

Communication

Highly Sensitive Electrochemiluminescence Analysis of miRNA-107 Using AIE-Active Polymer Dots as Emitters

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Abstract

The ultrasensitive detection of microRNA-17 (miRNA-107) is required for clinical diagnosis. In this work, an aggregation-induced electrochemiluminescence (AIECL) sensor was developed for the quantification of miRNA-107, in which AIECL-active polymer dots (Pdts) were characterized by transmission electron microscopy, ultraviolet–visible spectroscopy, and cyclic voltammetry and used as ECL emitters. Black hole quencher-labeled hairpin DNA (HP-BHQ) was modified on the Pdot surfaces, resulting in the ECL signal of the Pdts being in the “off” state due to the resonant energy transfer (RET) between the BHQ and Pdts. In the presence of miRNA-107, HP-BHQ opened through RNA–DNA hybridization. Subsequently, the introduced duplex-specific nuclease (DSN) facilitated the cleavage of DNA in the RNA–DNA hybrid chain and led to the detachment of HP-BHQ from the electrode surface. The ECL signal of the Pdts recovered, i.e., to the “on” state. The variation in the ECL signal was related to the concentration of the target miRNA-107. As a result, the AIECL biosensor exhibited a wide linear response to miRNA-107 concentrations ranging from 1.0 fM to 10.0 pM, and a low detection limit of 0.82 fM. This work provides a novel platform for the sensitive analysis of miRNA.

Keywords: aggregation-induced electrochemiluminescence; resonance energy transfer; polymer dots; duplex-specific nuclease; miRNA-107

1. Introduction

MicroRNA-107 (miRNA-107) is a non-coding molecule consisting of 23 nucleotides. miRNA-107 is widely recognized as a biomarker associated with various cancers, including gastric cancer [1], lung cancer [2], hepatocellular carcinoma [3], pancreatic ductal adenocarcinoma [4], and castration-resistant prostate cancer [5]. The aberrant expression of miRNA-107 has a significant impact on the onset and development of diseases. Consequently, it is clinically pivotal to establish a sensitive miRNA-107 detection platform for early cancer diagnosis and prognostic surveillance. Many methods have been employed for the detection of miRNA, such as surface-enhanced Raman spectroscopy (SERS) [6], surface plasmon resonance (SPR) [7], and electrochemiluminescence (ECL). ECL is triggered by an applied potential, inducing electron transfer to generate luminescence [8–13]. As an analytical method, ECL has been widely applied in the field of biosensing due to its high sensitivity, good selectivity, low background interference, and wide linear range [14–17],



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and it has attracted considerable attention in miRNA detection. Accurate measurement of miRNA using ECL technology still faces some challenges owing to miRNA's low molecular weight, low abundance, and high sequence similarity *in vivo* [18,19]. Several strategies have thus been developed to increase sensing sensitivity. The aggregation-induced ECL (AIECL) platform was fabricated to improve the ECL signal of luminophores due to the restricted molecular motion of luminophores in the aggregated state, increasing the radiative transition of excited species [20–23]. It is noteworthy that polymer dots (Pdots) were then applied AIECL due to their non-toxicity, facile synthesis, biocompatibility, substantial photostability, and high fluorescence quantum yields [24–26]. Wang et al. [27] successfully synthesized ABEI-Pdots, which exhibited low anode potential for ECL emission and were applied for the sensitive analysis of blood glucose with a detection limit of 3.3 μM .

Duplex-specific nuclease (DSN), through cleaving DNA in DNA–RNA double strands, is a promising approach for signal amplification, demonstrating high specificity and sensitivity in quantitative detection [28,29]. For instance, Xiao et al. [30] established an ECL sensor for ultrasensitive miRNA-107 detection by employing a novel silver metal–organic framework (AgMOF) coupled with a DSN-assisted target recycling amplification, achieving a low detection limit of 4.33 fM. Huo et al. [31] achieved a lower detection limit of 1 fM by applying a nanopore-based ECL sensor for ultrasensitive miRNA-107 detection, combined with DSN-assisted target recycling amplification. It is thus expected that the combination of AIECL with a DSN-mediated amplification strategy could improve sensors' analytical performance.

