Electronic Supplementary Material (ESI) for Chemical Communications

A novel label-free cascade amplification strategy based dumbbell probe-mediated rolling circle amplification-responsive G-quadruplex formation for highly sensitive and selective detection of NAD^+ or ATP

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EXPERIMENTAL SECTION

Materials and Reagents. Phi29 DNA polymerase, T4 DNA ligase, and dNTP were obtained from Fermentas (Lithuania). E. coli ligase was purchased from Takara Biotechnology Co., Ltd. (Dalian, China). The oligonucleotides with the following sequences were obtained from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (China). Nicotinamide adenine dinucleotide (NAD^+), the reduced form of nicotinamide adenine dinucleotide (NADH), reduced nicotinamide adenine dinucleotide phosphate (NADPH), adenosine diphosphate (ADP), and adenosine monophosphate (AMP) were obtained from Amresco. Adenosine triphosphate (ATP) was obtained from Sigma-Aldrich. The oligonucleotides with the following sequences were obtained from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (China): primer DNA (p-DNA): 5'-TTT GGC TAT TTT ATC GGT TT, the dumbbell probe (D-DNA): 5'-p-AAT AGC CAAAA CGA CAT CAA CCC AAAA CCC AAAA CCC AAAA CCC AAG ATG TCG CAC GCT AAA CCC AAAA CCC AAAA CCC AAAA CCC AAT AGC GTGAAAA CCG ATAA-3'. Other chemicals (analytical grade) were obtained from standard reagent suppliers. Water (≥18.2 M) was used and sterilized throughout the experiments. N-methyl mesoporphyrin IX (NMM) was purchased from J&K Scientific Ltd. (Beijing, China).

Apparatus. All the fluorescence measurements were performed on a Hitachi F-4500 spectrofluorimeter (Hitachi, Japan). The excitation wavelength was 399 nm, and the spectra are recorded between 595 and 625 nm. The fluorescence emission intensity

was measured at 607 nm.

Ligation Procedure

In a typical procedure, first, a 25 μl of a reaction mixture [40 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM dithiothreitol (DTT) (pH 7.8)], which contained 10 nM of p-DNA and 50 nM of dumbbell probe (D-DNA), was denatured at 90 ℃ for 3 min and cooled down to room temperature over a 10-min period. Then, the reaction mixture was incubated in 37℃ for 30 min to afford D-DNA/p-DNA hybrids. Second, the dumbbell probes were ligated for subsequent RCA by adding 5 U T4 DNA ligase and 2 μl different concentrations of ATP into the solution of the D-DNA/p-DNA duplex and allowing the ATP-triggered ligation reaction at 37 ℃ for 90 min. In the presence of ATP, the T4 DNA ligase could catalyze the ligation between 3’-OH and 5’-PO4 end of the dumbbell probes, triggering the formation of “circular” DNA template.

For the detection of NAD^+, a procedure similar to that used for ATP detection was conducted. First, a 25 μl of a reaction mixture [30 mM Tris-HCl（pH 8.0）, 4 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 1.2 mM EDTA], which contained 10 nM of p-DNA and 50 nM of dumbbell probe (D-DNA), was denatured at 90 ℃ for 3 min and cooled down to room temperature over a 10-min period. Then, the reaction mixture was incubated in 37℃ for 30 min to afford D-DNA/p-DNA hybrids. Second, the dumbbell probes were ligated for subsequent RCA by adding 0.5 μl 0.005% BSA, 20 U E. coli ligase and 2 μl different concentrations of NAD^+ into the solution of the D-DNA/p-DNA duplex and allowing the NAD^+-triggered ligation reaction at 37 ℃

for 90 min. In the presence of NAD^+, the E. coli ligase could catalyze the ligation between 3'-OH and 5'-PO_4 end of the dumbbell probes, triggering the formation of "circular" DNA template.

Different ligation conditions, including varying concentrations of DNA ligase, different molar ratios of D-DNA to the p-DNA (fixed at 10 nM) were employed in the above ligation procedures for optimization.

