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A highly sensitive ratiometric electrochemiluminescent biosensor for microRNA detection based on cyclic enzyme amplification and resonance energy transfer†

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A ratiometric electrochemiluminescent biosensor for the detection of microRNAs based on cyclic enzyme amplification and distance dependent resonance energy transfer was reported for the first time.

Mature microRNAs (miRNAs) are a class of single-stranded, short, non-protein-coding RNAs with a typical length of 19–23 nucleotides.[1] Over 2000 miRNAs have been identified in humans, and they regulate nearly 30% of all human genes through a combinatorial regulation mode.[2] Many research studies have proven that miRNAs play a significant role in post-transcriptional regulatory process in a broad range of animals and plants.[3] Therefore, miRNAs are valuable biomarkers for clinical diagnosis and the sensitive detection of multiplex miRNAs is of great significance. However, the expression analysis of miRNAs is still a challenge because of their unique characteristics including short length, low cellular abundance and sequence homology among family members. Besides traditional methods, such as Northern blot, microarrays and quantitative reverse transcriptase PCR (RT-PCR), new strategies are being developed to improve sensitivity and simplify the detection procedure.

Electrochemiluminescence (ECL) has attracted growing attention due to its inherent advantages. This technique has been widely applied in immunoassay, DNA detection, cancer screening and environmental analysis.[4] ECL signal generation is a synergistic effect of light-emitting materials and appropriate emission potential. The detection is usually based on the single emission intensity changes. False positive or negative errors may occur during the detection of trace level analytes due to instrumental efficiency or some environmental change. Ratiometric assay, in which the quantification depends on the ratio of two signals instead of absolute values, is an ideal approach to eliminate these interference factors and make the detection more convincing.[5] In recent studies, we have developed the first ECL ratiometric sensing approach based on the large absorption cross-section and catalytic ability of luminol-modified Pt NPs.[6] But this method requires the target analyte to be functionalized with the ECL reagent before use, which limits its practical applications.

Enzyme-assisted amplification is a powerful tool in bioanalysis. Many miRNA detection technologies include this process to increase sensitivity. However, we noticed that there were few reports that incorporated the advantages of enzyme amplification and ratiometry in the field of ECL sensors. Duplex-specific nuclease (DSN), a nuclease isolated from hepatopancreas of the Kamchatka crab, would selectively cleave double-stranded DNA or DNA in DNA–RNA heteroduplexes and show little activity for single-stranded DNA, single- or double-stranded RNA.[7] Using this feature, here we present an ECL ratiometric assay with cyclic enzymatic amplification for the first time. And the sensitivity is further improved by the distance dependent resonance energy transfer between gold nanoparticles (Au NPs), luminol–gold nanoparticles (L–Au NPs) and CdS semiconductor nanocrystals (NCs). As shown in Scheme 1, the CdS gel was drop-casted on the pretreated glassy carbon electrode (GCE) and modified with capture DNA. In consideration of clear expression, this capture DNA was divided into three sections and marked with different colors. The target miRNA is complementary to the red part. A DNA–RNA duplex formed in the presence of the target miRNA. So the red part of capture DNA would be cleaved off by DSN and the target miRNA would be released into the sample solution. This cycle would repeat with time under appropriate conditions,[7b] which lead to a negative correlation between the amounts of the remaining red part of capture DNA and the target miRNA. Then solutions of DNA 1 labeled Au NPs (Au-probe 1) and DNA 2 labeled L–Au NPs (L–Au-probe 2) were added in order. DNA 1 is complementary to the red and green part of capture DNA while DNA 2 is complementary to the green and blue part. If there had been no target miRNAs, Au-probe 1 would hybridize with capture DNA and enhance the ECL intensity of CdS. When L–Au-probe 2 was added, the green part was occupied by Au-probe 1. The blue part is too short to form a stable duplex with probe 2 at room temperature.

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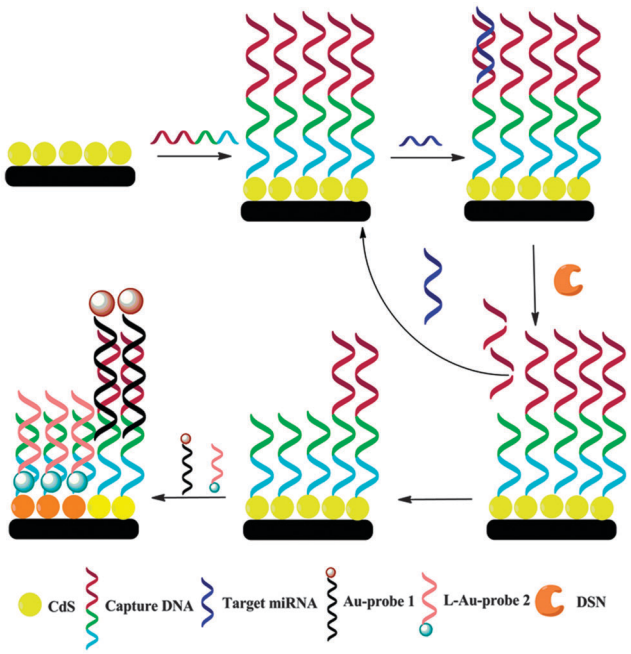

Scheme 1 Schematic representation of the ratiometric ECL biosensor for microRNAs based on cyclic enzyme amplification and resonance energy transfer.

