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View Article Online
DOI: 10.1039/C8CC06102E

Journal Name

COMMUNICATION

An Ultrasensitive Electrochemiluminescence Biosensor for Multiple Detection of microRNAs Based on a Novel Dual Circuit Catalyzed Hairpin Assembly

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

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A novel double-hairpin DNA inducing dual circuit catalyzed hairpin assembly (DC-CHA) strategy was proposed to fabricate electrochemiluminescence (ECL) biosensor for multiple targets (microRNA-21 and microRNA-155) ultrasensitive detection.

MicroRNA (miRNA) is found in eukaryotes, which has the function of regulation and control of a class of endogenous noncoding small RNA molecules with 19~23 nucleotides approximately.[1-3] By analyzing the site of miRNA in the genome, miRNA plays a crucial role in regulating cell growth and tissue differentiation processes. Recently, it has been found that the expression of miRNA has a direct relationship with many diseases for early diagnosis.[4,5] Two endogenous and noncoding RNAs, miRNA-21 with 22 nucleotides and miRNA-155 with 23 nucleotides are considered as significant tumor biomarkers for high expression in breast cancer, cervical cancer, prostate cancer and so on.[6,7] Nonetheless, due to the short length, sequence homology and low abundance of miRNA, accurate detection is still challenging. The traditional methods such as northern blotting,[8] cloning[9] and microarray-based techniques[10,11] are widely used for miRNA detection. However, these technologies often have some flaws, such as low sensitivity, poor specificity, high cost, time-consuming and complex instruments.[12] And for the moment, most of the existing researches are focused on the sensitive detection of single-target for miRNA.[13] Significantly, the multi-target detection can provide more reference information for the diseases and improve the diagnostic efficiency, hence it is crucial to research the sensitive detection of different miRNAs in the same system.

In recent years, electrochemiluminescence (ECL) biosensor research gradually become a hotspot in biomarker analysis and early cancer detection due to its unique advantages of simple operation, rapid responsibility, high sensitivity, strong controllability and low background signal.[14-16] In order to obtain more information about the cancer and improve the diagnose efficiency, the ECL detection of multi-component has received extensive attention from researchers. Jiang and co-workers constructed a biosensor for multi-component detection, which achieved the determination of two antigens at the cell surface based on the potential-resolved ECL.[17] Recently, we also adopted dual miRNAs-fueled DNA nanogears[6] and dual miRNA powered bi-directional DNA walking machines[18] to achieve the multiple sensitive detection of the miRNA biomarkers. Although those works realized the detection of multi-component, the use of biosensors in the detection of ultra-low concentrations of biomolecules was still limited due to the lack of signal amplification strategies. Hence, it was necessary to construct new biosensors for multi-component determination with high sensitivity. Here, an ultrasensitive ECL biosensor was constructed for multiple targets (miRNA-21 and miRNA-155) detection based on a double-hairpin DNA inducing dual circuit amplified strategy with ECL resonance energy transfer (ERET) and ECL double quenching effect.

For improving the ECL sensitivity, the amplification strategy of nucleic acids including polymerase chain reaction (PCR),[19] strand displacement amplification (SDA),[20] rolling circle amplification (RCA)[21] and catalyzed hairpin assembly (CHA)[22] are proposed due to the signal enhancement capability, which are mature in the construction of biosensors. Among them, CHA as an enzyme-free amplification strategy has attracted particular attention, because it can not only effectively enhance the sensitivity of miRNA detection with negligible background, but also greatly simplify the experimental conditions and reduce the experimental costs.[23,24] Zhen's group reported CHA, an enzyme-free DNA circuit, and an assisted graphene oxide (GO) amplified fluorescence anisotropy (FA) strategy for miRNA-21 detection.[25] Liu's group designed an enzyme-free ratiometric fluorescent sensor based on CHA with a high sensitivity and selectivity for miRNA-122 detection.[26] However, the traditional CHA didn't have a

