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Highly Sensitive Signal-on Biosensor for MicroRNA 142-3p Based on the Quenching of $Ru(bpy)_3^{2+}$-TPA Electrochemiluminescence by Carbon Dots and Duplex Specific Nuclease Assisted Target Recycling Amplification

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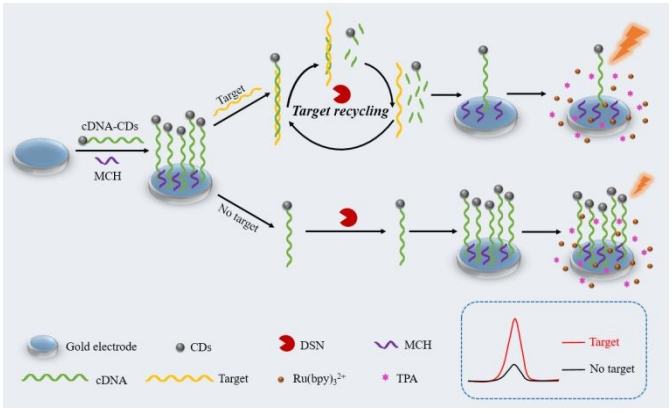
Carbon dots (CDs) has been used to quench the electrochemiluminescence (ECL) of $Ru(bpy)_3^{2+}$-tripropylamine (TPA) system for the first time. And which has been coupled with duplex specific nuclease assisted target recycling amplification to develop a highly sensitive ECL biosensor for microRNA.

Electrochemiluminescence (ECL) is a chemiluminescence phenomenon produced during electrochemical reactions on electrode surface. Which shows many outstanding advantages such as high sensitivity, easy operation and little sample consumption[1,2] $Ru(bpy)_3^{2+}$ (bpy = 2, 2'-bipyridyl)-tripropylamine (TPA) system possesses high ECL efficiency, good photochemical stability and good compatibility for analytes in aqueous medium, making it becoming one of the most popular ECL systems.[3] Mostly $Ru(bpy)_3^{2+}$ based ECL biosensors realize the quantification of the target through the regulation of the ECL indicator or the coreact compounds concentration. [4] Some compounds and materials which can effectively quench the ECL behavior of $Ru(bpy)_3^{2+}$-TPA has been used to construct ECL biosensors for different targets. For example, our group utilized ferrocene as quenching compound to inhibit the ECL emission of $Ru@SiO_2$ nanoparticles and develop a sensitive ECL biosensor for adenosine.[5] Tang and coworkers investigated a DNA sensor based on the quenching performance of multiwall carbon nanotube (MWNT) to ECL emission of $Ru(bpy)_3^{2+}$-TPA system, which because of the surface oxygen-containing groups and the intrinsic electron properties of MWNT.[6] Similarly, Huang and coworkers reported that graphene oxide (GO) could quench the ECL of $Ru(bpy)_3^{2+}$-TPA system, and the suppression of ECL mainly due to the oxygen-containing groups and poor electrical conductivity of GO.[7] As a kind of biocompatible, nontoxic, water-soluble and environmentally friendly nanomaterial, carbon dots (CDs) have been applied in constructing many biosensors, which is based on its unique properties such as strong luminescence, excellent catalytic properties.[8] CDs synthesized by chemical oxidation methods are rich in oxygen-containing functional groups, primary study showed that CDs also can quench the ECL of $Ru(bpy)_3^{2+}$-TPA system.

The abnormal expression of microRNA (miRNA) is related to cardiovascular disease, neurological diseases and multiple cancers.[9] MiRNA 142-3p has been proved to be closely related with cervical cancer,[10] colorectal cancer,[11] esophageal squamous cell carcinoma[12] and so on. Hence, its quantitative detection is of great clinical significance. Many effective methods , such as real-time PCR,[13] northern blotting,[14] and microarray analysis,[15] fluorescence and electrochemistry,[16] has been developed for miRNA detection. On account of the trace amount of miRNA, signal amplification strategy is needed to meet the accurate detection requirements. A series of signal amplification strategies such as rolling circle amplification (RCA),[17] hybridization chain reaction (HCR),[18] exonuclease-



Scheme 1. The signal-on biosensor for miRNA 142-3p based on the quenching of $Ru(bpy)_3^{2+}$-TPA ECL by carbon dots and duplex specific nuclease assisted target recycling amplification.