In this work, we fabricated a Pdots-based AIECL sensor for the ultrasensitive detection of miRNA-107 with the assistance of DSN. As shown in Figure 1, the prepared AIECL-active Pdots were first dropped onto the electrode surface. Black hole quencher-labeled hairpin DNA (HP-BHQ) was then linked with the Pdots through a condensation reaction between the amino groups and carboxyl groups. The ECL signal of the Pdots was successfully quenched by BHQ due to the resonance energy transfer (RET), consequently causing the signal to turn “off”. Upon the introduction of target miRNA-107, DSN severed the DNA fragment within the RNA–DNA hybrid chain. Subsequently, miRNA-107 was released and continued to open the next hairpin DNA, thereby achieving signal amplification. At the same time, the BHQ attached to the HP fell off the electrode surface as severed DNA fragments, leading the signal to turn “on”. The change in ECL intensity was proportional to the change in the concentration of miRNA-107. Accordingly, the AIECL sensor exhibited a good linear response in the miRNA-107 concentration range of 1.0 fM to 10.0 pM and a low detection limit of 0.82 fM.

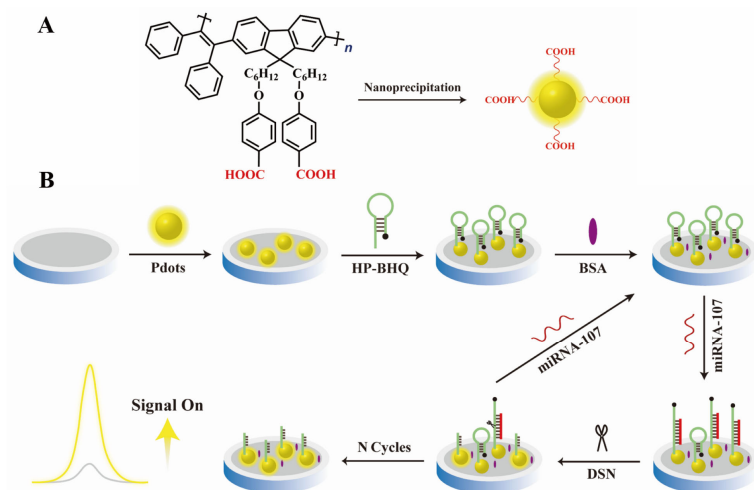


Figure 1. (A) Preparation of Pdots with AIECL activity. (B) Fabrication of AIECL sensor based on DSN-assisted target recycling amplification.

2. Materials and Methods

2.1. Apparatus

A JEM-2100 instrument (JEOL Ltd., Tokyo, Japan) was used to obtain the transmission electron microscopy (TEM) images. The UV–Vis spectra were acquired on a UV-3600 UV–vis–NIR spectrophotometer (Shimadzu, Kyoto, Japan). The fluorescence spectra were produced on a F-7000 fluorescence spectrometer (Hitachi Co., Tokyo, Japan). The ECL experiments were conducted with a MPI-A multi-functional electrochemical and chemiluminescent analytical system (Xi'an Remex Analytical Instrument Co., Xi'an, China). The electrochemical data were recorded on a CHI 760E electrochemical workstation (CHI Instruments Inc., Shanghai, China). The photoluminescence quantum yields and transient decay spectra were determined with an FLS980 spectrometer (Edinburgh Instruments, Livingston, UK).

2.2. Materials and Reagents

Triethylamine (TEA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), bovine serum albumin (BSA), tetrahydrofuran (THF, anhydrous, purity $\geq 99.9\%$) were purchased from Sigma-Aldrich Co. Ltd. (Shanghai, China). DSN and 10 $\times$ DSN master buffer (500 mM Tris-HCl, 50 mM MgCl₂, 10 mM D,L-dithiothreitol (DTT), pH 8.0) were purchased from Evrogen (Moscow, Russia). Each step in the construction of the ECL sensor was followed by a rinse with 0.1 M phosphate-buffered saline (PBS; pH = 7.4). The remaining reagents used were of analytical grade with higher purity. Ultrapure water was acquired from a Millipore water purification system (≥ 18 M Ω ·cm, Milli-Q, Millipore, Burlington, MA, USA). The nucleotide sequences employed in this work are shown in Table 1 and were synthesized by Sangon Biotech Co. Ltd. (Shanghai, China).

Table 1. Nucleotide sequences used in this work.

