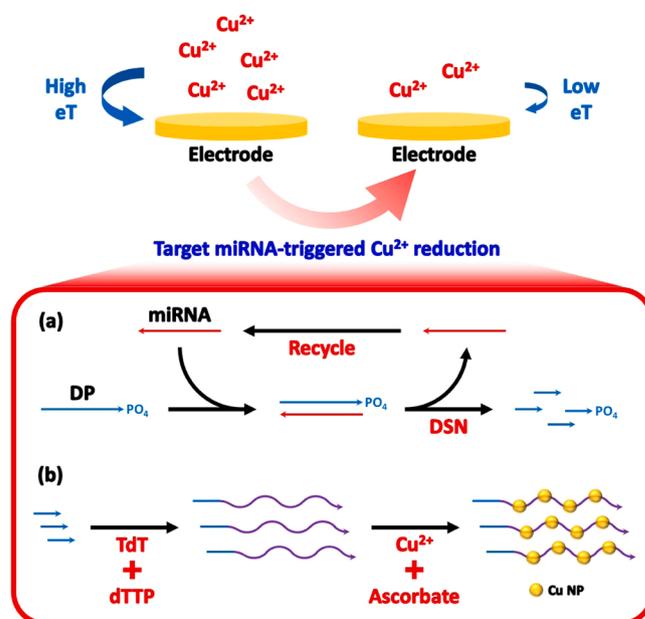


Fig. 1. Principle of electrochemical miRNA detection via miRNA-triggered Cu^{2+} reduction. (a) Target miRNA-induced cleavage of DP. (b) Cu^{2+} reduction by the synthesis of poly T-templated Cu NPs. Red, blue, and purple arrows indicate the 3' ends of miRNA, DP, and poly T, respectively.

3.2. Feasibility test and optimization

miR-141, a promising biomarker for human prostate cancer, was selected as a model target to verify the feasibility of the designed electrochemical miRNA detection method. First, we obtained and analyzed the cyclic voltammetry (CV) profiles of samples containing various combinations of the reaction components to investigate the accuracy of the designed strategy. The electrochemical signal intensity obtained from sample 7, in which all reaction components were present, considerably and exclusively decreased owing to the production of the poly T sequence and the concomitant synthesis of poly T-templated Cu NPs in the reaction solution (Fig. 2a). However, high electrochemical current intensities were generated from all other component mixture cases (samples 1–6 and 8; Fig. 2a) due to the high concentration of Cu^{2+} remaining in the reaction solution. This indicates the importance of each component for the miRNA-triggered Cu^{2+} reduction, wherein DP enables the recognition of the target miRNA, DSN plays a role in producing the TdT substrate, and TdT is essential for synthesizing poly T strands. Meanwhile, it is noteworthy that the CV traces obtained from the reactions show near-reversible electrochemical behavior at the SPE because Cu^{2+} undergoes fast outer-sphere electron-transfer [46].

The miRNA-triggered Cu^{2+} reduction method was subsequently analyzed by PAGE using a 15% polyacrylamide gel (Fig. 2b). When the target miRNA and DP were mixed, the 22 bases-long DNA/RNA hybrid product was observed in lane 3. This band is distinguishable from those of lanes 1 (miR-141) and 2 (DP), suggesting that miRNA and DP had been successfully hybridized. Although miR-141 and DP are 22 bases long, the band positions between lanes 1 and 2 are slightly different, which is ascribed to the electrophoretic mobilities of oligonucleotides and the effects of phosphate modification [47,48]. Essentially, the different conformational properties of DNA and RNA and the phosphate group attached only to the DP would affect the electrophoretic mobility, leading to the different band positions of miR-141 and DP. Bands corresponding to miR-141 and DP were observed in lanes 4 and 5, respectively, in the presence of DSN, suggesting the insubstantial cleavage activity of DSN against single-stranded RNA (miRNA) and DNA (DP). The strong cleavage preference of DSN only for the DNA in DNA/RNA hybrid was confirmed because the gel band intensity of the

