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Hairpin-Structured DNAzyme-Initiated Spherical Hybridization Chain Reaction Cascade Enables Highly Sensitive Electrochemical Detection of MicroRNA

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Abstract: Sensitive and accurate detection of low-abundance microRNAs (miRNAs) in complex biological matrices is critical for early disease diagnosis. Herein, we report a homogeneous cascade amplification-based electrochemical biosensing platform that integrates hairpin-structured DNAzyme-assisted target recycling amplification with spherical hybridization chain reaction (SHCR)-enhanced electrochemical signal transduction, enabling highly sensitive detection of miRNA-141 (miR-141). In this strategy, a hairpin recognition probe specifically recognizes miR-141, while DNAzyme-mediated catalytic amplification generates a large quantity of trigger strands to initiate SHCR, yielding amplification products densely labeled with electroactive species and thereby substantially enhancing the electrochemical signal output intensity. The fabricated biosensor exhibits a wide linear dynamic range from 0.1 fM to 1 nM with the limit of detection as low as 0.031 fM. Owing to its excellent selectivity and operational stability, the biosensor reliably discriminates miR-141 from structurally similar miRNAs and effectively detects the target in complex serum samples. Furthermore, practical applicability is demonstrated through analysis of miR-141 expression levels in lysates derived from multiple cancer cell lines. Collectively, this biosensing platform offers a robust and highly sensitive electrochemical assay for tumor-associated miRNAs, supporting early molecular diagnosis of related diseases.

Keywords: Hairpin-structured DNAzyme; spherical nucleic acids; hybridization chain reaction; homogeneous amplification; electrochemical detection

1. Introduction

MicroRNAs (miRNAs) are non-coding single-stranded RNA molecules approximately 21–23 nucleotides in length [1]. Their expression is frequently dysregulated in multiple malignancies, such as breast and prostate cancer, and they exhibit exceptional stability in biofluids as well as pronounced tissue- and disease-specific expression patterns [2,3]. Consequently, miRNAs have emerged as highly promising molecular biomarkers for cancer diagnosis and therapeutic monitoring [4]. Conventional miRNA detection methods, including Northern blotting [5], quantitative reverse transcription polymerase chain reaction (qRT-PCR) [6], and high-throughput sequencing [7], offer high sensitivity and broad profiling capability. Among these, qRT-PCR is widely regarded as the gold standard for miRNA detection. However, it typically requires laborious sample pretreatment, lengthy assay duration, and highly trained personnel [8]. Therefore, there is a pressing need to develop rapid, highly sensitive, and operator-friendly miRNA detection platforms.



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DNA-based electrochemical biosensors have been extensively utilized for the analysis of miRNA due to their rapid response, simple operation, and high specificity [9]. Unfortunately, conventional electrochemical sensing platforms based on the one-to-one recognition between the target and the signal-reporting unit still suffer from uncontrolled off-target recognition and inadequate sensitivity for the reliable detection of low-abundance miRNA in complex matrices [10]. Spherical nucleic acids (SNAs) are a class of three-dimensional nucleic acid nanostructures formed by the high-density conjugation of oligonucleotide strands onto nanoscale cores, such as gold nanoparticles (AuNPs) [11], magnetic beads (MBs) [12], and covalent organic frameworks (COFs) [13], conferring exceptional physiological stability and robust resistance to nuclease degradation [14]. These structural features yield a significantly expanded surface area and a high density of chemically addressable sites, enabling the high-density immobilization of molecular recognition elements, redox-active species, and other functional components within a single nanostructure, thereby enhancing interfacial assembly efficiency and signal transduction performance [15,16]. Therefore, SNAs have emerged as a versatile biosensing platform, and the SNA-based electrochemical assay demonstrates excellent single-base mismatch discrimination capability, with a detection limit reaching the femtomole level for miRNA detection in complex biological matrices [17]. Moreover, SNAs can be readily integrated with various isothermal amplification strategies, such as hybridization chain reaction (HCR) [18], catalytic hairpin assembly (CHA) [19], and DNAzyme-assisted amplification [20], thereby further enhancing target amplification capability and electrochemical detection sensitivity. For instance, integration of nuclease-assisted SNA amplification with the CHA reaction yielded a 2.6-fold enhancement of the electrochemical signal, thereby substantially improving detection sensitivity [21].

Given the unique biochemical properties of miRNA and the practical significance of miRNA analysis, cascading two or more signal amplification strategies can substantially lower the limit of detection to the attomolar range, thereby enabling ultrasensitive electrochemical detection of miRNA [22]. However, directly employing the target miRNA as an amplification primer carries a substantial risk of off-target hybridization, owing to the high sequence homology across the miRNA family [23]. This may initiate unintended cascade amplification in the absence of the target miRNA, leading to pronounced background signal and consequently compromising the accuracy and reliability of the detection methods [24]. It has been demonstrated that leak-proof hairpin structures incorporate structural locking to prevent target-independent signal leakage, thereby significantly reducing background noise [25]. Accordingly, the use of hairpin-structured target recognition probes, which are engineered to physically sequester the target miRNA from amplification components, can effectively suppress nonspecific activation.

The analytical performance of DNA-based electrochemical biosensors is constrained by signal amplification performed directly on the electrode surface (that is heterogeneous amplification). Specifically, steric hindrance arising from probe immobilization restricts conformational flexibility and may distort probe geometry, which impairs intermolecular recognition events on the modified electrode surface and reaction kinetics, ultimately diminishing amplification efficacy [26]. In addition, executing signal amplification directly on the electrode surface typically involves multi-step procedures and washing steps, which are time-consuming and exhibit considerable batch-to-batch variability, compromising detection stability and reproducibility [27]. In contrast, decoupling target amplification from electrochemical detection is achieved by performing amplification in a homogeneous solution followed by immobilization of the amplified products onto the electrode surface for signal output, yielding significantly enhanced and more reproducible electrochemical signals [28]. In our preliminary study, electrochemical signals obtained using CHA- and EDC-based homogeneous amplification strategies were 4-fold and 1.6-fold higher [29,30], respectively, than those generated via heterogeneous (electrode-confined) amplification. This approach enables efficient target amplification while eliminating layer-by-layer amplification reactions on the electrode surface, thereby facilitating high-sensitivity miRNA detection through specific hybridization between the capture probe-immobilized electrode and the amplification products.