RCA reactions

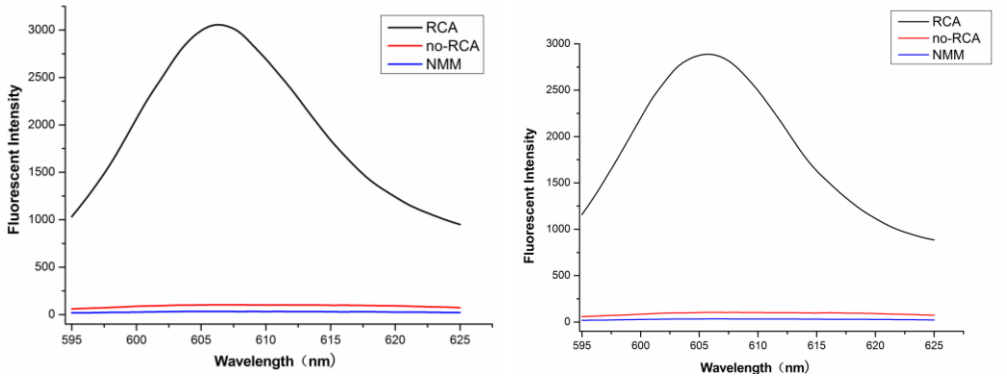
For RCA, the aforementioned ligation product was mixed with 4 μl 10 × reaction buffer [330 mM Tris–acetate, 100 mM $Mg(Ac)_2$, 660 mM Potassium Acetate (KAc), 1% Tween 20 and 10 mM DTT (pH 7.9), 2 μl (10 u/μl) phi29 DNA polymerase, 8 μl 10 mM dNTPs mixture and RNase-free water]. The reaction mixture was incubated at 37 ℃ for 90 min. Different RCA reaction times for ATP or NAD^+ were also employed in the above RCA reaction procedures for optimization.

Measurement of fluorescent spectra

The RCA amplification product was mixed with 4 μl 25 μM N-methyl mesoporphyrin IX (NMM) and 5 μl 100 mM KCl, then, diluted to final volume of 50 μl. The fluorescent spectra were measured using a spectrofluorophotometer. The excitation wavelength was 399 nm, and the spectra are recorded between 595 and 625 nm. The fluorescence emission intensity was measured at 606 nm.

RESULTS AND DISCUSSION

The verification of the sensing strategy based on dumbbell probe-mediated rolling circle amplification (D-RCA)-responsive G-quadruplex formation



A B

Fig. S1. The fluorescence emission spectra under different conditions: (A). NMM; p-DNA+D-DNA + T4 DNA ligase + Phi29 DNA polymerase + dNTPs + NMM; p-DNA+D-DNA + ATP（100 nM）+ T4 DNA ligase + Phi29 DNA polymerase + dNTPs + NMM. (B). NMM; p-DNA+D-DNA + E. coli DNA ligase + Phi29 DNA polymerase + dNTPs + NMM; p-DNA+D-DNA + NAD^+（100 nM）+ E. coli DNA ligase + Phi29 DNA polymerase + dNTPs + NMM.