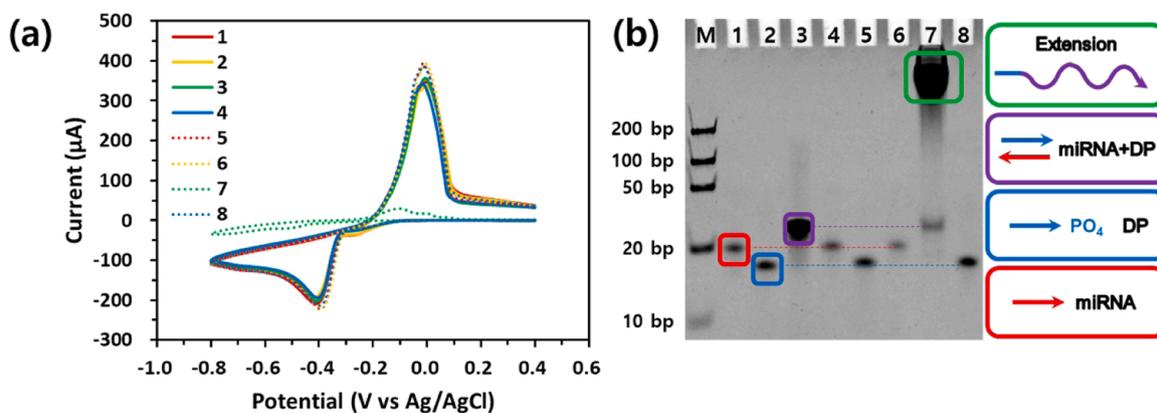


Fig. 2. (a) CV and (b) polyacrylamide gel analyses of target miRNA-triggered Cu^{2+} reduction conducted by varying the reaction components (1: miR-141, 2: DP, 3: miR-141 + DP, 4: miR-141 + DSN, 5: DP + DSN, 6: miR-141 + DP + DSN, 7: miR-141 + DP + DSN + TdT, and 8: DP + DSN + TdT). The concentrations of miR-141, DP, DSN, and TdT were 10 nM, 10 nM, 20 $\mu\text{M}/\mu\text{L}$, and 20 $\mu\text{M}/\mu\text{L}$, respectively.

DP significantly decreased while that of miR-141 was maintained (lane 6). The gel band corresponding to cleaved DP was not observed on the gel image because the cleaved products were less than 6 bases in size [49]. The lengthy reaction product, the poly T sequence, obtained only in the presence of all the reaction components, appeared in lane 7. A band corresponding to DP was observed in lane 8 since the designed reaction system cannot be promoted in the absence of the target miRNA, which confirmed the initiation of the developed method by the target miRNA and the importance of the combined activities of DSN and TdT. Additionally, the poly T-templated Cu NPs produced by the entire reaction were confirmed by TEM (Fig. S1).

After the verification of the feasibility of the miRNA-triggered Cu^{2+} reduction reaction, the reaction conditions including the component concentrations (DP, DSN, TdT, dTTP, and Cu^{2+}) and enzyme reaction time were optimized (Fig. S2). Overall, 10 nM DP, 20 $\mu\text{M}/\mu\text{L}$ DSN, 20 $\mu\text{M}/\mu\text{L}$ TdT, 500 μM dTTP, 0.5 mM Cu^{2+} , and 30 min of reaction time for DSN and TdT activities were determined as the optimal conditions for the proposed method and used for subsequent experiments.

3.3. Sensitivity

Among the various methods for measuring electrochemical signals, chronocoulometry offers a more reproducible electrochemical signal and background level than CV and chronoamperometry because the chronocoulometric data can be integrated at a given time [50]. Accordingly, in this study, chronocoulometry was adopted to obtain the