Herein, we developed a homogeneous cascade amplification strategy that integrated hairpin-structured DNAzyme-assisted target recycling amplification with a spherical hybridization chain reaction (SHCR)-enhanced electrochemical signal transduction system for highly sensitive detection of miR-141. The inherent stability of the hairpin-structured recognition probe, combined with the robust signal amplification capability of the DNAzyme-initiated SHCR cascade, conferred exceptional sensitivity, reproducibility, and operational stability to this electrochemical biosensing platform, thereby ensuring analytical accuracy and result reliability. Furthermore, the practicability of the biosensor was validated through quantitative detection of miR-141 in spiked fetal bovine serum and comparative analysis of miR-141 expression levels across multiple human cell lines.

2. Materials and Methods

2.1. Hairpin-Structured DNAzyme-Initiated SHCR Homogeneous Cascade Amplification Strategy

The homogeneous cascade amplification strategy developed herein comprises three sequential stages: (i) hairpin-structured probe-mediated target recognition; (ii) hairpin-structured DNAzyme-assisted target recycling amplification (Cycle I); and (iii) SHCR-amplified electrochemical signal transduction (Cycle II). For target recognition, miR-141 (2 μ L, 1 μ M) was mixed with the pre-annealed hairpin-structured recognition probe (HR, 2 μ L, 1 μ M) and incubated at 37 °C for 40 min. Subsequently, the L1/L2 hybrid (2 μ L, 1 μ M) formed by annealing equimolar single-stranded DNA 1 (L1) and single-stranded DNA 2 (L2) containing the DNAzyme catalytic strand was added, and the mixture was incubated for an additional 40 min at 37 °C to obtain the target transcript L2. For Cycle I, hairpin DNA 1 containing the DNAzyme substrate strand (H1, 1 μ L, 10 μ M) and Zn^{2+} (1 μ L, 20 mM) were added to the above mixture containing L2, and the reaction was incubated at 37 °C for 30 min to generate the single-stranded DNA cleavage fragment (L3), which served as the initiator for Cycle II. Specifically, the above reaction mixture was further incubated with prepared spherical nucleic acid (SNA, P/L4-L5-AuNP, 4 μ L, see Supporting Information), G-quadruplex-tethered hairpin DNA 2 (GH2, 2 μ L, 1 μ M), and G-quadruplex-tethered hairpin DNA 3 (GH3, 2 μ L, 1 μ M) at 37 °C for 4 h for HCR amplification and obtained the G-SHCR. Next, 10 μ L of 60 μ M methylene blue (MB) was added and incubated for 1 h under slight shaking. The resulting mixture was centrifuged at 12,000 rpm for 30 min to remove free DNA and MB. After three consecutive washes with PBS (10 mM, pH 7.4), the purified G-SHCR loaded with MB (GM-SHCR) was obtained. All the reaction processes and resultant products were analyzed by 12% native polyacrylamide gel electrophoresis (PAGE, Supporting Information).

2.2. Electrochemical Detection of miR-141

The gold electrode (AuE) was initially cleaned (see Supporting Information) and subsequently modified with DNA on its surface. Specifically, the TCEP-activated thiol-labeled capture DNA (SH-S, 1 μ M, 10 μ L) was dropped onto the cleaned AuE and incubated at 4 °C for 16 h, followed by incubation at 37 °C for 2 h; the S-modified AuE (S/AuE) was obtained. Following two washes with PBS (pH 7.4, 10 mM), 10 μ L of 1 mM 6-mercapto-1-hexanol (MCH) was added and incubated at room temperature for 1 h to block the remaining active sites, yielding MCH/S/AuE. The electrode was then rinsed with PBS (10 mM, pH 7.4), and 10 μ L of the homogeneous cascade amplification product (the SHCR containing G-quadruplex motif and loaded with MB, named as GM-SHCR) was dropped onto MCH/S/AuE and incubated at 37 °C for 2 h to obtain the GM-SHCR/MCH/S/AuE. After washing twice with PBS (10 mM, pH 7.4), the GM-SHCR/MCH/S/AuE was employed as the working electrode for subsequent electrochemical measurements (see Supporting Information).

2.3. Real Sample Analysis

For the recovery experiments, fetal bovine serum (FBS) was diluted 10-fold with PBS (10 mM, pH 7.4), and various known concentrations of miR-141 were spiked into the 10% FBS samples, corresponding to theoretical concentrations of 100 pM, 1 pM, and 100 fM. Subsequently, the miR-141 in the 10% FBS samples was amplified by using the designed homogeneous cascade amplification strategy, and the real miR-141 concentrations were measured with the electrochemical sensing platform. The recovery rates were calculated based on three parallel assays. For cell lysate detection, total RNA extracted from cervical cancer cells (HeLa cells) and human breast cancer cells (MDA-MB-231 cells) was amplified using the proposed strategy, followed by electrochemical detection to acquire quantitative signals (details of total RNA extraction are provided in the Supplementary Information).