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assisted cyclic amplification,[19] were introduced to increase the sensitivity of detection methods. The duplex specific nuclease (DSN) is known for its high specificity for cleaving double-stranded DNA or DNA in DNA-RNA duplexes, while does not work on single-stranded DNA and single or double-stranded RNA, which has been widely used to realize target recycling and signal amplification.

In this study, a sensitive signal-on ECL biosensor for miRNA 142-3p has been developed by coupling the quenching effect of CDs with the DSN-assisted target recycling amplification strategy. Scheme 1 showed the mechanism of the proposed biosensor. Firstly, CDs and complementary DNA (cDNA) modified with $-NH_2$ was connected by EDC/NHS, the prepared CDs-cDNA (see details in supplementary information, (SI)) was attached to electrode surface via Au-S bond and served as quenching probe. In the absence of the target, CDs-cDNA was free from hydrolyzation because DSN has no capacity for hydrolyzing single-stranded DNA and a large amount of CDs-cDNA remained on the electrode surface, leading to a weak ECL signal was detected when the modified electrode had been immersed in 2.0 mL of 10 mM PBS containing 25 nM $Ru(bpy)_3^{2+}$ and 20 μM TPA for test, which due to the quench effect of CDs toward the ECL system. In the presence of the target, cDNA binds with the target to form DNA-RNA duplexes via complementary base pairing principle, due to the high preference of DSN for cleaving DNA in DNA-RNA duplexes, cDNA was hydrolyzed and the target was released to bind with another cDNA modified on the electrode surface, then another cDNA was hydrolyzed and released target again, therefore, the DSN enzyme-assisted target recycling amplification strategy was induced, hydrolyzed cDNA and CDs then left the electrode surface after washing, so little amount of CDs remained on the electrode surface and a relative large ECL signal could be detected. Thus, a sensitive platform for the detection of microRNA 142-3p could be achieved.

The prepared CDs were characterized by TEM, AFM and FTIR, which verified the successful synthesis and abundant carboxyl groups of CDs. The reduced CDs (R-CDs) were also characterized by TEM and FTIR, confirming decline in the oxygen content (see details in Fig. S1 in SI). Afterwards, the quenching phenomenon of CDs to the ECL of $Ru(bpy)_3^{2+}$-TPA system was investigated. As illustrated in Fig. 1A, $Ru(bpy)_3^{2+}$-TPA system exhibited a strong anodic ECL signal at around +1.00 V (curve a), which decreased obviously as CDs were added into the solution (curve b). The corresponding cyclic voltammetry (CV) curves were showed in Fig. 1B, compared with the control (curve a), the oxidation current of the system in the range of 0.85~1.2 V was also inhibited obviously by the CDs (curve b). The results implied that CDs inhibited the ECL response of $Ru(bpy)_3^{2+}$-TPA system mainly through affecting the electrochemical reactions of the system. It is well known that the ECL reaction of $Ru(bpy)_3^{2+}$-TPA system involves the electra oxidation of both $Ru(bpy)_3^{2+}$ and TPA and the reaction between the formed $Ru(bpy)_3^{3+}$ and TPA radical.[20] To further investigate how the CDs affected the electrochemical processes of the ECL system, effects of CDs on the CV curves of only $Ru(bpy)_3^{2+}$ and TPA alone were tested, respectively. As