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Electronic supplementary information (ESI) available: Experimental details and additional figures. See DOI: 10.1039/x0xx00000x

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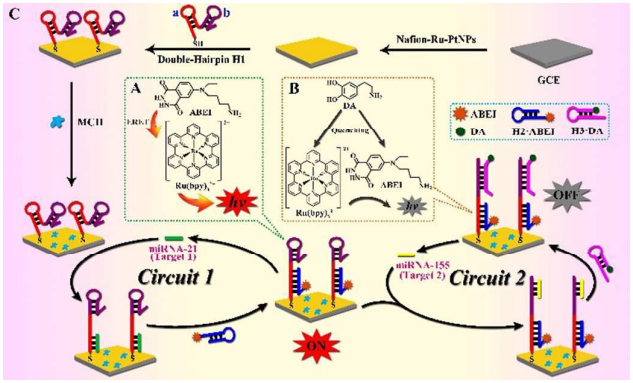
DOI: 10.1039/C8CC06102E

sufficiently high amplification efficiency.[27] Here, in order to further increase the amplification efficiency and detection sensitivity, a novel dual circuit CHA (DC-CHA) strategy induced by a special double-hairpin DNA was designed and applied to the construction of ultrasensitive ECL biosensor, achieving ultrasensitive targets (microRNA-21 and microRNA-155) detection. The superiorities of using the special double-hairpin DNA to induce DC-CHA were in two aspects. First, the designed double-hairpin DNA structure could achieve DC-CHA, and it could conveniently and efficiently detect multi-component in the process. Second, in the DC-CHA process, two targets could be cycled efficiently to achieve high amplification efficiency and detection sensitivity.

Herein, a novel double-hairpin DNA inducing DC-CHA strategy was proposed, based on which an ECL biosensor with ultrahigh detection sensitivity was constructed to achieve simple and effective detection of multiple miRNAs. Scheme 1 showed the construction of the proposed biosensor and the mechanism of signal-on and signal-off. When the mixture of $Ru(bpy)_3^{2+}$ and Pt nanoparticles (PtNPs) dispersed in Nafion solution (Nafion-Ru-PtNPs) was dropped onto a clean glass



Scheme 1 (A) The Signal Enhanced Mechanism, (B) the Signal Quenched Mechanism, (C) Construction of the ECL Biosensor.

carbon electrode (GCE), an initial ECL signal was generated. The synthetic procedures and characterization of Nafion-Ru-PtNPs could be found in electronic supplementary information (ESI). And then, H1 with a sulfhydryl was immobilized on the Nafion-Ru-PtNPs layer by the Pt-S bond. In the presence of target 1 miRNA-21, a-hairpin of H1 was opened. Then miRNA-21 could be replaced by H2 labeled with ABEI (H2·ABEI), and the released miRNA-21 would attend the next cycle, which finally made a large amount of H1-H2·ABEI be immobilized on the electrode surface. Simultaneously, ABEI, as an effective energy donor, could greatly enhance the ECL signal of $Ru(bpy)_3^{2+}$ (energy acceptor), achieving significantly enhanced ECL signal (signal-on) and reaching sensitive detection of target 1. Next, similar to the first circuit described above, target 2 miRNA-155 that hybridized with b-hairpin of H1 could be displaced from the structure by H3 labeled with DA (H3·DA). And the released miRNA-155 was available for initiating next cycle, resulting in hundreds of duplexes (H1-H2·ABEI-H3·DA) formation on the electrode. There was a distinct decline of the ECL signal (signal-off) due to the ECL double quenching effect of DA for both ABEI and $Ru(bpy)_3^{2+}$, achieving sensitive detection of target 2. The development of this novel DC-CHA strategy has provided a simple and ultrasensitive platform for the multiple ECL detection of miRNAs, which holds great potential for biomedical research and early clinical diagnosis. It's worth noting that all of the following ECL signals were measured in 2 mL detection phosphate buffer (PBS, 0.1 M, pH = 7.0) by scanning the potential from 0.2 to 1.25 V at a scanning rate of 100 mV/s.