resulting electrochemical data by sampling the charge intensity at 1.5 s after initiation of the signal measurement (Q) [51]. The sensitivity of the target miRNA-triggered Cu^{2+} reduction method was assessed by measuring the Q values from the samples containing target miRNA at varying concentrations (Fig. 3a). The resulting chronocoulometric signals rapidly increased with decreasing concentrations of miRNA. Furthermore, the plot of Q values versus miRNA concentrations shows an excellent linear relationship ($R^2 = 0.9997$) in a wide range of target miRNA concentrations (1 aM - 10 nM) and the limit of detection (LOD) was determined as 33.2 zM (1 copy/sample) based on the 3σ rule (σ : standard deviation of the negative control without the target miRNA) (Fig. 3b). This LOD is remarkably lower than those of previously reported electrochemical strategies for miRNA detection that require time-consuming and labor-intensive procedures to immobilize capture probes on sensing electrodes (Table S3). In addition, the relative standard deviation (RSD) values of the resulting signals from all samples with varying concentrations of target miRNA (1 aM - 10 nM) were all lower than 6%, demonstrating the high reproducibility of this electrochemical sensing strategy [52,53].

3.4. Selectivity

The high sequence similarity between different miRNAs in similar or the same families, which is a distinctive characteristic of miRNAs, hinders straightforward miRNA detection [54]. The target miRNA used in this study, miR-141, belongs to the miR-200 family, which also contains

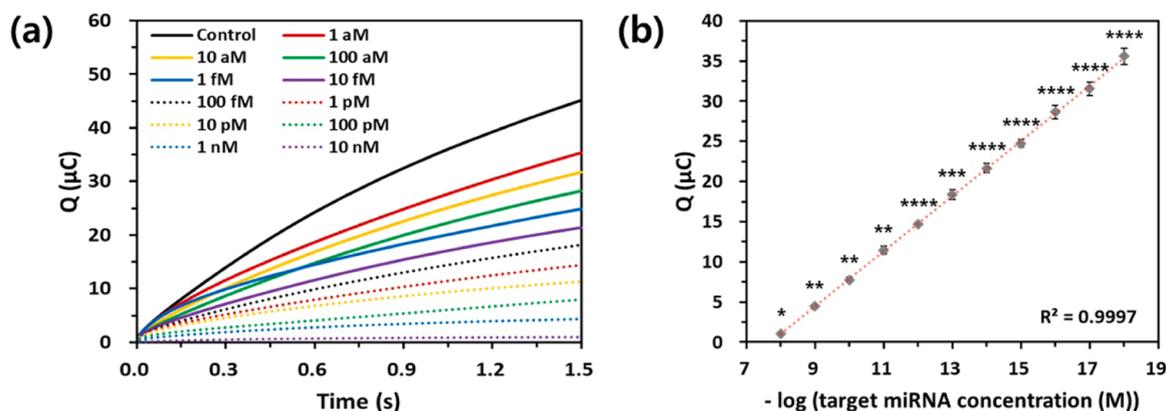


Fig. 3. (a) Chronocoulograms recorded at -0.4 V (vs Ag/AgCl) from the samples containing the target miRNA at varying concentrations (1 aM - 10 nM) and (b) corresponding plot of Q obtained at 1.5 s from the chronocoulogram as a function of the logarithmic miRNA concentration ($n = 3$, error bar = standard deviation). The asterisks indicate the RSD values ($6\% > * \geq 5\%$, $5\% > ** \geq 4\%$, $4\% > *** \geq 3\%$, and $**** < 3\%$). The concentrations of miR-141, DP, DSN, and TdT were 10 nM, 10 nM, 20 $\mu\text{M}/\mu\text{L}$, and 20 $\mu\text{M}/\mu\text{L}$, respectively.