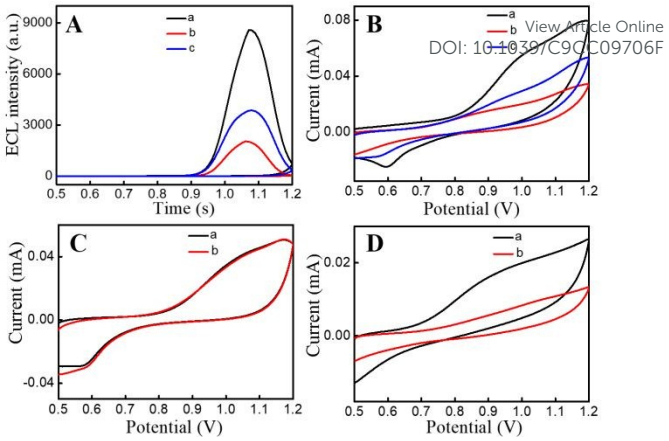

Figure 1. (A) ECL tests in 10 mM PBS containing 25 nM $Ru(bpy)_3^{2+}$ and 20 μM TPA without CDs (a), with 5 μg/mL CDs (b) and with 5 μg/mL R-CDs (c), respectively. (B) CV tests in 10 mM PBS containing 25 nM $Ru(bpy)_3^{2+}$ and 20 μM TPA without CDs (a), with 5 μg/mL CDs (b) and with 5 μg/mL R-CDs (c), respectively. (C) CV tests in 10 mM PBS containing 25 nM $Ru(bpy)_3^{2+}$ without CDs (a) and with 5 μg/mL CDs (b), respectively. (D) CV tests in 10 mM PBS containing 20 μM TPA without CDs (a) and with 5 μg/mL CDs (b), respectively. Scan range: 0.5 to 1.2 V, scan rate: 100 mV/s.

displayed in Fig. 1C, there was nearly no difference in the electrochemical behavior of $Ru(bpy)_3^{2+}$ before (curve a) and after (curve b) the addition of CDs, but in Fig. 1D, the oxidation current of TPA was inhibited significantly after the addition of CDs (curve b). The reason maybe lies in that CDs was negative catalyst for the electro-oxidation of TPA. It was usually considered that the zigzag edges are the active centers for the electrocatalysis.[21] And the electrocatalytic activities of the zigzag edges were affected by the connected oxygen-containing functional groups.[22] To verify this theory, in a control experiment, R-CDs, whose oxygen content was lower than that of CDs, were used instead of CDs as the quencher. As expected, R-CDs could also inhibit the ECL response (Fig. 1A, curve c) and CV behavior (Fig. 1B, curve c) of $Ru(bpy)_3^{2+}$-TPA system, but the quenching effect was weaker than that of CDs. The results suggested that the zigzag edge adjacent to several oxygen-containing functional groups of CDs should be the active centers for inhibiting the electrooxidation of TPA. And the possible quenching mechanism (Fig. S2 in SI) was that the interaction of CDs and TPA pulled down the electron-cloud density of TPA, which make the electrooxidation of TPA became more difficult.

Then the CDs with better quenching effect was applied to construct a highly sensitive ECL biosensor for miRNA 142-3p (see details in SI). To identify the feasibility of the DSN enzyme-assisted recycling amplification strategy, polyacrylamide gel electrophoresis (12%) characterization was carried out. As shown in Fig. 2A, the complex of cDNA and target miRNA was formed successfully in the absence of DSN enzyme (lane c), while the cDNA in cDNA-target duplex was hydrolyzed in the presence of DSN enzyme, and released target miRNA (lane d), which confirmed the feasibility of this signal amplification strategy.