But when target miRNAs existed and the red part was cleaved off by DSN, Au-probe 1 could not be immobilized onto the electrode because of the same reason that only the green part is not long enough. Instead, L–Au-probe 2 would hybridize with the remaining green and blue part. The closely contacted luminol–gold nanoparticles quenched the ECL signal from CdS NC and brought the ECL signal of luminol. So the increase of target miRNAs resulted in the quenching of the ECL signal from CdS and the increase of the signal from luminol.

A series of studies have been reported by using CdS NCs as ECL donors and metal nanoparticles as ECL acceptors. In a traditional approach, the binding of target molecules changes the conformation of rigid structures, resulting in the quenching or enhancement of ECL from CdS. When the NCs and metal nanoparticles are at close proximity, the emission of S–NCs can be easily quenched through nonradiative energy dissipation in the metal. When they are separated at a certain distance, ECL emission from the CdS NC film could induce surface plasmon resonance (SPR) of Au NPs, and the induced SPR in turn enhances the ECL response of CdS.[8] Here we present a novel strategy to achieve ratiometric detection by double-assisted ECL signal amplification by the synergistic effect of three kinds of nanoparticles. The preparation of these nanoparticles (CdS, Au NPs, and L–Au NPs) was according to previous studies.[9] The detailed experimental process, characterization and optimization are provided in the ESI.† Unlike the hairpin structure, target RNAs or DNAs would not trigger the conformation change but will play the role of occupying the binding site and will lead to the cyclic cleavage of the binding site. Then two kinds of probes will competitively hybridize with capture DNA at appropriate temperature. For the hairpin structure, the signal will increase depending on the concentration of targets. In this work, on the surface, the initial state is bare NCs, which could be referred to as the ground state. As shown in Scheme 1, a part of NCs is quenched by L–Au-probe 2 while the other part is enhanced by Au-probe 1. It is hard to judge whether the signal will increase or decrease from the ground state. But in fact, this so called "ground state" is just an intermediate state. With no target, the hairpin will maintain the closed conformation and the signal is "signal off". In the same situation, in this work, it is obvious that NCs will be enhanced by Au-probe 1. So essentially, the initial state of NCs is the "signal on" state. The luminescence intensity of NCs is at peak when there are no target miRNAs. Then it will decline with the increase of target concentration, whereas the signal from luminol will be enhanced.

Luminol is chosen because it could emit ECL at a positive potential. The signals from luminol and CdS (at −1.25 V) could be obtained and clearly distinguished in one potential scan in the presence of their common coreactant H_2O_2. Fig. 1 shows four different signal states. Curve a represents the DNA-modified CdS, in which there is only one cathode signal. Then Au-probe 1 was added. After complete hybridization between Au-probe 1 with capture DNA, we can see that the signal from CdS is around 4-fold higher than the emission in curve a (curve b). Although Au-probe 1 was firstly added and fully reacted, it could not occupy every binding site. Due to the remaining capture DNA, the following L–Au-probe 2 was also partially immobilized and brought a weak anode signal and a slightly decreased cathode signal (curve c). To test the performance of L–Au-probe 2, we added it into the electrode without adding Au-probe 1. As shown in curve d, the signal from luminol is much stronger than that in curve c, whereas the cathode signal is effectively quenched.

On the other hand, curves c and d can also be respectively regarded as the blank and saturated states. When there is no target, the signal would be similar to curve c. When the concentration of target miRNAs is high enough to cleave most of the capture DNA, it would behave like curve d. The ratio of the signal from luminol to the signal from CdS increases with the increase of concentrations of targets. MiR-21 is found to be overexpressed in 80% of the tumor samples,[10] such as glioblastoma tumor, neuroblastoma, breast cancer, and lung cancer. Here we chose it as a detection model to demonstrate the capability of this method. Firstly, we optimized