Name	Sequence (5'-3')
HP-BHQ	NH ₂ -(CH ₂) ₆ -TTTTTGAGGCGCAGTCTATGATAGCCCTG-TACAATGCTGCTTAGACTGCG-BHQ
miRNA-107	AGCAGCAUUGUACAGGGCUAUC
miRNA-122	UGGAGUGUGACAAUGGUGUUUG
miRNA-141	GGUAGAAAUGGUCUGUCACAAU
miRNA-21	UAGCUUAUCAGACUGAUGUUGA

2.3. Preparation of Pdots

According to previous research [32–34], the nano-coprecipitation approach was adopted to prepare the Pdots (Figure 1A). First, 100 μ g/mL of polymer stock solution in THF was prepared. Then, 2 mL of the prepared solution was promptly injected into 10 mL of ultrapure water under sonication conditions, and the mixture was further sonicated for 3 min. To obtain 2 mL of Pdot concentrate, excessive THF and water were eliminated by rotary evaporation under reduced pressure. Finally, the solution was filtered through a 0.22 μ m poly(ether sulfone) (PES) syringe filter, affording carboxylated Pdots with uniform dispersion.

2.4. Polyacrylamide Gel Electrophoresis (PAGE)

Both 100 μ L of miRNA-107 and HP solutions were separately prepared and then annealed at 95 $^{\circ}$ C for 5 min. After cooling to room temperature, 50 μ L each of the miRNA-107 and HP solutions was taken out, mixed thoroughly, and incubated at 37 $^{\circ}$ C for

2 h to form RNA–DNA heteroduplexes. Afterward, 50 μL of the mixture was reacted with sufficient DSN at 42.5 $^{\circ}\text{C}$ for 1.5 h to cleave the DNA in the RNA–DNA hybrid chain and release the target miRNA-107 into the circulation. Then, 10 μL of each of the miRNA-107, HP, the mixture of miRNA-107 and HP, and the mixture treated with DSN was separately mixed with 6 $\times$ loading buffer and finally loaded onto the apparatus for polyacrylamide gel electrophoresis (PAGE) analysis.

2.5. Construction of the ECL Sensor

The construction of the ECL sensor is shown in Figure 1B. A total of 10 μL of Pdots was dropped on the electrode surface and dried naturally at room temperature. To activate the carboxyl groups of the Pdots, the modified electrode was reacted with 0.1 M PBS (pH 7.4) containing 20 mM NHS and 10 mM EDC for 1 h at 37 $^{\circ}\text{C}$. Next, 1 μM HP-BHQ was introduced and incubated with the electrode for 12 h. Then, the electrode was flushed with 0.1 M PBS to remove the unbound HP, followed by blocking non-specific binding sites with 100 μM BSA. Afterward, the acquired electrode was immersed in 80 μL of miRNA-107 at different concentrations and subsequently incubated for 2 h at 37 $^{\circ}\text{C}$. Finally, the obtained electrode was incubated with sufficient DSN at 42.5 $^{\circ}\text{C}$ for 1.5 h and rinsed with 0.1 M PBS for the subsequent ECL scan.

3. Results and Discussion

3.1. Characterization of Polymer and Pdots

We first investigated the aggregated photoluminescence (PL) behaviors of the polymer in the THF/ H_2O mixtures with various water fractions (*fw*). As depicted in Figure S1, the polymer showed a weak PL signal when *fw* was below 50%. At *fw* = 60%, an obvious PL signal was observed, and the PL signal enhanced as *fw* increased. When *fw* = 95%, a maximum PL emission was reached. Afterward, the mixture exhibited a slight decrease in PL emission, which may have been due to the decrease in solubility [17]. Next, TEM was applied to investigate the morphology of the Pdots. Figure 2A shows that the prepared Pdots exhibited a typical spherical morphology with a uniform size overall and high dispersibility without any apparent agglomeration. Furthermore, 100 randomized nanoparticles were statistically analyzed with the intention of exactly determining the particle size distribution of the Pdots. The result indicated that the average size of the Pdots was around 3.3 nm (inset in Figure 2A).

In addition, we collected UV–Vis and PL spectra to explore the photophysical properties of the Pdots. As presented in Figure 2B (black line), the UV–Vis spectrum of the Pdots possessed obvious absorption peaks in the vicinities of 250 nm and 355 nm. The former was caused by the π – π^* transition of the side-chain *p*-hydroxybenzoic acid units, whereas the latter was ascribed to the π – π^* transition of the main polymer chain [33]. As presented in Figure 2B (red line), the PL spectrum revealed that the Pdots displayed a distinct emission peak at 500 nm, with an absolute PL quantum yield up to 23.29%, as measured by the integrating sphere. Additionally, the PL transient spectrum of the Pdots was measured, and the corresponding luminescent lifetime was determined to be 2.75 ns, which indicated that Pdots are a typical fluorescent dye (Figure S2).