Electrochemical impedance spectroscopy (EIS) is common and effective method to characterize the surface of electrode by using the redox couple $[Fe(CN)_6]^{3-/4-}$. The semicircle appears at higher frequencies, which is related to the charge transfer-

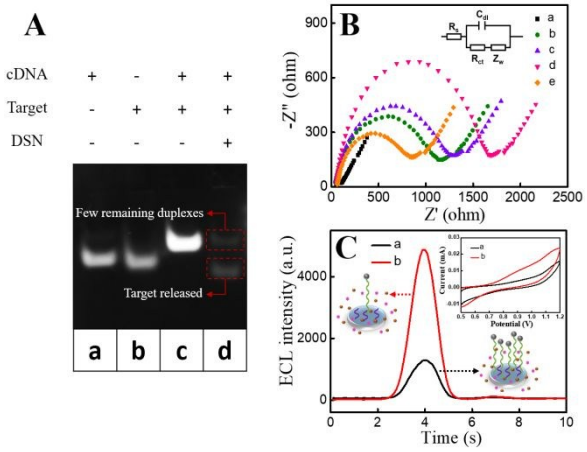

Fig. 2 (A) 12% Gel electrophoresis image of 10 μM cDNA (lane a), 10 μM target miRNA (lane b), 5.0 μM cDNA mixed with 5.0 μM target miRNA (lane c), 5.0 μM cDNA mixed with 2.0 μM target miRNA and DSN enzyme (lane d). (B) EIS on different electrodes in 0.1 M KCl aqueous solution containing 5.0 mM $[Fe(CN)6]^{3-/4-}$ and 100 mM KCl, (a) Bare Au electrode, (b) CDs-cDNA/Au electrode, (c) MCH/CDs-cDNA/Au electrode, (d) Target/CDs-cDNA/MCH/Au electrode (e) DSN/target/CDs-cDNA/MCH/Au electrode, respectively. (C) ECL test without target (a) and with 1.0 pM target (b), respectively, insert: the corresponding results of CV test. Scan range: 0.5 to 1.2 V, scan rate: 100 mV/s.

limited process. Consequently, the increase of diameter of the semicircle reflects an increase of electron transfer resistance (R_{ct}). Thus, successful modifications on electrode surface could be estimated according to the change of the diameter. As shown in Fig. 2B, there was almost no semicircle of bare gold electrode (curve a), revealing the fleet charge transfer process on Au electrode surface. After the immobilization of CDs-cDNA, the R_{ct} was obviously increased to 1200Ω (curve b), owing to the electrostatic repulsion between electronegative cDNA and $[Fe(CN)_6]^{3-/4-}$ ions, which indicated CDs-cDNA was attached to the electrode surface successfully. The R_{ct} increased to 1300Ω (curve c) if MCH had been used to block the nonspecific binding sites. In the presence of 1.0 pM target, the R_{ct} increased to 1700 Ω (curve d), showing the binding of target and cDNA. In the presence of 1.0 pM target and DSN, R_{ct} decreased distinguished (curve e), confirming that the DSN enzyme-assisted recycling amplification process was induced, CDs-cDNA was hydrolyzed and then left the electrode surface by washing. The above results showed that each step of electrode modification was successful, and the feasibility of the experiment was verified again. The ECL tests were carried out with 1.0 pM target and without target, as shown in Fig. 2C, in the absence of the target, the ECL intensity was feeble due to the quencher CDs on the electrode surface (curve a), in the presence of the target, the ECL intensity conspicuous enlarged (curve b) due to the target induced DSN hydrolysis to cDNA, and the corresponding CV test verified the quenching mechanism again (insert picture in Fig.2C), which indicated the feasibility of the experiment.

Several key factors which could obviously affect the performance of proposed biosensor had been optimized. Firstly, the length of cDNA was optimized by changing the number of complementary bases of cDNA and the target. As shown in Fig. S3A in SI, when the length of cDNA increased from 25 bp to 33 bp, the *ΔECL* (the difference in the ECL intensity between the presence and absence of target) enlarged gradually, which was

