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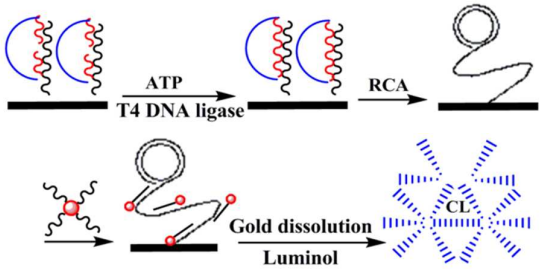

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ARTICLE TYPE

A cascade amplification strategy based on rolling circle amplification and hydroxylamine amplified gold nanoparticles enables chemiluminescent detection of adenosine triphosphate

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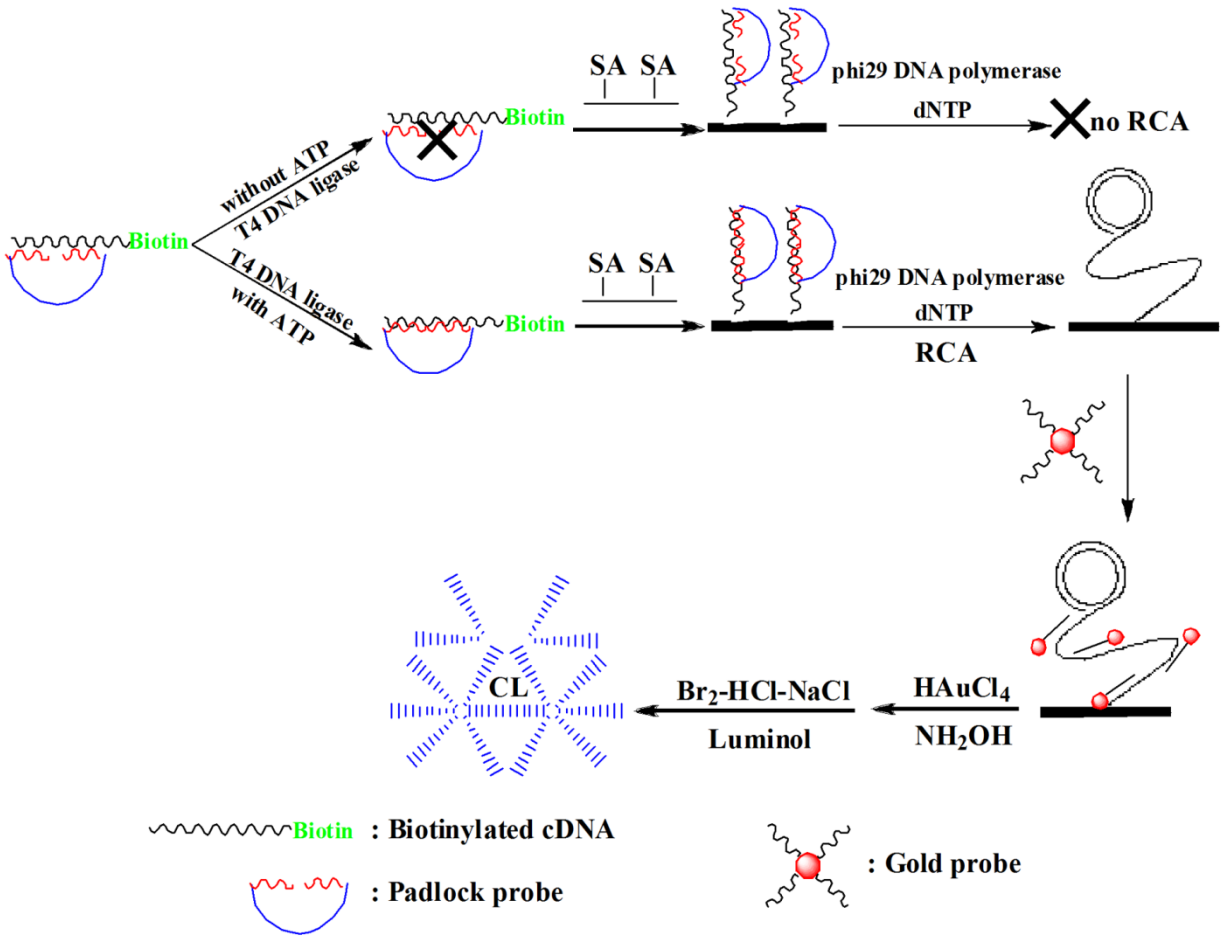
A highly sensitive and selective chemiluminescent (CL) biosensor for adenosine triphosphate (ATP) was developed by taking advantage of the ATP-dependent enzymatic reaction (ATP-DER), the powerful signal amplification capability of rolling circle amplification (RCA), and hydroxylamine-amplified gold nanoparticles (Au NPs). The strategy relies on the ability of ATP, a cofactor of T4 DNA ligase, to trigger the ligation-RCA reaction. In the presence of ATP, the T4 DNA ligase catalyzes the ligation reaction between the two ends of the padlock probe, producing a closed circular DNA template that initiates the RCA reaction with phi29 DNA polymerase and dNTP. Therein many complementary copies of the circular template can be generated. The ATP-DER is eventually converted into detectable CL signal after a series of processes, including gold probe hybridization, hydroxylamine amplification, and oxidative gold metal dissolution coupled with a simple and sensitive luminol CL reaction. The CL signal is directly proportional to the ATP level. The results showed that the detection limit of the assay is 100 pM of ATP, which compares favorably with those of other ATP detection techniques. In addition, by taking advantage of ATP-DER, the proposed CL sensing system exhibited extraordinary specificity towards ATP and could distinguish the target molecule ATP from its analogues. The proposed method provides a new and versatile platform for the design of novel DNA ligation reaction-based CL sensing systems for other cofactors. This novel ATP-DER based CL sensing system may find wide applications in clinical diagnosis as well as environmental and biomedical fields.