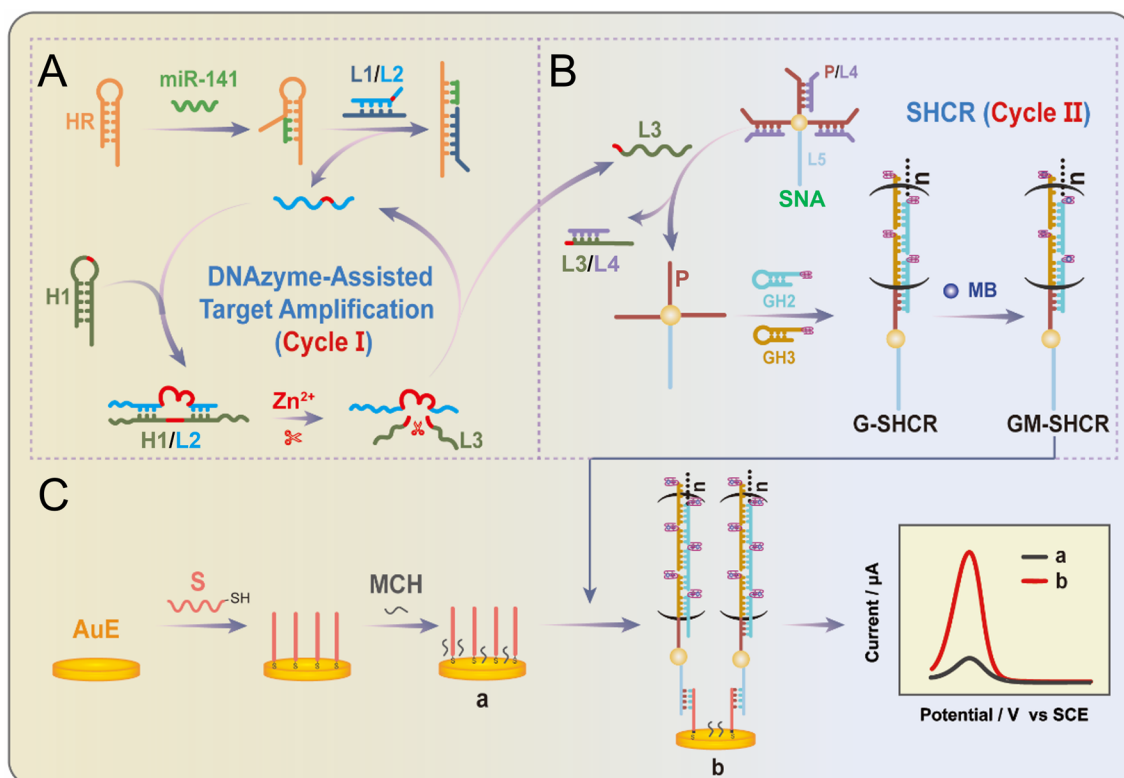
3. Results and Discussion

3.1. Principle of the Hairpin-Structured DNAzyme-Initiated SHCR Homogeneous Cascade Amplification Strategy

The homogeneous cascade amplification strategy mainly includes hairpin-structured probe-mediated target recognition and hairpin-structured DNAzyme-initiated SHCR cascade. As shown in Scheme 1A, miR-141 triggers the opening of the hairpin-structured probe (HR) and subsequently releases the DNAzyme catalytic strand (L2) from the L1/L2 hybrid via the strand displacement reaction, thereby obtaining a stable single-stranded DNA, which can initiate the cascade amplification. Briefly, the released L2 binds to the loop region of hairpin DNA 1 (H1), which contains the DNAzyme substrate sequence, and cleaves the designated recognition site within H1 in the presence of Zn^{2+} , yielding single-stranded DNA L3. Meanwhile, L2 is regenerated and hybridizes with additional H1 to initiate successive rounds of catalytic cleavage, thereby generating a mass of L3 (Cycle I). Subsequently,

the SHCR-amplified electrochemical signal transduction is activated by L3 (Cycle II). As illustrated in Scheme 1B, L3 hybridizes with L4 immobilized on the SNA probe (P/L4-L5-AuNP), initiating a strand displacement reaction that releases the P strand. The liberated P then unfolds G-quadruplex-tethered hairpin DNA 2 (GH2) and exposes a previously sequestered single-stranded region, which specifically hybridizes with G-quadruplex-tethered hairpin DNA 3 (GH3). In turn, GH2 can be opened since the newly exposed fragment of GH3 can recognize GH2. This self-sustaining and SNA-confined hybridization cascade, termed the spherical hybridization chain reaction (SHCR), progressively generates a nicked double-helical architecture to enable secondary target amplification. In addition, the G-quadruplex motif in SHCR facilitates high-density loading of methylene blue (MB) via π - π stacking interactions [31], yielding the MB-loaded SHCR (GM-SHCR), thereby enhancing the electrochemical signal output intensity.

For the electrochemical detection (Scheme 1C), the GM-SHCR is subsequently immobilized on the S-modified electrode surface through specific hybridization between L5 anchored on the GM-SHCR and the capture strand S, thereby substantially enhancing the electrochemical signal (curve b). In contrast, only a negligible electrochemical response is observed in the absence of miR-141 (curve a). Compared with electrode-interface-based amplification strategies that are directly initiated by the target, the conversion of the target miRNA into a stable DNA strand, achieved using a hairpin-structured recognition probe at the initial amplification stage, enhances the stability and accuracy of the amplification process. Moreover, performing the amplification in homogeneous solution eliminates steric and kinetic constraints imposed by the electrode interface, thereby enabling efficient signal amplification. Overall, the proposed cascade amplification strategy offers a robust platform for the accurate detection of tumor-associated miRNAs.



Scheme 1. Scheme of the hairpin-structured DNAzyme-initiated SHCR homogeneous cascade amplification strategy for highly sensitive electrochemical detection of miR-141. (A) hairpin-structured DNAzyme-assisted target recycling amplification. (B) SHCR-amplified electrochemical signal transduction. (C) The fabrication of the electrochemical biosensor for miR-141 detection.

3.2. Feasibility of the Homogeneous Cascade Amplification Strategy

Native polyacrylamide gel electrophoresis (PAGE) was employed to investigate the feasibility of the homogeneous cascade amplification. For the hairpin-structured DNAzyme-assisted target recycling amplification (Figure 1A), the HR and L1/L2 hybrid could coexist stably without generating additional bands (lane 10), whereas the emergence of a distinct L2 band upon addition of the target miR-141 confirmed that miR-141 has been successfully converted to stable L2 via the strand displacement reaction (lane 12). As designed, the L2

incorporating DNAzyme catalytic sequence could hybridize with H1 containing the complementary DNAzyme substrate sequence to form the functional catalytic core of the DNAzyme (lane 9). Upon further addition of Zn^{2+} , the H1 underwent site-specific cleavage, resulting in the appearance of a distinct L3 fragment that served as the initiator for HCR-mediated secondary target amplification (lane 14). As illustrated in Figure 1B, the P hybridized with and opened H2, which in turn triggered the opening of H3, thereby initiating the HCR and generating a ladder of discrete bands corresponding to stepwise HCR products of increasing molecular weight (lane 11). However, when P was sequestered by L4 to form the P/L4 hybrid, the HCR was inhibited, and no new bands appeared (lane 12). In contrast, upon addition of L3, which specifically bound L4 and displaced P via a strand displacement reaction, the liberated P initiated HCR, thereby yielding abundant long DNA concatemers and a distinct L3/L4 hybrid band (lane 13). These results collectively demonstrate that the hairpin-structured DNAzyme-initiated HCR cascade amplification strategy can successfully execute the task as designed, facilitating the highly sensitive detection of low-abundance miR-141 in subsequent electrochemical detection.