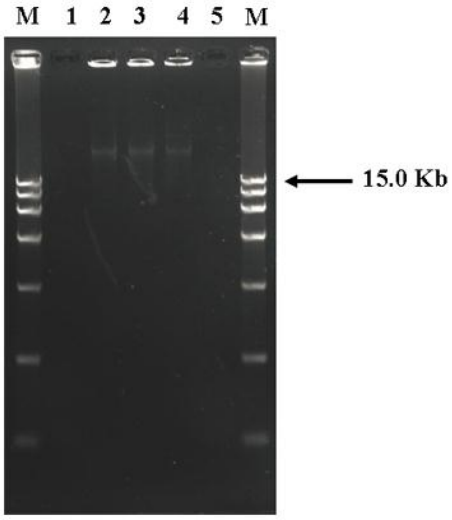
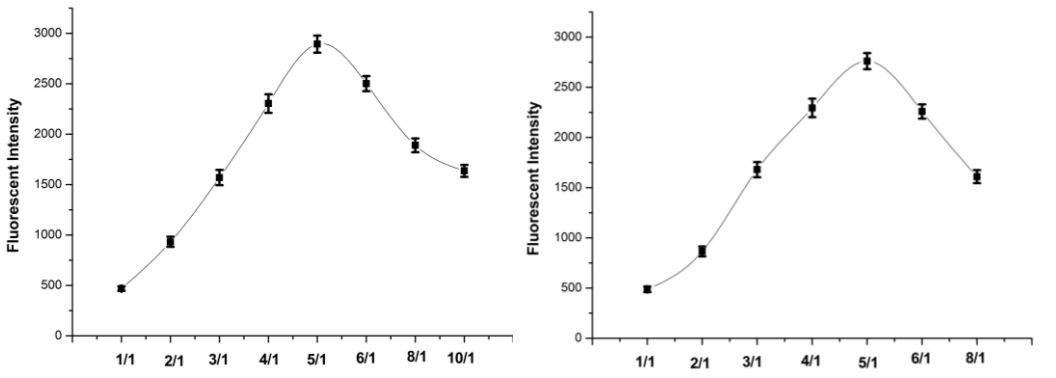


Fig. S2. Agarose gel (0.7%) electrophoresis experiments. The products of RCA reaction (1.5 h) (indicated by 1-5) were denatured at 95 °C for 5 min and quenched with ice-cooled water for 10 min. The marker was indicated by M. The high molecular weight RCA products are observed in lines in lane 2, 3 (100 nM NAD^+) and 4 (100 nM ATP)

Typical fluorescence spectra characteristics of the sensing strategy in response to NAD^+ or ATP are shown in Fig. 1. Compared with the background fluorescence of the NMM, 100 nM NAD^+ or ATP resulted in a significant fluorescence enhancement while a control experiment without NAD^+ or ATP only exhibited a negligible fluorescence change. The result provided a convincing proof of the detection mechanism of the proposed sensing strategy shown in Scheme1.

The feasibility of the fluorescent biosensor was also verified by agarose gel electrophoresis and ethidium bromide staining. As shown in Fig. 2, the products of D-RCA triggered by NAD^+ or ATP were observed in lane 2, 3 (100 nM NAD^+) and 4 (100 nM ATP), respectively, in which the RCA products showed extremely low

mobility. A brighter band was observed in lane 2, 3, 4, indicating that the higher concentration of NAD^+ or ATP would result in generating more RCA products. In contrast, no band appeared in the absence of NAD^+ or ATP in lane 1 and lane 5, indicating no RCA products were generated in the control experiment. Although the experiments might not give a reliable assessment of the molecular weight of the RCA products due to difference in electrophoretic behavior between single-stranded and double-stranded DNA, they provided immediate evidence for the high molecular weight of the ssDNA products.[1-2] These results indicated that NAD^+ or ATP acted as a trigger of the RCA reaction and the signal enhancement had a positive correlation with the NAD^+ or ATP level.



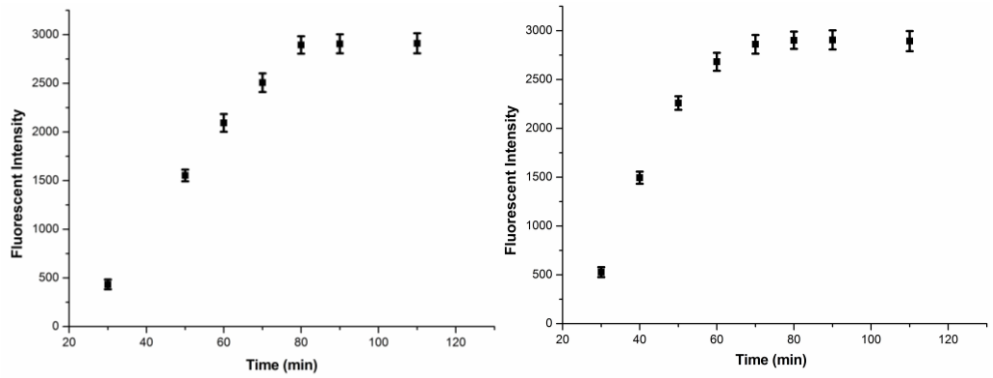
A B

Fig. S3. The effect of the molar ratio of D-DNA to the p-DNA on the fluorescence response of (A) ATP, (B) NAD^+ sensing system. The concentration of ATP and NAD^+ is 100 nM, respectively.