1. Introduction

ATP as a biologically important molecule is often used as a universal energy storage molecule in all living organisms and also as an indicator of living organisms for cell viability and cell injury[1-4]. It is well known that ATP is the primary energy carrier of the living systems and the aberrant ATP levels are associated with a variety of genetic disease such as hypoxia, hypoglycemia, Parkinson's disease and some malignant tumors[5-8]. Therefore, the sensitive and selective detection of ATP is of great significance for biochemical studies and clinical diagnosis. In recent years, various aptamer-based biosensors for ATP have been developed using electrochemiluminescence (ECL)[9-11], electrochemical[12, 13], fluorescent[14-18] and colorimetric method[19, 20]. However, these methods show moderate sensitivity because the anti-ATP DNA aptamer is a low-affinity species with a relatively low association constant with ATP[21]. Furthermore, most of these methods cannot discriminate ATP from their analogues, such as AMP.

Instead of aptamer-based ATP sensors, some enzyme reactions show specific dependence on cofactors ATP, namely ATP-DER, providing an efficient platform for constructing highly selective sensing systems for ATP. Using ATP as a cofactor to T4 DNA ligase, several fluorescent and electrochemical sensing systems have been reported for the detection of ATP, most of which were based on dually labeled molecular beacon[22, 23] or molecular beacon-like DNA[24]. The dually labeled oligonucleotide probe brings about complexity and high cost which may limit its practical use. Yu group developed a highly selective fluorescent biosensor for ATP detection using single-labeled probe based on ATP-DER and the different adsorption affinity between graphene oxide and DNA structures[25]. Wang group developed a non-covalent labeling strategy for ATP based on inhibition of template-directed G-quadruplex assembling by ligation reaction[26]. T4 DNA ligase is one of the key enzymes involved in the RCA assays, in which the hybridized padlock probes are circularized by ATP-activated T4 DNA ligase and are then copied into linear concatamers by DNA polymerases under isothermal conditions [27]. The concatamers are frequently thousands of nucleotides in length, leading to a large amplification of the initial circular "signal". Using ATP-triggered RCA, several fluorescent sensing schemes for detection of ATP have been developed[28, 29]. For example, Jiang group developed highly sensitive detection of ATP based on dumbbell probe-mediated RCA-responsive G-quadruplex formation[28]. Those ATP-DER-based methods are proven to be highly sensitive and more selective than the aptamer-based methods due to the unique role of ATP in the



Scheme 1 The schematic diagram of ATP triggered ligation-RCA assay for CL detection of ATP.

enzymatic reaction. However, we noticed that most of those methods focused on using fluorescent methods. As one of the most sensitive methods, CL promises high sensitivity, simplicity, and low cost. It has become an attractive bioanalytical method. In recent years, sereval CL mechanisms have been desigened for the determination of ATP. For example, in previous work, our group developed a label-free CL aptasensor for the detection of ATP with a detection limit of 70 μM by using graphene oxide nano-platform[30]. Based on chemiluminescence resonance energy transfer (CRET), Zhao group developed an aptasensor for ATP with a detection limit of 185 nM[31]. Because of the reliance on anti-ATP DNA aptamer, the above designs for the CL determination of ATP suffered from low sensitivity as well as low descrimination of ATP and its analogues. To overcome these problems, the present work developed a novel CL assay for the determination of ATP based on ATP-DER, rolling circle amplification and hydroxylamine amplified Au NPs. To the best of our knowledge, ATP-DER-based CL strategy for ATP detection has not been reported yet.