To further verify the availability of the DC-CHA strategy, the biosensor was used for the ECL detection with different concentrations of the two targets. As depicted in Figure 1A, the ECL signal of the proposed biosensor enhanced with the concentration of miRNA-21 ranging from 50 aM to 1 nM (curve a to i). The graph showed that there was a positive correlation between ECL intensity and the logarithmic value of miRNA-21 concentration, and the linear regression equation was I_1 = 4033.02+1315.83 lgc_1 (the ECL intensity was replaced by I_1 and the concentration of miRNA-21 was replaced by c_1). In addition, the squared correlation coefficient was calculated to be 0.992 and the estimated limit of detection (defined as LOD = 3SB/m, where m is the slope of the corresponding calibration curve and S_B is the standard deviation of the blank) was 14.8 aM according to reference.[28] Simultaneously, it could be observed from Figure 1B that increasing the concentration of miRNA-155 (from 20 aM to 1 nM, curve a to i) would lead to prominent decrease in ECL response under the constant concentration of miRNA-21 (1 nM). Furthermore, the linear regression equation for miRNA-155 was I_2 = 8501.13—1104.17 lgc_2 (the ECL intensity was replaced by I_2, and the concentration of miRNA-155 was replaced by c_2) with the squared correlation coefficient of 0.994 and LOD of 5.3 aM.

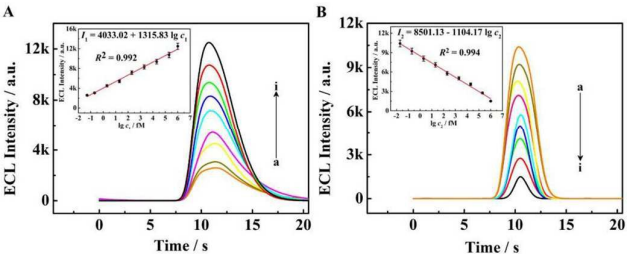


Figure 1 (A) ECL intensities detection of the proposed biosensor modified with target 1 (miRNA-21) for different concentrations: (a) 50 aM, (b) 200 aM, (c) 2 fM, (d) 20 fM, (e) 200 fM, (f) 2 pM, (g) 20 pM, (h) 200 pM, (i) 1 nM. (B) ECL responses detection for target 2 (miRNA-155) at various concentrations: (a) 20 aM, (b) 200 aM, (c) 2 fM, (d) 20 fM, (e) 200 fM, (f) 2 pM, (g) 20 pM, (h) 200 pM, (i) 1 nM. All ECL signals were measured in 2 mL PBS (0.1 M, pH = 7.0) by scanning the potential from 0.2 to 1.25 V at a scanning rate of 100 mV/s.

Stability and reproducibility were two other important aspects of the biosensor performance. So as to fully demonstrate the linear accuracy of this system, the continuous cyclic potential scans were implemented for 10 cycles, and the concentrations of miRNA-21 and miRNA-155 were 1 nM and 20 pM, respectively. As shown in Figure 2A and Figure 2B, the ECL intensity of each cycle exhibited no significant fluctuation, indicating the favorable stability of the biosensor. Moreover,

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from Figure 2C, RSD of intra-assay and inter-assay were 2.64% and 2.69% for the mRNA-155 detection, respectively, which proved the reproducibility of this biosensor was acceptable.