Parameters-Dependent signal amplification of the sensing strategy based on D-RCA –responsive G-quadruplex formation

In this novel strategy, RCA was a crucial step, which mediated the generation and amplification of the fluorescence signal. In order to achieve the system's best sensing performance, several experimental parameters affecting RCA were investigated (Fig. S3). The NAD^+ or ATP concentration was set at 100 nM for the subsequent optimizations. The NAD^+ or ATP-triggered ligation reaction has a significant influence on the RCA process. The full hybridization equilibrium between the D-DNA and p-DNA is a key factor for ensuring effcient ligation reaction. Therefore, the effect of molar ratio of the D-DNA to p-DNA was evaluated using a fixed concentration of p-DNA, namely 10 nM. As shown in Fig. S3, the fluorescence intensity increased with the increase in molar ratio. When the ratio value reached 5:1, the maximum fluorescence intensity was achieved. Thereafter, the fluorescence response exhibited a gradual decrease with a further increase in the molar ratio. This was probably because a large excess of dumbbell probes disturbed their hybridization with the c-DNA in a head-to-tail fashion and the subsequent D-RCA reaction. As a result, the molar ratio of 5:1 was selected for further investigation. Finally, 50 nM was selected as the optimal concentration of dumbbell probes due to its strongest fluorescence intensity. In theory, more complementary copies (duplex G-quadruplexes DNA) of the dumbbell probes template are generated with the elongation of RCA reaction time; stronger signal amplification will be produced. So the effect of RCA reaction time on the fluorescence signal was examined, which is shown in Fig. S4. The fluorescence intensity enhanced quickly with the increase in reaction time, and nearly reached a plateau after 90 min. This might be attributed to the fact that the RCA reaction had

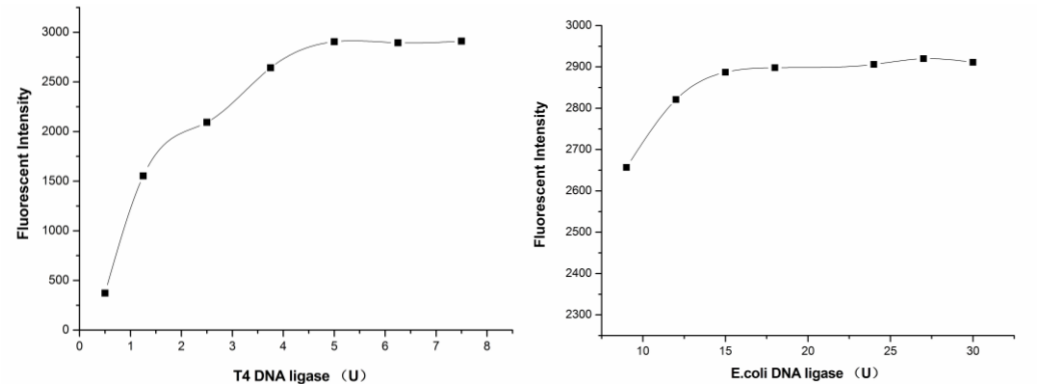
reached equilibrium caused by exhaustion of the RCA substrates or inactivation of phi29 DNA polymerase. Therefore, 90 min was chosen as the optimum time for the RCA reaction. This time was in agreement with the reported RCA reaction time of 1-2 h.



(A) (B)

Fig. S4. Influence of D-RCA reaction time on the fluorescent intensity signal responding to (A) 100 nM ATP and (B) 100 nM NAD^+.