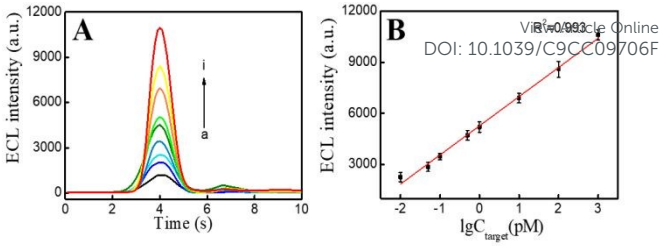

Fig. 3 (A) ECL signal curves of the target at various concentrations from a to i: control, 10 fM, 50 fM, 100 fM, 500 fM, 1.0 pM, 10 pM, 100 pM and 1.0 nM. (B) Linear plot of the ECL intensity vs logarithmic concentration of the target (R^2 = 0.993). Scan range: 0.5 to 1.2 V, scan rate: 100 mV/s.

due to the number of complementary bases increased, making the hybridization product of cDNA and the target more stable,and the cleaving activity of DSN was more active when the complementary base was bigger than 15bp, which led to a bigger ECL enhancement after adding the target. However, when the length of cDNA was higher than 31 bp, *ΔECL* began to decrease because the CDs were far away from the electrode surface, and the quenching effect become weaker, so *ΔECL* decreased. Consequently, 31 bp was chosen as the best condition. The amount of CDs used in the synthesis of CDs-cDNA was the foundation to make sure cDNA connected with CDs as much as possible, as shown in Fig. S3B in SI, with the increase of CDs, the quenching effect enlarged, consequently, *ΔECL* became more obvious, however, excess unconnected carbon may cause a little nonspecific adsorption to affect the results, so when the concentration was bigger than 10 μg/mL, *ΔECL* decreased slightly, therefore, the optimal concentration of CDs was 10 μg/mL. Moreover, the concentration of DSN also affected the performance of the sensor greatly. Enough amount of DSN could make sure the hydrolysis efficiency, as shown in Fig. S3C in SI, *ΔECL* increased with the enhanced DSN concentration firstly and reached a plateau when the concentration of DSN was 10 mU/μL. Thus, which had been chosen as the optimized concentration. Finally, enough time for the DSN enzyme-assisted recycling process was directly related to the signal amplification efficiency, as shown in Fig. S3D, 50 min was enough for the enzyme-assisted recycling process.

Under the optimal conditions, the analysis capability of proposed biosensor was evaluated. As shown in Fig. 3A, ECL response increased with the enhancing of the target concentration from 10 fM to 1.0 nM. Fig. 3B showed the linear relationship between ECL signal value and the logarithmic concentration of the target. The linear equation could be expressed as follow:

I(a.u.) = 1713.12 Log C_{target} (pM) +5268.70

Where I is the ECL intensity of the system and C_{target} is concentration of the target. The correlation coefficient (R^2) is 0.993 and the limit of detection (LOD) was 10 fM (the lowest concentration that can be really detected in the linear range).

In addition, the performance of the proposed biosensor was compared with other methods for miRNA detection. As shown in Table S1 in SI, the proposed sensor presented a wider linear range and lower LOD for miRNA detection. Several highly similar interferents, including single-base mismatched target (SM-target), three-base mismatched target (TM-target), miRNA 21,

miRNA 155, and the mix of all these interferents with the target were used to test the selectivity of the sensor. Concentration of interferents were 10 pM while the target concentration was 1.0 pM. As shown in Fig. S4A in SI, interfering substances had scarcely any effect. The result reflected the good selectivity and anti-interference capability of the sensing platform. The reproducibility of the sensor was evaluated by five different electrodes with the same concentration of the target (1.0 pM) under the same experimental conditions. As shown in Fig. S4B in SI, the results of ECL test was numerically close and a relative standard deviation (RSD) was 4.96% (n = 5), revealing good reproducibility of the biosensor.