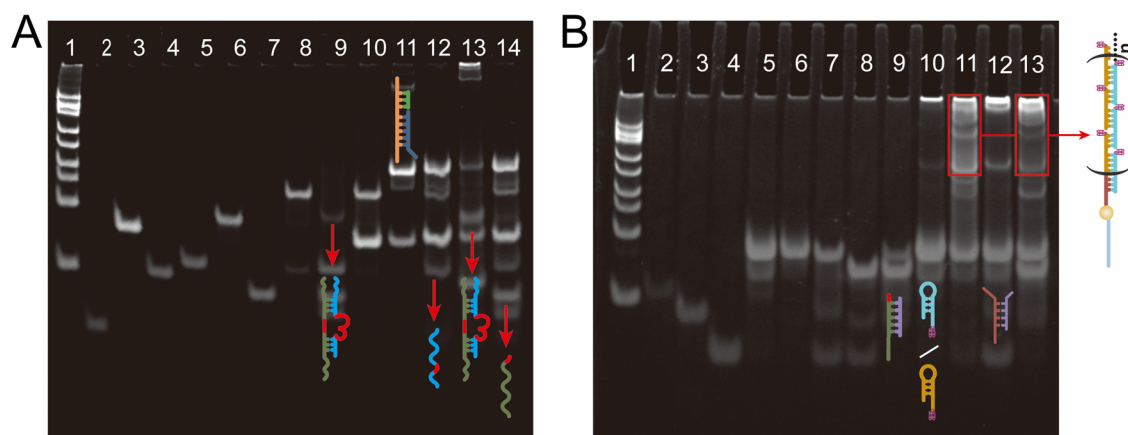


Figure 1. PAGE characterization of the homogeneous cascade amplification strategy. **(A)** The hairpin-structured DNAzyme-assisted target recycling amplification: lane 1, marker; lane 2, miR-141; lane 3, HR; lane 4, L1; lane 5, L2; lane 6, H1; lane 7, L3; lane 8, L1 + L2; lane 9, L2 + H1; lane 10, L1 + L2 + HR; lane 11, L1 + HR + miR-141; lane 12, L1 + L2 + HR + miR-141; lane 13, L1 + L2 + HR + miR-141 + H1; lane 14, L1 + L2 + HR + miR-141 + H1 + Zn^{2+} . **(B)** the HCR-mediated secondary target amplification: lane 1, marker; lane 2, P; lane 3, L3; lane 4, L4; lane 5, H2; lane 6, H3; lane 7, P + L4; lane 8, L3 + L4; lane 9, P + L3 + L4; lane 10, H2 + H3; lane 11, P + H2 + H3; lane 12, P + L4 + H2 + H3; lane 13, P + L4 + L3 + H2 + H3.

3.3. Characterization of the Fabricated Electrochemical Biosensor

The assembly process of spherical nucleic acids (SNAs) and spherical hybridization chain reaction (SHCR) was monitored by UV-vis absorption spectra. As shown in Figure 2A, a characteristic absorption spectrum was observed for the as-prepared gold nanoparticles (AuNPs), exhibiting a maximum absorption peak at 521 nm. Upon modification with P/L4 and L5 via an Au-S bond, the resulting construct designated P/L4-L5-AuNP (termed SNA) displayed a distinct DNA-specific absorption band at 260 nm, accompanied by a redshift of the AuNP surface plasmon resonance peak to 530 nm, indicating successful immobilization of DNA onto the AuNP surface. Notably, the absorbance intensity at 260 nm increased slightly upon formation of the HCR product mediated by GH2 and GH3, yielding the G-SHCR. Subsequent loading of MB, an additional characteristic absorption peak attributable to MB appeared at 663 nm [32], thereby confirming the complete assembly of the GM-SHCR. In addition, the morphology and dispersity of AuNPs and SNA were characterized by transmission electron microscopy (TEM). The AuNPs exhibited spherical morphology with an average diameter of approximately 13 nm. In contrast, the SNA exhibited improved dispersibility, attributable to the negatively charged phosphate backbone of the surface-immobilized DNA, which strengthened interparticle electrostatic repulsion and thereby suppressed aggregation (Figure S1).

The layer-by-layer assembly process on the working electrode was characterized by electrochemical impedance spectroscopy (EIS), with the semicircle diameter in the Nyquist plot quantitatively reflecting the charge-transfer resistance value (R_{et}). As shown in Figure 2B and Table S2, the bare gold electrode (AuE) exhibited a narrow semicircle in the Nyquist plot ($R_{et} \approx 170.2 \Omega$), attributable to its high intrinsic conductivity. Following immobilization of the thiol-modified capture DNA probe (S/AuE), the R_{et} increased to 8954 Ω . Subsequent blocking of nonspecific binding sites with MCH (MCH/S/AuE), the semicircle diameter in the Nyquist plot progressively increased ($R_{et} \approx 19,769 \Omega$), confirming stepwise functionalization of the electrode surface.

Remarkably, upon anchoring the GM-SHCR generated via the hairpin-structured DNAzyme-initiated SHCR homogeneous cascade amplification strategy onto the modified electrode (GM-SHCR/MCH/S/AuE), the charge-transfer resistance increased further ($R_{et} \approx 25,167 \Omega$). This is attributed to intensified electrostatic repulsion between the negatively charged phosphate backbone of DNA on the electrode surface and the $[\text{Fe}(\text{CN})_6]^{3-/4-}$, impeding electron transfer from the probe to the electrode interface. These results are consistent with the cyclic voltammetry (CV) (Figure S2), collectively supporting the successful fabrication of the electrochemical biosensor.