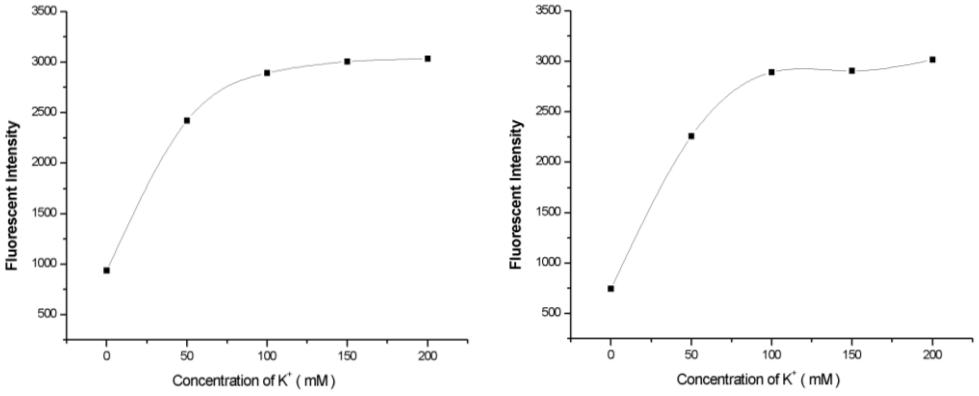
The full effcient ligation reaction equilibrium of the D-DNA is also a key factor for ensuring RCA reaction. Therefore, the effect of the dosage of DNA ligase was also evaluated. As shown in Fig. S5. Experimental results of DNA ligase showed that the following conditions could provide maximum fluorescent signal for the sensing system: 5 U of T4 DNA ligase, 20 U of E. coli DNA ligase.



(A) (B)

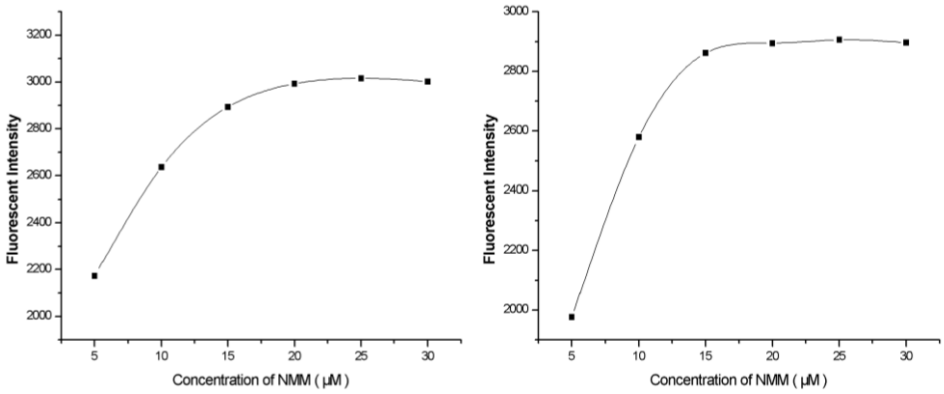
Fig. S5. The effect of (a) the concentration of T4 DNA ligase, (B) *E. coli* DNA ligase concentration on the fluorescence response of the ATP and NAD^+sensing system.

To achieve the best sensing performance, the concentration of NMM and K^+ were also optimized. As shown in Fig. S6 and S7. The experimental results indicated that a concentration of 100 mM of K^+ and 25 μM of NMM could provide maximum S/N ratio for the sensing system.



(A) (B)