In order to verify the practical application, the ECL biosensor was applied to detect miRNA 142-3p in cervical cancer cells (Hela) lysate with low expression, and human colorectal cancer cells (SW480) lysate with high expression of the target. As shown in Fig. S5 in SI, the rise of *ΔECL* was in accordance with the increased number of cells. The *ΔECL* enhanced slowly in the range of 10^2 to 10^4 cells in Hela cell lysate while increased rapidly in SW480 cell lysate of the same cell number. Those results showed that the uptrend of the *ΔECL* closely coincided with the increase of cell number and expression level, revealing that the proposed ECL biosensor has the potential in monitoring microRNA expression in cancer cells.

In summary, the quenching effect of CDs to the ECL signal of $Ru(bpy)_3^{2+}$-TPA system was utilized for the first time. Based on this finding, an ultrasensitive signal-on ECL biosensor for the detection of miRNA 142-3p was constructed. The linear range was from 10 fM to 1.0 nM, and the LOD was 10 fM. Selectivity and anti-interference tests testified that the sensing platform could selectively detect the content of the target in the presence of several interferents. The proposed biosensor also had good reproducibility and the potential in practical application. In addition, a research on the quenching mechanism was carried out by CV and ECL tests, the results indicated that the quenching is possibly derived from the restraint towards electrooxidation of TPA by CDs. The proposed sensing platform opens up a new application for CDs as ECL quenchers. By using CDs as beacon molecules to modify different sequences, detection of many different targets can be achieved easily.

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Conflicts of interest

There are no conflicts to declare.