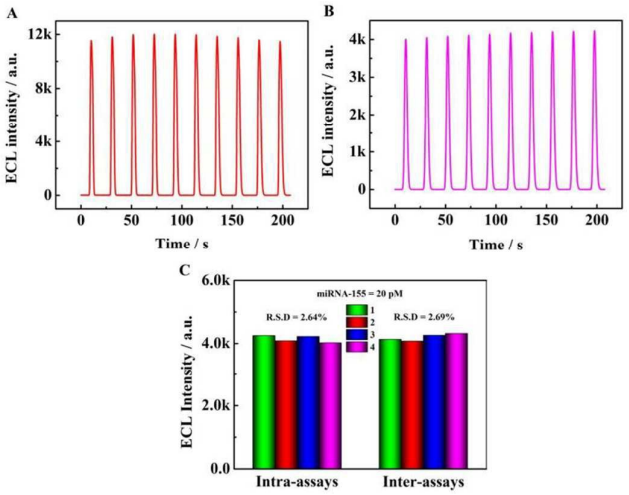

Figure 2 Stability of the biosensor under a continuous cyclic potential scans for (A) 10 cycles (1 nM miRNA-21) and (B) 10 cycles (20 pM miRNA-155) in 2 mL PBS (0.1 M, pH = 7.0) by scanning the potential from 0.2 to 1.25 V at a scanning rate of 100 mV/s. (C) Reproducibility of the proposed biosensor.

Selectivity was another important indicator to investigate the performance of this proposed biosensor. For the first circuit, interferences including miRNA-141 (2 nM), miRNA-let-7a (2 nM) and miRNA-155 (2 nM) were used to explore the biosensor selectivity, and the experimental results could be seen in Figure 3A. The first CHA process was initiated by miRNA-21 due to the specific recognition of the sequence, and then the following amplification reaction could be generated. So we could observe that ECL intensity was significantly enhanced when miRNA-21 existed, no matter the pure miRNA-21 (20 pM) solution or the mixture containing miRNA-21 (20 pM). Nevertheless, under the same experimental conditions, the ECL responses showed no significant differences between the blank group and other interfering miRNA due to the sequence of interfering miRNA could not specifically recognize a-hairpin of H1. Similarly, for the second circuit, the first circuit has been completed at the moment. And in order to make the phenomenon more obvious, we chose the maximum concentration of miRNA-21 (1 nM) in the linear range. Based on the completion of the first CHA process, the second CHA process was initiated by miRNA-155 due to the specific recognition of the sequence. As could be seen from Figure 3B, when the pure miRNA-155 (20 pM) and the mixture containing miRNA-155 (20 pM) were detected, there was a remarkable reduction in ECL intensity. As expected, the ECL intensity was almost the same as the blank group while the interference substances were detected under the same experimental conditions due to the sequence of interfering miRNA could not specifically recognize b-hairpin of H1. All the above results indicated that there were significant changes in the ECL intensity if the target existed comparing to the blank group and separate interferences, it suggested that the detection of miRNA-21 and miRNA-155 had excellent selectivity and specificity.

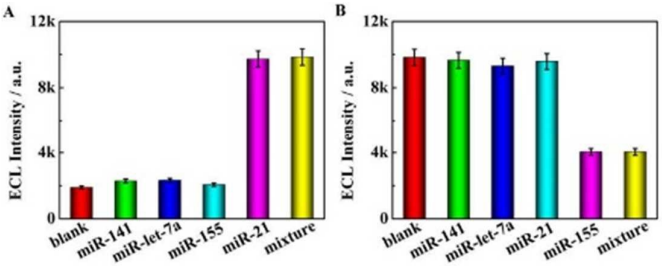

Figure 3 Selectivity of the miRNA biosensor with different targets: (A) blank, miRNA-141 (2 nM), miRNA-let-7a (2 nM), miRNA-155 (2 nM), miRNA-21 (20 pM), and the mixture containing miRNA-21(20 pM). (B) blank, miRNA-141 (2 nM), miRNA-let-7a (2 nM), miRNA-21 (2 nM), miRNA-155 (20 pM), and the mixture containing miRNA-155 (20 pM). All ECL signals were measured in 2 mL PBS (0.1 M, pH = 7.0) by scanning the potential from 0.2 to 1.25 V at a scanning rate of 100 mV/s.