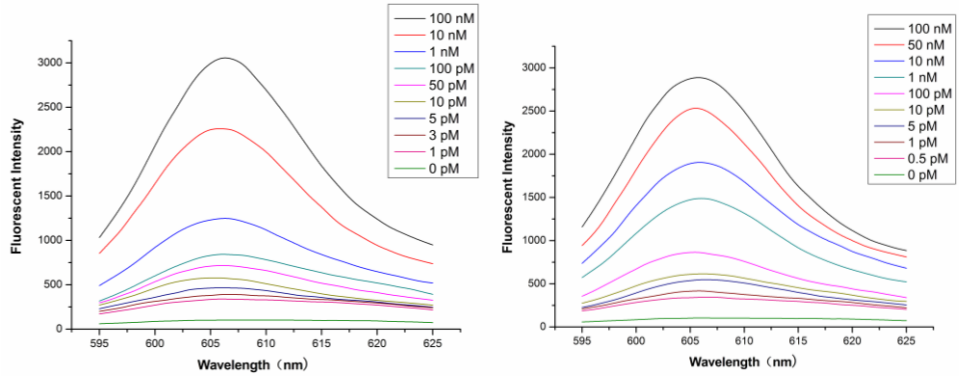
Fig. S6. The effect of the concentration of K^+ on the fluorescence response of the sensing system. (A) ATP,（B）NAD^+. The concentration of ATP and NAD^+ is 100 nM, respectively.



(A) (B)

Fig. S7. The effect of the concentration of NMM on the fluorescence response of the sensing system. (A) ATP,（B）NAD^+. The concentration of ATP and NAD^+ is 100 nM, respectively.

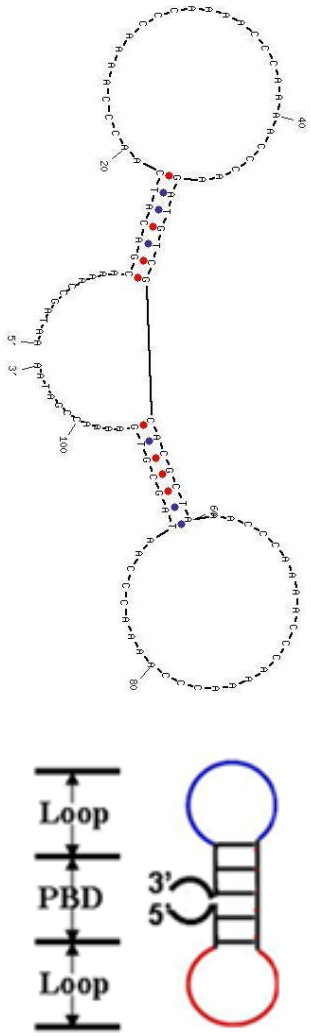
Application of the label-free cascade amplification strategy based on D-RCA-responsive G- quadruplexe formation toward ATP and NAD^+ Detection