miR-200a, miR-200b, and miR-200c. Therefore, the specificity of this assay was examined using these candidates along with miR-21, which is known to be considerably over-expressed in breast tumors in a similar manner to miR-141. Charge differences, $Q_0 - Q$, were calculated from the samples containing different types of miRNAs, where Q_0 and Q are the charge intensities obtained at 1.5 s from the chronocoulograms in the absence and presence of the miRNAs, respectively (Fig. 4). A significantly high value of $Q_0 - Q$ was observed only in the presence of the target miRNA, miR-141. This result demonstrates the sufficiently high specificity of the proposed method for distinguishing the target miRNA not only from the non-complementary miRNA, miR-21, but also from miRNAs belonging to the same family. Notably, miR-200a, which contains only double base mismatches with the employed DP, was clearly distinguished from the target miRNA, verifying the excellent specificity of this strategy. To prove the statistical difference between the $Q_0 - Q$ values of the target and non-specific miRNAs, we calculated the p-value (unpaired two-tailed *t*-test) of the $Q_0 - Q$ values for the samples with non-specific miRNAs and compared it to that of the samples with the target miRNA. The results showed that all p-values were lower than 0.0001, demonstrating the target miRNA-induced signal is significantly different from those of non-specific miRNAs. Furthermore, we conducted melting curve analysis for the hybridization of DP with each miRNA and confirmed that the high selectivity of this strategy can be ascribed to the much higher melting temperature (T_m) of the DP/miR-141 complex ($\sim 65^\circ\text{C}$) compared to the reaction temperature (50°C), while that of the complexes between DP and mismatched miRNAs ($\sim 35^\circ\text{C}$) was much lower than the reaction temperature, leading to the efficient formation of a complex between the miRNA and DP only when their sequences are perfectly complementary (Fig. S3). In addition to the selectivity test with miRNAs included in the miR-200 family, we also performed this assay using a target miRNA from a different family, the Let-7 family, as a model. As evidenced by the results in Fig. S4, we successfully confirmed the high specificity of our method for the detection of the target miRNA, Let-7a, demonstrating the expansibility of the developed assay system by simply redesigning the

DP to be complementary with the miRNA of interest.

3.5. Practical applicability

The practical applicability of the developed electrochemical miRNA detection method was examined by determining miR-141 in the total RNA extracted from various cell lines. MCF-7, HeLa-141, and LNCaP-LN3 were selected as miR-141-positive cell lines, whereas HeLa, RAG, and TCMK-1 were adopted as miR-141-negative cell lines. Fig. 5a shows the plot of $Q_0 - Q$ values corresponding to the investigated cell lines, where Q_0 and Q are the charge intensities obtained at 1.5 s from the chronocoulograms in the absence and presence of total RNA, respectively. As shown in the results, considerable $Q_0 - Q$ values were obtained only in the presence of the miR-141-positive cell lines. Moreover, the assay results were compared with the corresponding $C_{t0} - C_t$ values obtained with the RT-PCR method, where C_{t0} and C_t are the threshold cycle numbers in the absence and presence of total RNA, respectively (red-colored data in Fig. 5a). Surprisingly, the electrochemical signals derived from the developed approach are in perfect agreement with those obtained by RT-PCR. Furthermore, we also validated the ability of our strategy to quantify the target miRNA included in the total RNA (Fig. 5b). For this validation experiment, we measured the electrochemical signals from the samples containing HeLa cell-derived total RNA spiked with target miRNA at varying concentrations. The resulting Q values showed an excellent linear relationship with the concentration of target miRNA even in samples with total RNA, confirming the practical utility of this method for reliable and accurate detection of a target miRNA even in complex biological samples.

3.6. Discussion

A label-free electrochemical miRNA detection method was established using the target-triggered Cu^{2+} reduction reaction promoted by the combined activities of DSN and TdT. This method offers certain benefits for electrochemical miRNA detection. First, the developed strategy exhibits a remarkably higher sensitivity compared to those of previous methods, enabling the precise identification of small amounts of miRNA expressed in cells. Zeptomolar sensitivity is essential for direct profiling of the unamplified miRNA expression, particularly in biofluids with a relatively low (typically sub-nanomolar) concentration of individual miRNA [55,56]. Considering that a fraction of miRNAs is encapsulated in exosomes or bound to protein complexes, the absolute concentration of freely circulating biomarkers that can be directly detected in biological samples is even lower, which necessitates the use of more sensitive tools [57]. The developed system with zeptomolar sensitivity and minuscule sample volume ($1\ \mu\text{L}$) enables miRNA to be detected in complex biological samples such as urine and blood.