Au NPs are particularly attractive in bioassay by virtue of their facile synthesis, large specific surface area, high chemical stability, and biocompatibility[32-34]. As an amplification tag, Au NPs have been the subject of research directed at gene analysis and antibody or antigen detection[35-38]. In current work, an ATP-DER-based CL assay using hydroxylamine amplified Au NPs as label was developed for the determination of ATP with high sensitivity and specificity. Scheme 1 shows the sensing mechanism. The 28 3'- and 5'- terminal bases of the 81-mer padlock probe were designed to be complementary to the biotinylated cDNA. In the presence of T4 DNA ligase together with its cofactor ATP, the 3'-OH and 5'-PO_4 ends of the padlock probe could be ligated to produce a closed circular DNA template in an amount that is positively related to the concentration of the cofactor ATP. The ligation products were then immobilized on the surface of 96-well plate via biotin-streptavidin reaction. The immobilized cDNA acted as primers to initiate the RCA reaction in the presence of phi29 DNA polymerases and dNTP. The RCA reaction process produced long single-stranded DNA by replicating the circular template hundreds to thousands of times, which could serve as an excellent template for the binding of gold probe. Au NPs assembled on the surface of 96-well plate were then enlarged through a redox reaction of $HAuCl_4$ and NH_2OH, in which gold metals were catalytically deposited onto the surfaces of the Au NPs[39, 40]. Finally, the gold metal contained in the bound phase was dissolved in an oxidative Br_2-HCl-NaCl solution, and the gold ions thus released into solution were

quantitatively measured by the Au^{3+} catalyzed luminal CL reaction. The CL signal was proportionally correlated to the concentration of ATP in the standard or sample. In contrast, in the absence of ATP, the T4 DNA ligase was inactive and could not catalyze the ligation reaction of padlock probe and subsequent RCA reaction. Therefore, no Au NPs could be immobilized on the surface of 96-well plate. We successfully converted the ATP-DER into detectable CL signals by this way.

2. Experimental

2.1 Materials and chemicals

All chemicals were of analytical reagent grade and were used as received. Distilled water (18.2 MΩ cm^{-1}) was used throughout the current work. T4 DNA ligase, phi29 DNA polymerase, and deoxynucleotides (dNTPs) were obtained from Thermo Fisher Scientific (China) Co., Ltd. Bovine serum albumin (BSA), dithiothreitol (DTT), and human serum were obtained from Puboxin Biotechnology Co., Ltd (Beijing, China). Calmodulin was purchased from Sigma-Aldrich (St. Louis, MO). Hydroxylamine hydrochloride ($NH_2OH·HCl$), hydrogen tetrachloroaurate (III) tetrahydrate ($HAuCl_4·4H_2O$), tween 20, and other chemical reagents were obtained from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). Luminol was purchased from Alfa Aesar (Tianjin, China). Citrate-stabilized Au NPs (13-nm diameter) were prepared according to the methods described in the literature[41, 42]. ATP, GTP, UTP, CTP, AMP and all oligonucleotides were acquired from Sangon Biological Engineering Technology & Service Co., Ltd (Shanghai, China). All oligonucleotides were purified by high-performance liquid chromatography (HPLC) and are listed in Table 1. 96-well plate coated with a layer of reactive N-oxysuccinimide esters, referred to as NOS group surface, was purchased from Corning Inc. (New York, United States).

2.2 Apparatus

CL measurements were performed with a BPCL chemiluminescence analyzer (Beijing, China). The hydrodynamic sizes of Au NPs were evaluated by a Zetasizer Nano ZS system (Malvern Instruments Ltd., Worcestershire, UK).

2.3 Preparation of the reporter probe-Au NP conjugates (gold probes).

Streptavidin modified Au NPs were prepared via slightly modifying a previously published method[43]. The streptavidin (10 % more than the minimum amount, which was determined using a flocculation test) was added to 1 mL colloidal gold suspension (pH 7.0, adjusted by 0.1 M K_2CO_3) followed by incubation at room temperature for 30 min. The conjugates were centrifuged at 4 ℃ (25,000 g, 60 min). After carefully removing the supernatant, the red soft sediment was re-suspended in buffer A (20 mM Tris-HCl and 0.5 M NaCl, pH 8.0) containing 0.025 % tween 20 and 1 % BSA. Subsequently, 3.09 μL of biotinylated reporter probe (16.2 μM) was added and incubated for 30 min with gentle mixing at 37 ℃. The final conjugates were isolated by centrifugation (25,000 g, 60 min). The red oily precipitate was re-suspended in 1 mL of buffer A. The addition of BSA with a final concentration of 1% allowed storage of the gold probe at 4 ℃ for several days.