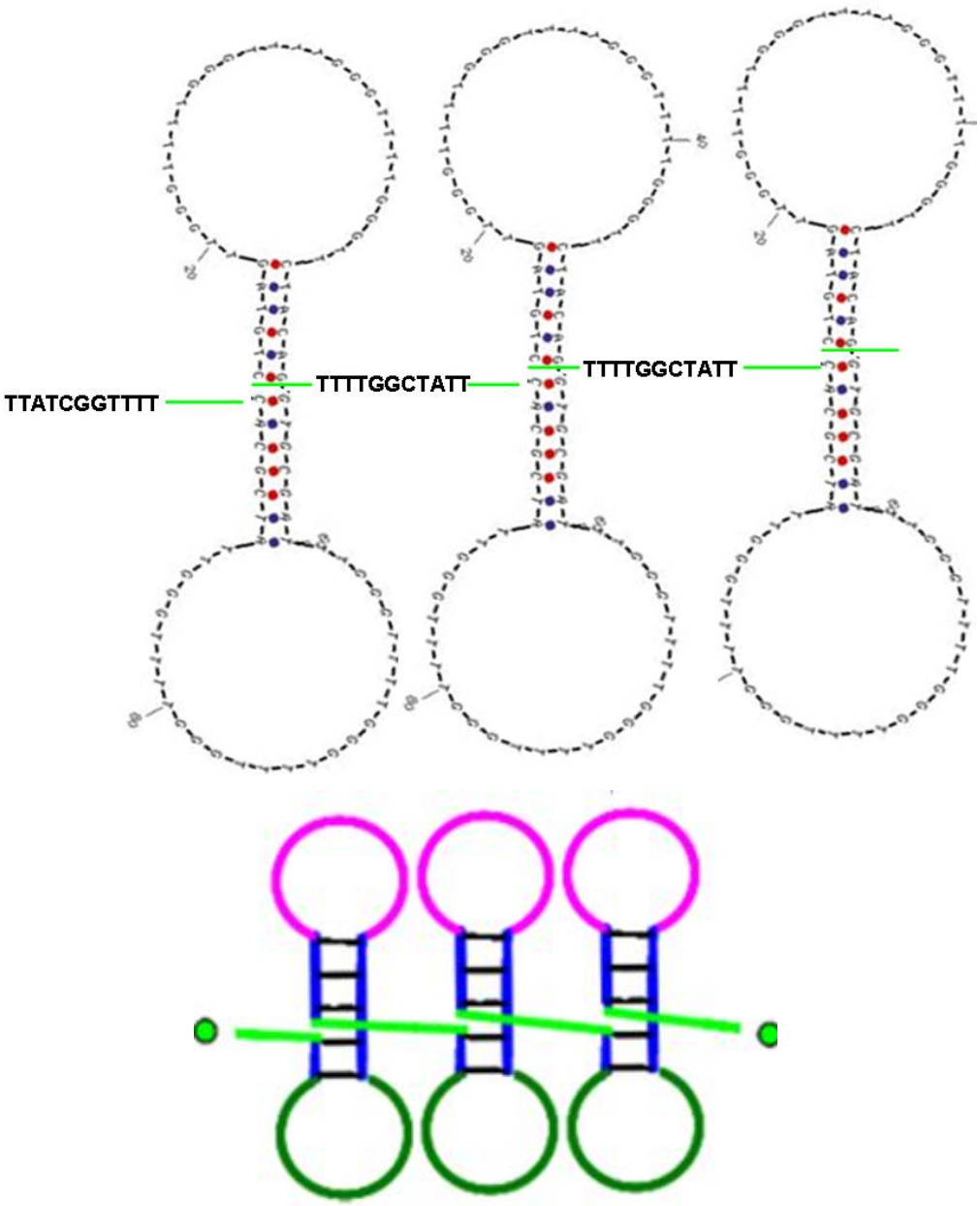
(A) (B)

Fig. 8. (A) Fluorescence emission spectra obtained in the label-free cascade amplification strategy for detection of (A) ATP and (B) NAD^+ with varying

concentrations from 0-100 nM.



（A）



(B)

Fig. S9. Structure of （A）the dumbbell probe (D-DNA) and (B) a part of the repetitive structure of the RCA-product.

To demonstrate the performance of the proposed method for real sample analysis, we take analysis of cellular ATP as an example to demonstrate the performance of the proposed method for real sample analysis. Analysis of cellular ATP from HeLa cell lysate was implemented. A suspension of 2.2×10^6 cells was used for ATP. The results of the determination were listed in Table 1. The detected concentration of ATP in the

HeLa lysate was about 3.72 μM, which was in good agreement with that obtained using the previous reported method.[3] These results demonstrated that the developed method could be used to monitor the content of ATP in cell extracts without the interference of other substances in the cell.

Table 1 Comparisons of the D-RCA assay with the CESA-Based Method[2] and the HPLC Method[2] for the Detection of ATP in the HeLa Lysate

sample[a]	D-RCA(μM)	RSD	CESA Method[3] (μM)	RSD	HPLC[3] (μM)	RSD
1	3.57	3.8%	3.69	3.9%	3.86	3.2%
2	3.85	3.4%	3.94	3.1%	3.78	3.7%
3	3.74	3.1%	3.83	4.2%	3.61	4.1%

[a]Each sample was analyzed in triplicate, and the results are the average values.

Notes and references

1. L. Zhou, L. J. Ou, X. Chu, G. L. Shen and R. Q. Yu, Anal. Chem., 2007, 79, 7492.
2. Q. Xue, L.Wang and W. Jiang, Chem. Commun., 2012, 48, 3930
3. J. Li, H. E. Fu, L. J. Wu, A. X. Zheng, G. N. Chen and H. H. Yang, Anal. Chem, 2012, 84, 5309-5315.