The feasibility of the constructed electrochemical biosensor was evaluated using differential pulse voltammetry (DPV). As illustrated in Figure 2C, compared with the bare AuE (curve a), the DPV signals for S/AuE (curve b) and MCH/S/AuE (curve c) showed only weak increases, attributable to nonspecific adsorption of MB at the modified electrode interfaces. A significantly enhanced DPV signal was observed when the L2 strand directly released from the target was captured onto the electrode interface (that is, no amplification, curve d), which originates from MB loaded onto the DNA phosphate backbone via electrostatic interactions. Following incubation with strand L3 generated from hairpin-structured DNAzyme-assisted target recycling amplification (Cycle I, curve e), the further enhanced DPV signal demonstrates its excellent amplification capability. In SHCR-mediated target amplification, employing GH2 alone (curve f) or GH3 alone (curve g) resulted in only marginal enhancements of the DPV current relative to the no amplification strategy (curve d), with GH2 eliciting a significantly higher signal intensity than GH3. This difference is primarily because GH2 can be captured onto the electrode surface and consequently results in greater loading of MB. Notably, the MB-loaded SHCR (GM-SHCR) generated by the hairpin-structured DNAzyme-initiated SHCR homogeneous cascade amplification strategy (Cycle I + Cycle II, curve i) yielded the highest DPV signal, confirming the high signal amplification efficiency of the proposed cascade strategy. Moreover, in comparison with the electrochemical signals obtained from homogeneous cascade amplification in solution and amplification directly on the modified electrode surface, a more stable and higher DPV response was observed via the homogeneous cascade amplification (Figures 2D and S3), ensuring the reliability and accuracy of electrochemical detection.

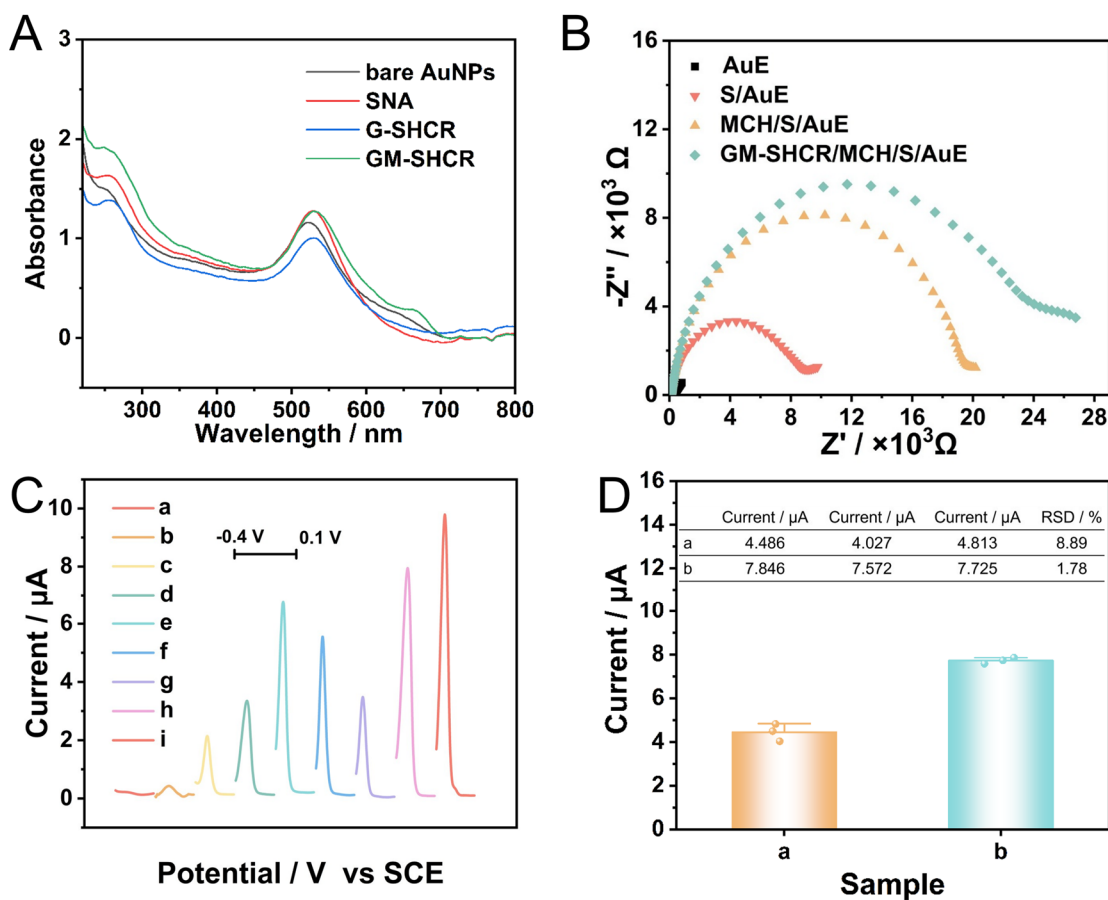


Figure 2. (A) UV–vis absorption spectra of bare AuNPs, SNA, G-SHCR, and GM-SHCR. (B) EIS characterization of the electrode modification process. (C) DPV responses of MB on different modified electrodes. (a) bare AuE; (b) S/AuE; (c) MCH/S/AuE; (d) target recognition without amplification; (e) DNAzyme-assisted target recycling amplification (Cycle I); (f) SHCR with GH2 only; (g) SHCR with GH3 only; (h) complete SHCR amplification

(Cycle II); (i) DNAzyme-assisted target recycling amplification+SHCR (Cycle I+Cycle II). **(D)** DPV responses comparison of (a) the electrode interface in situ amplification and (b) homogeneous amplification and then immobilization on the electrode interface. The concentration of miR-141 is 1 pM. Error bars indicate standard deviations from three repeated measurements.