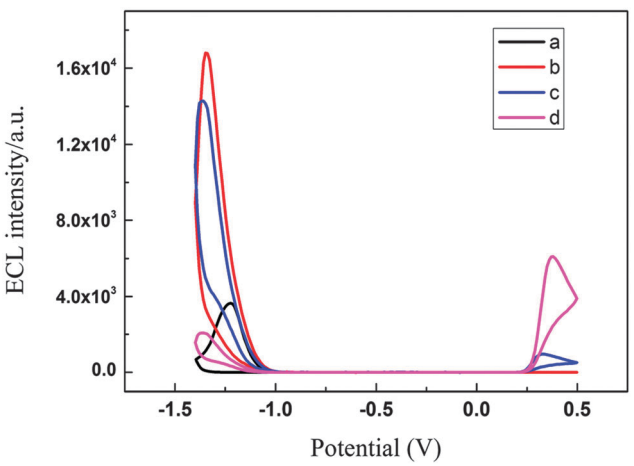

Fig. 1 Cyclic ECL curves of the DNA modified CdS NC film on the GCE (a); enhanced by Au-probe 1 (b); synergistic effect of Au-probe 1 and L–Au-probe 2 (c); quenched by L–Au-probe 2 (d).

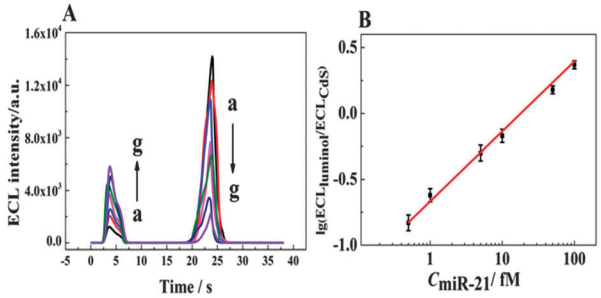

Fig. 2 (A) ECL signal–time curve at the dual-potential ECL sensing interface with different concentrations of oncogene miR-21 (from a to g): 0 fM, 0.5 fM, 1 fM, 5 fM, 10 fM, 50 fM, and 100 fM, respectively. (B) Relationship between the ratio of anode to cathode ECL peak intensity and the concentration of miR-21. Scan rate: 100 mV s^{-1}. Scan direction: 0 V → 0.5 V → −1.4 V → 0 V.

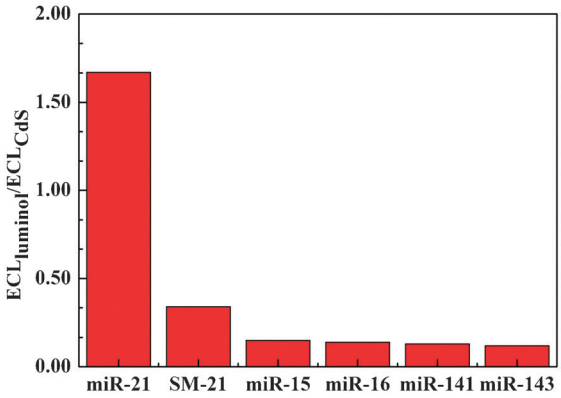

Fig. 3 Specificity of this ECL miRNA sensor for miR-21 over SM-21, miR-15, miR-16, miR-141, and miR-143.

detection conditions, including the amount of DSN enzyme and incubation time. As shown in Fig. S3 (ESI†), we found that 30 min incubation with 0.1 U is sufficient for the detection. Under these conditions, we investigated the sensitivity of this ratiometric assay based on cyclic enzyme amplification and resonance energy transfer. As shown in Fig. 2A, with the increase of miR-21 concentration, cathode ECL peak intensity decreased and anode ECL peak intensity increased correspondingly. The ECL emission is stable under continuous potential scanning (Fig. S4, ESI†). The logarithmic value of $ECL_{luminol}/ECL_{CdS}$ linearly depended on the logarithm of miR-21 concentration in the range of 0.5–50 fM with a correlation coefficient of 0.996, and with a detection limit of 0.24 fM at the S/N ratio of 3 (Fig. 2B). We measured the concentration of miR-21 in Hela cell extracts. The average copy number of miR-21 per cell was around 2.1×10^3, which is consistent with previous reports.[11] The recovery rates of standard addition is between 94% and 102%. These results indicated that the proposed method is capable of detecting miRNAs in real samples.

One major challenge to miRNA assay is the highly homogeneous sequences of multiple miRNAs. Distinguishing these miRNAs is of significance to profile their expression and understand their biological functions. DSN shows a good ability of discriminating between perfectly and nonperfectly matched short duplexes. Specificity of this ECL detection was evaluated using four other miRNA sequences (miR-15, miR-16, miR-141, and miR-143) and single-base mismatched miR-21(SM-21). As shown in Fig. 3, for the same concentration of various miRNAs (50 fM), there is a significant difference in the ratio of anode to cathode ECL peak intensity between target miR-21 and other miRNAs. This result clearly suggests that the proposed method is highly selective and has potential to discriminate the miRNA family members.