Additionally, to explore the ECL behavior of the Pdots, cyclic voltammetry (CV) and ECL experiments were further conducted. There were two irreversible oxidation peaks observed in the CV curve (Figure 2C). The lower peak at +0.9 V corresponded to the oxidation process of the co-reactant, TEA; the higher at +1.16 V originated from the oxidation reaction of the Pdots. This was consistent with the Pdots generating a significant ECL signal at a +1.2 V potential (Figure 2D). As reported in previous research [35], the potential mechanism through which Pdots generate ECL is outlined in the Supporting

Information (Equations (S1)–(S5)). The above results demonstrate that the Pdots are viable for ECL.

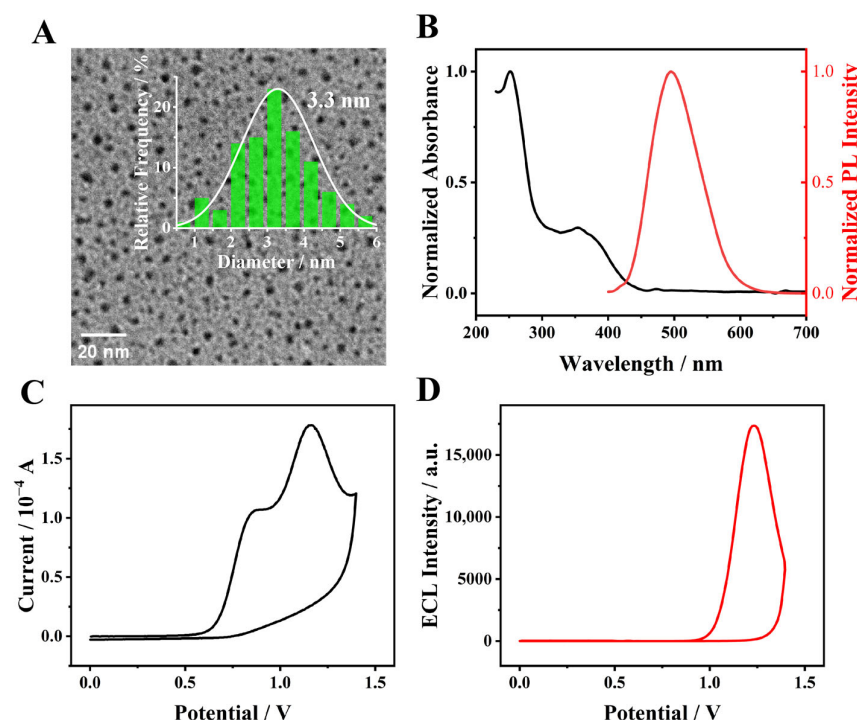


Figure 2. (A) TEM image of Pdots (inset: particle size distribution). (B) UV–Vis spectrum (black line) and PL spectrum (red line) of Pdots. (C) CV curve and (D) ECL intensity–potential curve of Pdots in presence of 25 mM TEA as co-reactant.

3.2. Feasibility of Sensing Strategy

To verify the feasibility of DSN-assisted target recycling amplification, we used PAGE to elucidate the reaction system. Figure 3A illustrates every step of the DSN signal amplification reaction, with Figure 3B correspondingly demonstrating the experimental result. Lane 1 (miRNA-107) and lane 2 (HP) showed the typical migration bands of single-stranded nucleic acid. Compared with lane 1 and lane 2, a new band with a slower migration rate appeared in lane 3 (miRNA-107 and HP hybrids), indicating the successful formation of hybrid duplexes between miRNA-107 and HP. In contrast to lane 3, the band of hybrid duplexes disappeared, while the band for miRNA-107 reappeared in lane 4 (miRNA-107 and HP hybrids treated with DSN). This demonstrated that the DSN was able to specifically cleave the DNA fragments of the hybrid duplexes, consequently releasing the entire miRNA-107. Following its introduction into the circulation, the miRNA-107 combined with the remaining HP to create more hybrid duplexes, which eventually vanished completely following an identical process. Thus, the sensing strategy is reasonable and feasible.