Second, the electrochemical miRNA detection method based on target-triggered Cu^{2+} reduction provides an excellent quantitative relationship ($R^2 = 0.9997$) and a wide dynamic range ($1\ \text{aM} - 10\ \text{nM}$). miR-141 is present at levels of $\sim 2 \times 10^4$ to $\sim 8 \times 10^4$ copies/sample in exosomes extracted from the serum of prostate cancer patients [58]. Because the proposed technique entirely covers this range, it is expected to be used for the liquid biopsy of prostate cancer, possibly enabling the metastasis, prognosis, and treatment of the disease to be predicted.

The excellent sensitivity and quantitative behavior of this system are attributable to 1) DSN permitting target miRNA recycling for exponential amplification, thereby enabling high sensitivity; 2) the stable and intense metal ion-derived electrochemical signals facilitating precise detection of target miRNA even in biological samples; and 3) the unrestrained presence of the reaction probe in the solution without the steric hindrance of the reaction components. Typically, solid phase-immobilized nucleic acid probes exhibit relatively lower sensitivities compared to those of free nucleic acid amplification-based probes [59]. Furthermore, the preclusion of DNA probe immobilization on the electrode eliminates the labor-intensive and time-consuming procedures

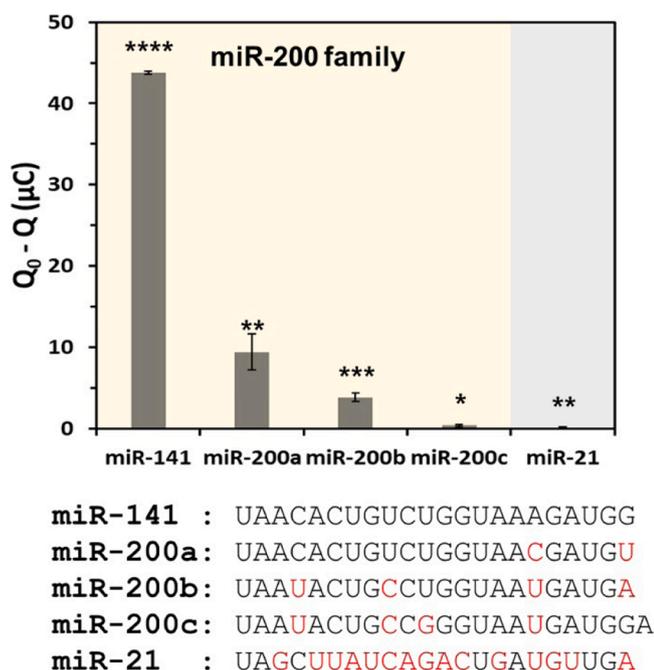


Fig. 4. Plot of $Q_0 - Q$ as a function of miRNAs (miR-141, miR-200a, miR-200b, miR-200c, and miR-21) ($n = 3$, error bar = standard deviation). The asterisks indicate the RSD values (* $\geq 30\%$, $30\% > ** \geq 20\%$, $20\% > *** \geq 10\%$, and $**** < 10\%$). The concentrations of miRNA, DP, DSN, and TdT were 10 nM, 10 nM, 20 mU/ μL , and 20 mU/ μL , respectively.