In conclusion, we have successfully presented a ratiometric ECL biosensor for the detection of microRNAs based on cyclic enzyme for the first time. DSN will cleave a part of the DNA capture probes in the presence of target miRNAs and release miRNAs to the next circle. Two probes, Au-probe 1 and L–Au-probe 2, can competitively and selectively hybridize with the capture DNA, depending on the completeness of this DNA. Au-probe 1 will enhance cathode emission from CdS NCs, whereas L–Au-probe 2 will quench the cathode emission and bring anode emission from luminol. Hence the target may be quantified by the ratio of ECL intensities at two excitation potentials. This novel ratiometric method is highly sensitive and selective, which may be a powerful tool for biomedical research and clinical diagnostics.

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Notes and references

1 P. Ramnani, Y. Gao, M. Ozsoz and A. Mulchandani, *Anal. Chem.*, 2013, **85**, 8061.
2 C. Hilton and F. Karpe, *Clin. Chem.*, 2013, **59**, 729.
3 (*a*) C.-Z. Chen, L. Li, H. F. Lodish and D. P. Bartel, *Science*, 2004, **303**, 83; (*b*) J. Brennecke, D. R. Hipfner, A. Stark, R. B. Russell and S. M. Cohen, *Cell*, 2003, **113**, 25; (*c*) C. Arenz, *Angew. Chem., Int. Ed.*, 2006, **45**, 5048; (*d*) G. A. Calin and C. M. Croce, *Nat. Rev. Cancer*, 2006, **6**, 857.
4 (*a*) J. Wang, Y. Shan, W.-W. Zhao, J.-J. Xu and H.-Y. Chen, *Anal. Chem.*, 2011, **83**, 4004; (*b*) H. Zhou, J. Liu, J.-J. Xu and H.-Y. Chen, *Chem. Commun.*, 2011, **47**, 8358; (*c*) L.-J. He, M.-S. Wu, J.-J. Xu and H.-Y. Chen, *Chem. Commun.*, 2013, **49**, 1539; (*d*) M.-S. Wu, H.-W. Shi, L.-J. He, J.-J. Xu and H.-Y. Chen, *Anal. Chem.*, 2012, **84**, 4207.
5 (*a*) K. Ren, J. Wu, F. Yan and H. Ju, *Sci. Rep.*, 2014, **4**, 4360, DOI: 10.1038/srep04360; (*b*) Y. Cheng, Y. Huang, J. Lei, L. Zhang and H. Ju, *Anal. Chem.*, 2014, **86**, 5158; (*c*) K. Zhang, H. Zhou, Q. Mei, S. Wang, G. Guan, R. Liu, J. Zhang and Z. Zhang, *J. Am. Chem. Soc.*, 2011, **133**, 8424; (*d*) K. Aslan, J. R. Lakowicz and C. D. Geddes, *Anal. Chem.*, 2005, **77**, 2007.
6 H.-R. Zhang, J.-J. Xu and H.-Y. Chen, *Anal. Chem.*, 2013, **85**, 5321.
7 (*a*) X. Lin, C. Zhang, Y. Huang, Z. Zhu, X. Chen and C. J. Yang, *Chem. Commun.*, 2013, **49**, 7243; (*b*) B.-C. Yin, Y.-Q. Liu and B.-C. Ye, *J. Am. Chem. Soc.*, 2012, **134**, 5064; (*c*) S. Wang, B. Fu, J. Wang, Y. Long, X. Zhang, S. Peng, P. Guo, T. Tian and X. Zhou, *Anal. Chem.*, 2014, **86**, 2925; (*d*) F. Degliangeli, P. Kshirsagar, V. Brunetti, P. P. Pompa and R. Fiammengo, *J. Am. Chem. Soc.*, 2014, **136**, 2264; (*e*) Y. Ren, H. Deng, W. Shen and Z. Gao, *Anal. Chem.*, 2013, **85**, 4784.
8 Y. Shan, J.-J. Xu and H.-Y. Chen, *Chem. Commun.*, 2009, 905.
9 H.-R. Zhang, M.-S. Wu, J.-J. Xu and H.-Y. Chen, *Anal. Chem.*, 2014, **86**, 3834.
10 H. Dong, J. Lei, L. Ding, Y. Wen, H. Ju and X. Zhang, *Chem. Rev.*, 2013, **113**, 6207.
11 G. Meister, M. Landthaler, A. Patkaniowska, Y. Dorsett, G. Teng and T. Tuschl, *Mol. Cell*, 2004, **15**, 185.