To further verify the practical applicability of this proposed biosensor, we examined the expression of miRNA-21 and miRNA-155 in different biological cell lysates. The experimental procedures for cell analyses could be found in ESI. The cell samples were processed by a commercial miRNA extraction kit after cell counting. As presented in Figure 4A, the ECL intensity of the biosensor incubated with miRNA-21 in human breast cancer cells (MCF-7) increased distinctly with the number of MCF-7 increasing from 0 to 10^5. However, the figure showed a negligible change of ECL in cervical cancer cells (Hela) lysates which was similar to the blank one. Similarly, for the second circuit, in order to ensure the accuracy of the miRNA-155 analysis, we introduced the maximum number of MCF-7 cells (10^5) in advance to complete the first circuit. Based on the completion of the first CHA process, the second CHA process was initiated by miRNA-155. As shown in Figure 4B, when the number of MCF-7 cells incubated on the sensing platform increased from 0 to 10^5, the ECL signal of the biosensor incubated with miRNA-155 was reduced prominently. However, the lysate from Hela cells depicted a significant ECL response (b) which was in accordance with the blank detection (a). And the slope of the decline with MCF-7 was slightly more pronounced than Hela (including the blank detection). Therefore, the above consequence showed that miRNA-21 was overexpressed in

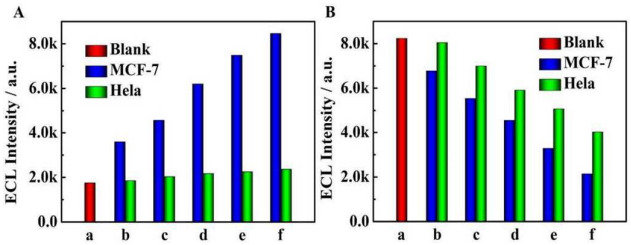

Figure 4 ECL intensity for the detection of (A) miRNA-21 and (B) miRNA-155 from MCF-7 and Hela cells: (a) blank detection without miRNA, (b) 10, (c) 10^2, (d) 10^3, (e) 10^4, (f) 10^5 in 2 mL PBS (0.1 M, pH = 7.0) by scanning the potential from 0.2 to 1.25 V at a scanning rate of 100 mV/s.

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DOI: 10.1039/C8CC06102E

MCF-7 instead of Hela, and miRNA-155 was expressed in MCF-7 slightly more than Hela. It showed a good agreement with the previously published related literature.[6] Hence, this ECL biosensor provided a feasible and promising strategy for miRNA detection in cancer cells.

Conclusions

In summary, an ultrasensitive ECL biosensor was fabricated for multiple detection of miRNAs (miRNA-21 and miRNA-155). A novel double-hairpin DNA inducing DC-CHA strategy was used to achieve the quantitative detection for multiple targets with ultrahigh detection sensitivity and high selectivity. The advantages and characteristics mainly included the following two aspects. On the one hand, the DC-CHA strategy combined two CHA processes with dual circuit amplification of targets achieved ultrahigh detection sensitivity in ECL biosensor construction. On the other hand, in the process of DC-CHA, the ECL quantitative detection of miRNAs was realized effectively. Meanwhile, the biosensor was simple to construct and the experiment was easy to operate. Considering the outstanding sensitivity, stability, and selectivity, the use of a special DNA structure design and a novel construction strategy of ECL biosensor to achieve multi-component detection was very ingenious. Meanwhile, the construction of ECL biosensor to achieve multi-component ultrasensitive detection not only could expand the application of ECL, but also could provide more reference information for diseases and improve the efficiency of diagnose.

Conflicts of interest

There are no conflicts to declare.

Acknowledgement

This work was financially supported by the National Natural Science Foundation of China (Grant Nos. 21575116, 21675129, 51473136, and 21775124), the Fundamental Research Funds for the Central Universities (XDJK2017C023, XDJK2018AA003), the Chongqing Research Program of Basic Research and Frontier Technology (cstc2017jcyjA1305), the China-Singapore Plan (Chongqing) and the Chongqing Postdoctoral Science Foundation (Xm2017016).

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