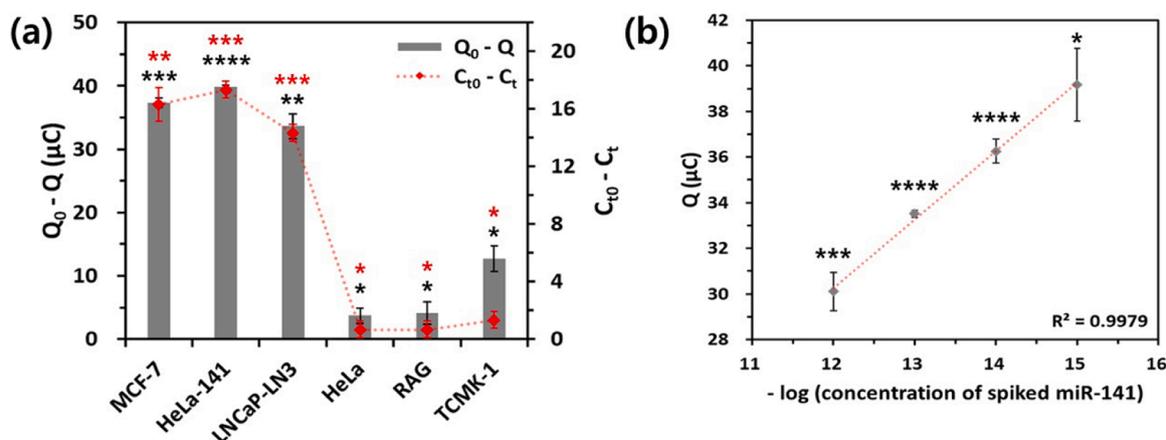


Fig. 5. (a) Comparative plots of $Q_0 - Q$ and $C_{10} - C_t$ values as functions of total RNA extracted from various cell lines ($n = 3$, error bar = standard deviation). The asterisks indicate the RSD values ($* \geq 10\%$, $10\% > ** \geq 5\%$, $5\% > *** \geq 2\%$, and $**** < 2\%$). (b) Plot of the Q values obtained at 1.5 s from the chronocoulograms as a function of the logarithmic concentration of miRNA spiked into the total RNA extracted from the HeLa cell line ($n = 3$, error bar = standard deviation). The asterisks indicate the RSD values ($5\% > * \geq 4\%$, $4\% > ** \geq 3\%$, $3\% > *** \geq 2\%$, and $**** < 2\%$). The concentrations of total RNA, DP, DSN, and TdT were 0.1 $\mu\text{g}/\mu\text{L}$, 10 nM, 20 mU/ μL , and 20 mU/ μL , respectively.

used for preparing sensing electrodes.

The specificity exhibited by the proposed method for target miRNA can be ascribed to the following: 1) DSN, which is capable of discriminating a perfectly matched DNA/RNA hybrid from a mismatched one (even a single nucleotide mismatch), can specifically recognize a DNA/RNA hybrid and facilitate the recycling reaction only when the miRNA is completely complementary to the DP, and 2) even a short target miRNA can efficiently form a complex with DP in this reaction system because of the higher T_m ($\sim 65^\circ\text{C}$), even compared to the considerably high reaction temperature (50°C). Contrary to the target miRNA, a non-target miRNA–DP duplex exhibiting a T_m ($\sim 35^\circ\text{C}$) significantly lower than the reaction temperature would be easily denatured under the reaction conditions (Fig. S3).

Importantly, since this electrochemical miRNA detection method avoids complicated reverse transcription procedures and sophisticated instrumentation, it could be suitable for POC diagnostics. Moreover, this method is expected to be widely applied to electrochemically detect diverse biomolecules by simply replacing the DP with a DNA aptamer sequence that can specifically bind to the target analytes of interest.

However, the conceived approach still has room for improvement. Despite the successful detection of miR-141, a multiple miRNA detection system for expression profiling needs to be urgently established because of the intimate association between aberrant expressions of various miRNAs and cancer initiation/metastasis. Additionally, clinical samples, such as model or actual patient samples, should be tested on this platform. These aspects will be considered in future research on this method, which is anticipated to be extensively applicable for biomarker diagnostics in liquid biopsy.

4. Conclusion

A label- and wash-free electrochemical detection method was developed for miRNA detection with zeptomolar sensitivity and excellent specificity against mismatched miRNAs. The practical diagnostic capability of this strategy was verified by reliably identifying the target miRNA in total RNA samples extracted from various cancer cell lines. This system can presumably be further used to diagnose biomarkers in real samples.

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CRedit authorship contribution statement

Hyo Yong Kim: Conceptualization, Methodology, Validation, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Jayeon Song:** Conceptualization, Methodology, Validation, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Hyun Gyu Park:** Writing – review & editing, Supervision, Project administration. **Taejoon Kang:** Writing – review & editing, Supervision, Project administration, Funding acquisition. **Hyo Yong Kim and Jayeon Song** contributed equally to this work. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Not applicable.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.snb.2022.131666](https://doi.org/10.1016/j.snb.2022.131666).

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