3.4. Optimization of Electrochemical Test Conditions

To maximize the analytical performance of the electrochemical biosensor, several test conditions were optimized, with the performance reflected by the change in DPV peak current ($\Delta I = I - I_0$), where I and I_0 represent the peak currents in the presence and absence of miR-141, respectively. The concentration of MB critically influences the electrochemical signal intensity; thus, optimization of MB concentration is essential to achieve optimal detection performance. As shown in Figure 3A, the DPV response increased progressively with rising MB concentration and reached a maximum at 60 μM . A further increase in MB concentration led to a rapid decline in the DPV signal, which is mainly attributed to aggregation of the GM-SHCR induced by excessive MB.

Moreover, the Zn^{2+} concentration and reaction time critically influence the signal amplification efficiency of the DNAzyme. The change in ΔI increased progressively with increasing Zn^{2+} concentration and peaked at 20 mM (Figure 3B). Similarly, the maximum ΔI was observed after a reaction time of 30 min (Figure 3C). Accordingly, 20 mM Zn^{2+} and a 30 min reaction time were selected as the optimal conditions for hairpin-structured DNAzyme-mediated target recycling amplification. For the optimization of SHCR reaction time, ΔI increased progressively with prolonged reaction time and peaked at 4.0 h. A subsequent decline was observed at 5.0 h, likely attributable to substrate depletion (Figure 3D). Consequently, 4 h was determined as the optimal SHCR reaction time.

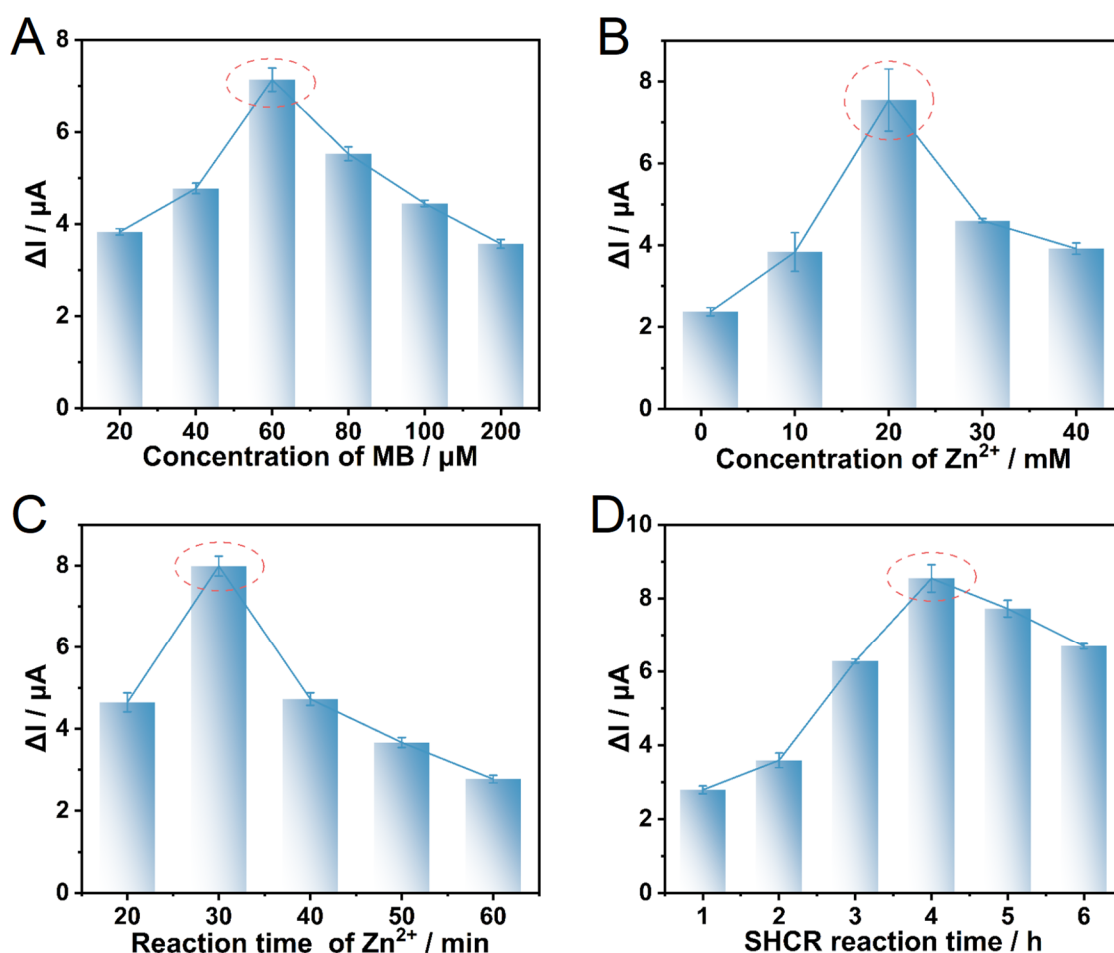


Figure 3. Optimization of the experimental parameters. **(A)** The MB incubation concentration. **(B)** The Zn^{2+} concentration and **(C)** reaction time in the DNAzyme amplification system. **(D)** The SHCR reaction time. The miR-141 concentration is 100 pM. Error bars represent the standard deviations from three repetitive measurements.

3.5. Evaluation of Analytical Performance of the Proposed Biosensor for miR-141 Detection

The analytical performance of the designed biosensor was evaluated by quantifying varying concentrations of miR-141 under optimized electrochemical test conditions. As shown in Figure 4A, the DPV response increased progressively with increasing miR-141 concentration over the range of 0.1 fM to 1 nM. A linear relationship was observed between the peak current at -0.28 V and the logarithm of miR-141 concentration (Figure 4B) with a limit of detection of 0.031 fM ($S/N = 3$), which was competitive with or even superior to these previously reported DNA cascade amplification-based biosensors for miRNA detection (Figure 4C and Table S3). Moreover, control experiments were conducted using hairpin-structured DNAzyme-assisted target recycling amplification (Cycle I) and SHCR (Cycle II) alone for the electrochemical detection of miR-141 across a range of concentrations (Figures S4 and S5). The corresponding limits of detection were determined to be 0.58 fM and 0.27 fM, respectively, which were approximately 19-fold and 9-fold higher than that achieved by the proposed homogeneous cascade amplification strategy. These findings confirm that the biosensor employing the integrated cascade amplification approach delivers significantly enhanced analytical sensitivity for the electrochemical detection of low-abundance microRNA-141.