The ECL emitter Pdots had extremely stable and effective luminescence. When the Pdots were dropped on the electrode surface, they exhibited a strong initial ECL signal. After the addition of HP, the HP-modified BHQ attached to the Pdots due to the condensation reaction between the amino and carboxyl groups. Meanwhile, the BHQ modification at the 3' terminus of the HP quenched the ECL signal of the Pdots through RET. In this instance, the signal turned “off”. Upon the introduction of the target miRNA-107, HP and miRNA-107 formed complementary DNA–RNA hybrid duplexes. With the cleavage of the DSN, the miRNA-107 was released from hybrid duplexes and entered the subsequent circulation, resulting in BHQ moving away from electrode surface, so the signal turned “on”. As the miRNA-107 concentration increased, the BHQ amount on the electrode surface

decreased, while the ECL signal significantly enhanced. Accordingly, the concentration of miRNA-107 was detected by monitoring the intensity of the ECL signal change.

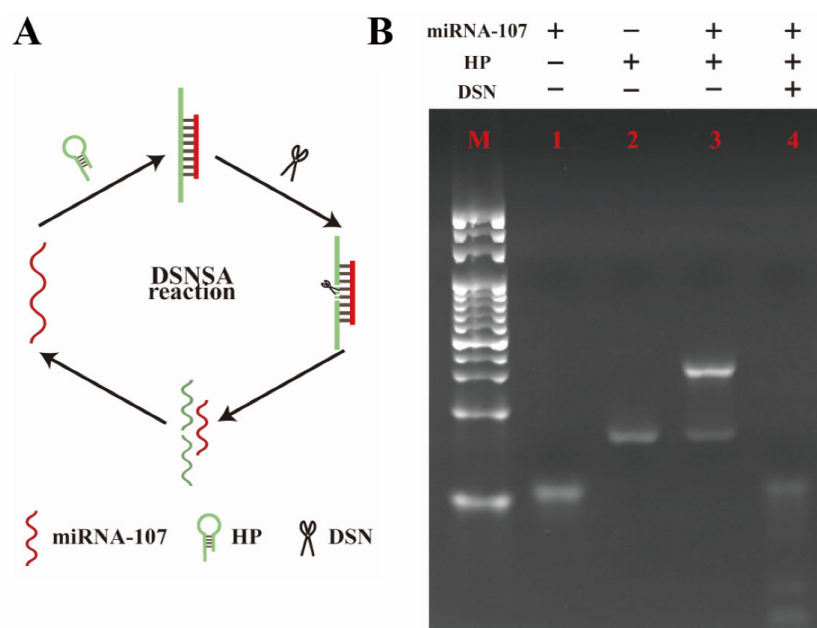


Figure 3. (A) Schematic illustration of DSN signal amplification reaction. (B) PAGE reaction system: (1) miRNA-107, (2) HP, (3) miRNA-107 + HP, (4) miRNA-107 + HP + DSN. M indicates marker.

3.3. Performance of ECL Sensor

We evaluated the analytical performance of the AIECL sensor in terms of linearity, reproducibility, and selectivity. As the concentration of miRNA-107 gradually increased from 1.0 fM to 10.0 pM, the ECL response intensity exhibited a distinct rising trend (Figure 4A). The ECL changes (ΔECL) showed an excellent linear relationship with the logarithm of the miRNA-107 concentration, as described by the linear fitting equation $\Delta\text{ECL} = 33,227.669 + 2179.83 \times \lg\text{CmiRNA-107}$ ($R^2 = 0.997$), and the LOD was calculated to be 0.82 fM based on an S/N of three (Figure 4B). These data were significantly lower than those in previous reports (Table S1).

The relative standard deviation (RSD) of the ΔECL of three AIECL sensors that we assembled under the same experimental conditions was estimated to be 2.9%, indicating that the sensor possessed excellent reproducibility (Figure 4C). Moreover, utilizing the sensor to separately detect miRNA-122, miRNA-141, miRNA-21, and miRNA-107, we observed that the ΔECL demonstrated a prominent change only in for miRNA-107, while the signal change in the other miRNAs was negligible (Figure 4D). This result indicated that the sensor showed favorable selectivity.

To confirm the sensor's detection ability in practical applications, we employed the standard addition method to quantitatively analyze miRNA-107 using fetal calf serum as the real sample. As shown in Table 2, the recovery ranged from 85% to 114%, and RSD ranged from 2.7% to 8.1%. The results indicate that this AIECL biosensor possesses good accuracy for clinical testing.

Table 2. Detection results of miRNA-107 in spiked fetal calf serum samples ($n = 3$).