Notes and references

1. (a)Z. Xu, L. Liao, Y. Chai, H. Wang and R. Yuan, *Anal. Chem.*, 2017, **89**, 8282-8287;(b)A. Zhang, W. Guo, H. Ke, X. Zhang, H. Zhang, C. Huang, D. Yang, N. Jia and D. Cui, *Biosens. Bioelectron.*, 2018, **101**, 219-226.
2. (a)Q. Zhai, H. Xing, X. Zhang, J. Li and E. Wang, *Anal. Chem.*, 2017, **89**, 7788-7794;(b)L. Yang, Y. Zhang, R. Li, C. Lin, L. Guo, B. Qiu, Z. Lin and G. Chen, *Biosens. Bioelectron.*, 2015, **70**, 268-274.
3. (a)D. Wang, Y. Li, Z. Lin, B. Qiu and L. Guo, *Anal. Chem.*, 2015, **87**, 5966-5972;(b)Q. Q. Gai, D. M. Wang, R. F. Huang, X. X. Liang, H. L. Wu and X. Y. Tao, *Biosens. Bioelectron.*, 2018, **118**, 80-87.
4. L. Guo, L. Xue, B. Qiu, Z. Lin, D. Kim and G. Chen, *Phys. Chem. Chem. Phys.*, 2010, **12**, 12826-12832.
5. L. Chen, Q. Cai, F. Luo, X. Chen, X. Zhu, B. Qiu, Z. Lin and G. Chen, *Chem. Commun.*, 2010, **46**, 7751-7753.
6. X. Tang, D. Zhao, J. He, F. Li, J. Peng and M. Zhang, *Anal. Chem.*, 2013, **85**, 1711-1718.
7. X. Huang, X. Huang, A. Zhang, B. Zhuo, F. Lu, Y. Chen and W. Gao, *Biosens. Bioelectron.*, 2015, **70**, 441-446.
8. (a)N. L. Li, L. P. Jia, R. N. Ma, W. L. Jia, Y. Y. Lu, S. S. Shi and H. S. Wang, *Biosens. Bioelectron.*, 2017, **89**, 453-460;(b)Y. Sun, C. Ding, Y. Lin, W. Sun, H. Liu, X. Zhu, Y. Dai and C. Luo, *Talanta*, 2018, **186**, 238-247;(c)L. L. Xu, W. Zhang, L. Shang, R. N. Ma, L. P. Jia, W. L. Jia, H. S. Wang and L. Niu, *Biosens. Bioelectron.*, 2018, **103**, 6-11.
9. S. Lin and R. I. Gregory, *Nat Rev Cancer*, 2015, **15**, 321-333.
10. B. Deng, Y. Zhang, S. Zhang, F. Wen, Y. Miao and K. Guo, *Tumour Biol*, 2015, **36**, 8065-8073.
11. Y. Mao, Z. Fu, Y. Zhang, L. Dong, Y. Zhang, Q. Zhang, X. Li and C. Wang, *J. Cell. Physiol.*, 2019, **234**, 6810-6819.
12. R. J. Lin, D. W. Xiao, L. D. Liao, T. Chen, Z. F. Xie, W. Z. Huang, W. S. Wang, T. F. Jiang, B. L. Wu, E. M. Li and L. Y. Xu, *J Surg Oncol*, 2012, **105**, 175-182.
13. J. Li, B. Yao, H. Huang, Z. Wang, C. Sun, Y. Fan, Q. Chang, S. Li, X. Wang and J. Xi, *Anal. Chem.*, 2009, **81**, 5446-5451.
14. A. Valoczi, C. Hornyik, N. Varga, J. Burgyan, S. Kauppinen and Z. Havelda, *Nucleic Acids Res.*, 2004, **32**, e175.
15. L. P. Lim, N. C. Lau, P. Garrett-Engele, A. Grimson, J. M. Schelter, J. Castle, D. P. Bartel, P. S. Linsley and J. M. Johnson, *Nature*, 2005, **433**, 769-773.
16. (a)Y. Zhang, G. Xu, G. Lian, F. Luo, Q. Xie, Z. Lin and G. Chen, *Biosens. Bioelectron.*, 2020, **147**, 111789;(b)X. Zhang, Z. Yang, Y. Chang, M. Qing, R. Yuan and Y. Chai, *Anal. Chem.*, 2018, **90**, 9538-9544;(c)W. Yang, Y. Shen, D. Zhang, C. Li, R. Yuan and W. Xu, *Anal. Chem.*, 2019, **91**, 7782-7789;(d)T. Hou, N. Xu, W. Wang, L. Ge and F. Li, *Anal. Chem.*, 2018, **90**, 9591-9597.
17. Y. He, X. Yang, R. Yuan and Y. Chai, *Anal. Chem.*, 2017, **89**, 2866-2872.
18. Y. H. Yuan, B. Z. Chi, S. H. Wen, R. P. Liang, Z. M. Li and J. D. Qiu, *Biosens. Bioelectron.*, 2018, **102**, 211-216.
19. L. Lu, H. Su and F. Li, *Anal. Chem.*, 2017, **89**, 8328-8334.
20. W. J. Miao, J. P. Choi and A. J. Bard, *J. Am. Chem. Soc.*, 2002, **124**, 14478-14485.
21. I.-Y. Jeon, S. Zhang, L. Zhang, H.-J. Choi, J.-M. Seo, Z. Xia, L. Dai and J.-B. Baek, *Adv. Mater.*, 2013, **25**, 6138-6145.
22. Z. Lu, G. Chen, S. Siahrostami, Z. Chen, K. Liu, J. Xie, L. Liao, T. Wu, D. Lin, Y. Liu, T. F. Jaramillo, J. K. Nørskov and Y. Cui, *Nat. Catal.*, 2018, **1**, 156-162.

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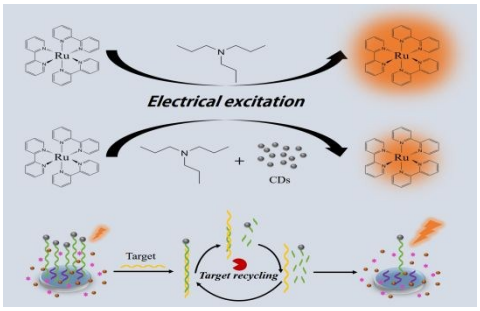

CDs have been used to quench the ECL of $Ru(bpy)_3^{2+}$-TPA for the first time and a sensitive biosensor was developed.