Next, the selectivity and specificity of the biosensor were evaluated by measuring DPV responses at -0.28 V to various miRNAs (Figures 4D and S6). Compared with the DPV peak current containing miR-141 (l and m), the DPV signals of other RNAs (b-k) were similar to that of the blank (a), indicating that this biosensor possesses excellent sequence discrimination capability and can accurately distinguish miR-141 from various interferences, even single-base mismatched miR-141. The exceptional sensitivity and selectivity of this electrochemical biosensor stem primarily from two synergistic design features: (1) a rationally engineered hairpin-structured recognition probe, whose optimized thermodynamic stability effectively suppresses nonspecific background signals and ensures high-fidelity target discrimination, and (2) a homogeneous cascade amplification strategy that integrates DNAzyme-assisted target recycling amplification with a SHCR, thereby achieving exponential signal amplification while preserving system robustness against nonspecific interference and baseline noise.

Moreover, the reproducibility and operational stability of the biosensor were evaluated by measuring the DPV responses of five independently prepared electrode batches, all of which underwent the cascade amplification and surface modification procedures, toward miR-141 under the same experimental conditions. The DPV responses obtained from these electrodes exhibited no statistically significant variation, with a relative standard deviation (RSD) of 2.32%, confirming the excellent reproducibility of the biosensor (Figures 4E and S7). Furthermore, after storage of the capture DNA-immobilized gold electrode (S/AuE) at 4°C for five days, the DPV signal retained 92.56% of its initial signal intensity (Figure S8), confirming the satisfactory operational stability and storage stability of the fabricated biosensor. Therefore, the fabricated biosensor demonstrates satisfactory selectivity, specificity, and reproducibility, attributes that collectively enable accurate detection of miR-141 in complex biological matrices.

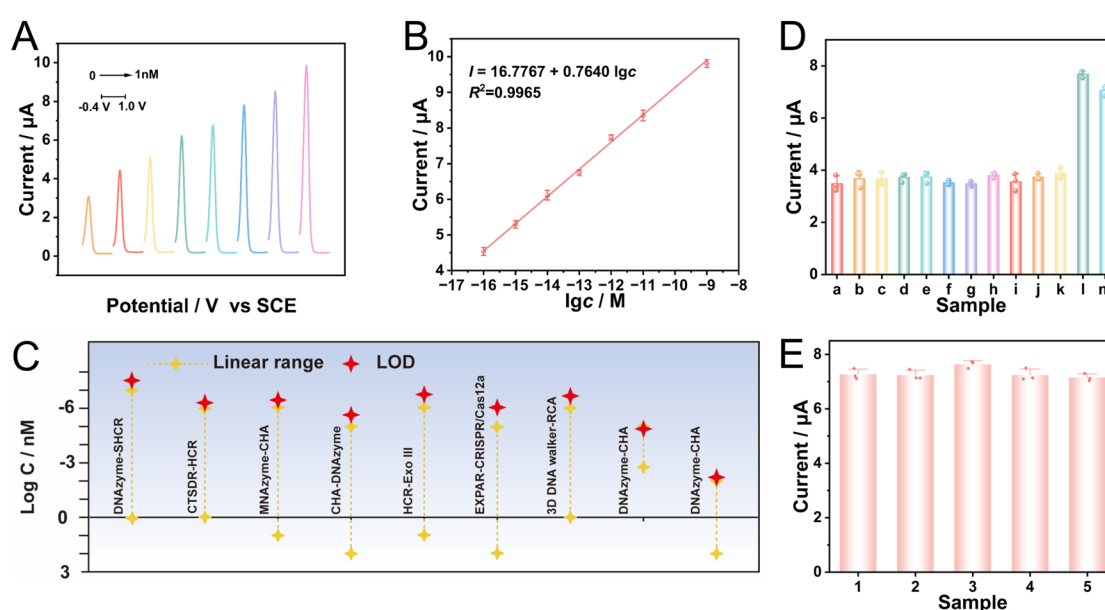


Figure 4. (A) DPV curves for different concentrations of miR-141 under the cascade amplification strategy (miR-141 concentrations: 0, 0.1 fM, 1 fM, 10 fM, 100 fM, 1 pM, 10 pM, and 1 nM). (B) Corresponding calibration curve

of peak currents versus the logarithm of miR-141 concentrations. (C) Comparison of this work with other cascaded amplification strategies. (D) Selectivity of the electrochemical biosensor: a. blank; b. survivin mRNA; c. miR-34b; d. miR-34c; e. miR-122; f. miR-107; g. let-7a; h. miR-144; i. TK1 mRNA; j. mis141-1; k. mis141-3; l. miR-141; m. mixture (miR-141 concentration: 1 pM, other RNAs: 100 pM). (E) Reproducibility analysis of the electrochemical biosensor (miR-141 concentration: 1 pM. Error bars represent the standard deviation of three replicate measurements).

3.6. Real Sample Analysis

To evaluate the reliability and applicability of the proposed electrochemical biosensor in real sample analysis, spike-and-recovery experiments were performed using 10% fetal bovine serum (FBS) as representative complex matrices. According to the calibration curve established using the standard miR-141 solution, three representative concentrations, namely high (0.1 nM), medium (1 pM), and low (0.1 pM), were spiked into preprocessed real sample matrices. As shown in Figure 5A, Figure S9, and Table S4, the recovery rates of spiked miR-141 ranged from 78.2% to 127%, with RSDs between 0.62% and 1.37%, indicating that the proposed electrochemical sensing platform exhibits satisfactory accuracy for the detection of miR-141 in complex biological samples. Furthermore, the electrochemical biosensor was applied to assess miR-141 expression levels in cervical cancer cells (HeLa cells) and human breast cancer cells (MDA-MB-231 cells). As shown in Figures 5B and S10, the DPV signal increased progressively with cell number across the range of 2×10^2 to 2×10^4 for MDA-MB-231 and HeLa cells relative to the blank control, confirming detectable expression of miR-141 in both cell lines. Notably, MDA-MB-231 cell lysates yielded a significantly higher DPV response than HeLa cell lysates, indicating elevated miR-141 expression in MDA-MB-231 cells, a finding consistent with previously published reports [33].