Sample	Added	Detected	Recovery (%)	RSD (%)
1	1.00 fM	1.07 fM	107	7.6
2	100 fM	1.14 fM	114	8.1
3	1.00 pM	8.53 pM	85	2.7

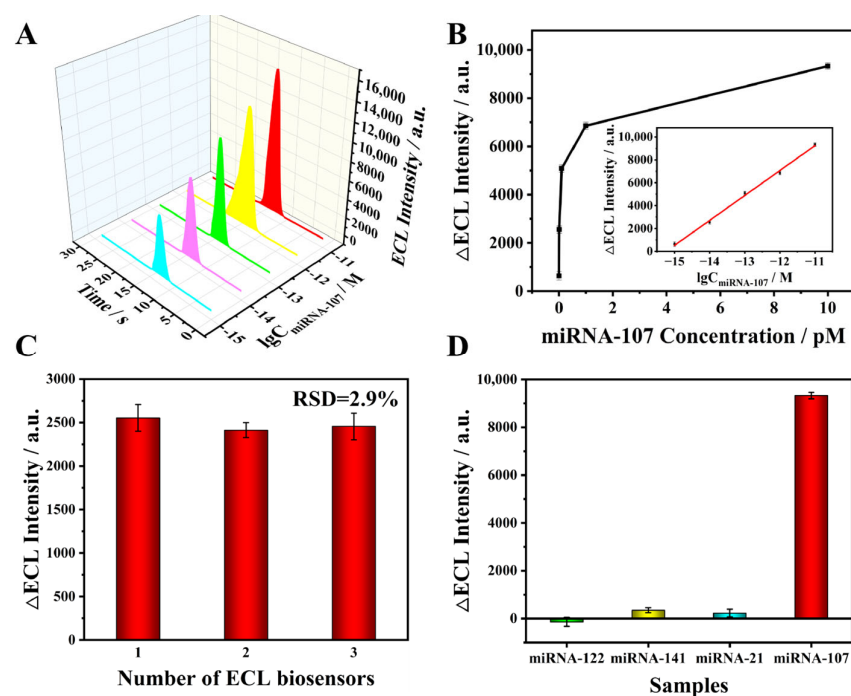


Figure 4. (A) ECL response to 1.0 fM–10 pM miRNA-107. (B) Relationship of 1.0 fM–10.0 pM miRNA-107 and ΔECL intensity (inset: linear relationship). (C) Reproducibility of ECL sensor detection of 10 fM miRNA-107. (D) ECL signal comparison of 10 pM miRNA-107 and other miRNAs. (Error bars indicate standard deviation of three sets of parallel experiments.)

4. Conclusions

In summary, a biosensing platform for the ultrasensitive detection of miRNA-107 was successfully fabricated utilizing AIECL-active Pdts as the signal probe and BHQ as the quencher. Upon the introduction of the target and DSN, RNA–DNA hybridization and DNA cleavage triggered the detachment of BHQ from the surface of the Pdts, resulting in the switching of the ECL signal from “off” to “on”. The quantitative analysis of the target miRNA-107 was performed by measuring the changes in ECL intensity. The proposed AIECL sensor exhibited highly sensitive quantification of miRNA-107 with a detection limit of 0.82 fM. This biosensing system could be adapted to detect other miRNAs by adjusting the base sequence of the capture probe, providing a promising reference for clinical analysis.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/bios16020099/s1>. The possible ECL generation mechanism of Pdts; Figure S1: PL behaviors of polymer in THF/H₂O mixtures; Figure S2: Fluorescent lifetime of Pdts; Table S1: Comparison of this method with reported methods in terms of miRNA detection limit and dynamic range; References [32,36–46] are cited in the Supplementary Materials.

Author Contributions: Conceptualization, Z.-H.X. and H.G.; methodology, Z.-H.X., X.W., R.-M.L., H.T. and Y.G.; investigation, Z.-H.X., X.W., R.-M.L., H.T. and Y.G.; formal analysis, Z.-H.X., X.W., R.-M.L., H.T., Y.G., L.-S.Y. and Q.X.; data curation, L.-S.Y. and Q.X.; writing—original draft preparation, Z.-H.X., L.-S.Y. and Q.X.; writing—review and editing, L.-S.Y., Q.X. and H.G.; supervision, Q.X. and H.G.; project administration, Q.X. and H.G.; funding acquisition, Z.-H.X., L.-S.Y. and H.G. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Dataset available on request from the authors.

Conflicts of Interest: The authors declare no conflicts of interest.

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