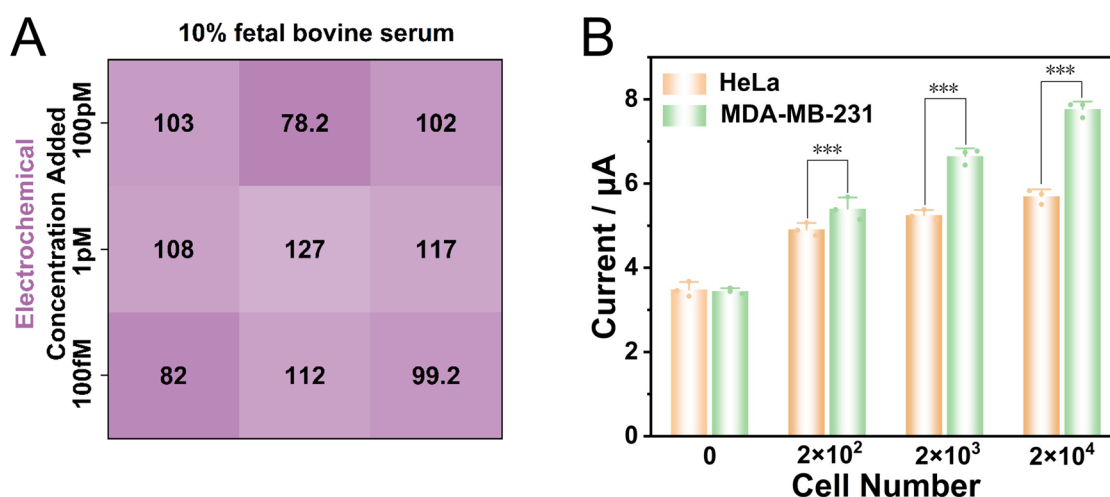


Figure 5. (A) Heat map of miR-141 recovery rates in a 10% FBS sample. (B) DPV current responses of miR-141 expression levels in cell lysates. Error bars represent the standard deviation of three replicate measurements (***) $p < 0.001$).

4. Conclusions

In summary, a highly sensitive electrochemical biosensing platform by synergistically integrating hairpin-structured DNzyme-assisted target recycling amplification with SHCR has been constructed for accurate detection of miR-141. This strategy achieves decoupling of target recognition, homogeneous amplification, and electrochemical signal transduction while incorporating an anti-leakage hairpin probe design, thereby simultaneously suppressing nonspecific signals and mitigating steric hindrance commonly encountered in conventional heterogeneous amplification systems, and endowing the biosensor with excellent accuracy and reliability for miR-141 detection in complex biological samples. Moreover, the proposed biosensor is readily adaptable for the multiplexed detection of microRNAs through simple substitution of the hairpin recognition probe sequences. Therefore, further shortening detection time and combining with portable electrochemical instrumentation, this homogeneous cascade amplification electrochemical sensing platform holds significant potential for rapid and large-scale clinical diagnosis of miRNA-associated diseases.

Supplementary Materials

The additional data and information can be downloaded at: <https://media.sciltp.com/articles/others/2608311017428450/NENP-26060196-SM.docx>. Figure S1: TEM images of (A) bare AuNPs and (B) SNA. Figure S2: CV characterization of the stepwise electrode modification process. Figure S3: DPV response of (a) the electrode interface in situ amplification and (b) homogeneous amplification and then immobilization on the electrode interface (miR-141 concentration: 1 pM). Figure S4: (A) DPV curves of this biosensor based on only the hairpin-structured DNAzyme-assisted target recycling amplification (miR-141 concentrations: 0, 1 fM, 10 fM, 100 fM, 1 pM, 10 pM, and 1 nM). (B) Corresponding calibration curve of peak currents versus the logarithm of miR-141 concentrations. Error bars indicate standard deviations from three repeated measurements. Figure S5: (A) DPV curves of this biosensor based on only the SHCR amplification (miR-141 concentrations: 0, 1 fM, 10 fM, 100 fM, 1 pM, 10 pM, and 1 nM). (B) Corresponding calibration curve of peak currents versus the logarithm of miR-141 concentrations. Error bars indicate standard deviations from three repeated measurements. Figure S6: DPV curves of the biosensor in response to a. blank; b. survivin mRNA; c. miR-34b; d. miR-34c; e. miR-122; f. miR-107; g. let-7a; h. miR-144; i. TK1 mRNA; j. mis141-1; k. mis141-3; l. miR-141; m. mixture (miR-141 concentration: 1 pM, other RNAs: 100 pM). Figure S7: DPV curves of this biosensor response to five batch-modified electrodes under the same condition; the concentration of miR-141 is 1 pM. Figure S8: (A) DPV curves and (B) peak current of the S DNA-immobilized electrodes stored for different days under 4 °C for the detection of miR-141 (1 pM). Error bars indicate standard deviations from three repeated measurements. Figure S9: DPV curves of miR-141 detection in fetal bovine serum. Figure S10: DPV curves of miR-141 expression levels in cell lysates. Table S1: All the DNA oligonucleotide sequences involved in this work. Table S2: Electron-transfer resistance (R_{et}) values obtained from EIS measurements during the stepwise electrode modification process. Table S3. Comparison of this work with other cascaded amplification strategies. Table S4: Spike-recovery experiments for miR-141 detection in 10% fetal bovine serum. References [34–41] are cited in supplementary materials.

Author Contributions

J.Y.: investigation, methodology, software, data curation, writing—original draft preparation; Y.Z. (Yuelin Zhou): methodology, verification, data curation; J.W.: data curation; S.Y.: conceptualization, supervision, investigation, funding support, writing—reviewing and editing; Y.Z. (Yuanzhen Zhou): supervision, funding support. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement

Data are contained within the article.

Conflicts of Interest

The authors declare no conflict of interest.

Use of AI and AI-Assisted Technologies

No AI tools were utilized for this paper.

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