2.4 Preparation of streptavidin-bound 96-well plate.

Streptavidin was diluted to 30 ng in 1 mL of coupling buffer (0.05 M Na_2HPO_4-NaH_2PO_4, pH 8.5). The diluted streptavidin solution were divided equally into the wells (50-μL per well) of a 96-well plate which had a NOS surface, and incubated at 37 ℃ for 60 min with gentle shaking. After removing the solution, 200 μL of Tris solution (10 mM in coupling buffer) was added into each well of the 96-well plate and incubated at 37 ℃ for another 60 min to block the remaining NOS groups. The wells were then washed twice with washing buffer (7 mM Tris, 0.17 M NaCl and 0.05% tween 20, pH 8.0).

2.5 ATP-DER for ATP detection

For the ligation reaction with T4 DNA ligase, the reaction mixture consisted of ligation buffer (40 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, pH 7.8), 0.5 U of T4 DNA ligase, 0.25 pmol of padlock probe, 1.25 pmol of cDNA, and different concentration of ATP in a reaction volume of 25 μL. Before the addition of T4 DNA ligase, ATP, and ligation buffer, the solution was heated to 90 ℃ and maintained for 3 min, and then cooled down to room temperature. After adding ligation buffer and designed concentration of ATP, the mixture was incubated at 16℃ for 30 min with gentle shaking. T4 DNA ligase was then added and the reaction mixture was incubated at 16 ℃ for 90 min. T4 DNA ligase together with the cofactor ATP catalyzed the formation of a circular DNA template in an amount that is positively related to the concentration of the cofactor ATP. The reaction mixture was then heated at 65 ℃ for 10 min to denature the enzymes and cooled down to room temperature for the hybridization between cDNA and the circular DNA template.

The ligation products were transferred to each well of the streptavidin-bound 96-well plate, and then incubated at 16 ℃ for 30 min with gentle mixing. After washing in triplicate with 2×SSC buffer (0.3 M NaCl, 0.03 M $C_6H_5Na_3O_7 • 2H_2O$, pH 7.0), the RCA reaction was performed at 30 ℃ for 90 min in a volume of 50 μL of solution containing 33 mM Tris-acetate, 10 mM $Mg(Ac)_2$, 66 mM KAc, 1 mM dNTPs, 1 U phi29 DNA polymerase, and 1 mM DTT. The wells were then washed triple with buffer AT (buffer A containing 0.05% tween 20). An aliquot of 50 μL of gold probe (0.928 nM in buffer A containing 1% BSA) was hybridized with the RCA products for 40 min at 30 ℃. The excess gold probe was washed away with washing buffer, and the Au NPs assembled on the surface of 96-well plate were catalytically enlarged in the presence of 0.5 mM NH_2OH and 0.05 mM $HAuCl_4$ at room temperature for 4 min. Then, the wells were rinsed three times with the washing buffer. After carefully removing the rinsing solution, 200 μL of gold metal oxidative solution (final concentration was 0.25 mM Br_2 - 2.5 mM HCl - 0.15 M NaCl) was pipetted into the wells and reacted for 10 min. The resultant mixture was placed in the water bath at 60 ℃ for 4 min. Finally, 60 μL of the solution was transferred into 14×40 mm glass tubes containing 100 μL of $5×10^{-6}$ M luminol (in 0.75 M NaOH) and the CL signal was then displayed in the CL analyzer (n=3).

Table 1 . Oligonucleotides Synthesized in the Present Study

cDNA
5'-biotin-AAAAAAAAAATGTCCGTGCTAGAAGGAAACAGTTACCA-3'
Padlock probe
5'-PO_4-TAGCACGGACATATATGATGGTAC*CGCAGTATGAGTATCTCC*TATCACTACTAAGTGGAAGAAATGGTAACTGTTTCCTTC-3'
Reporter probe
5'-biotin-AAAAAAAAAACGCAGTATGAGTATCTCC-3'

The underlined sequences in cDNA can hybridize the two ends of padlock probe and italic sequence in padlock probe is similar to the end sequence of detect probe.

3. Results and discussions

3.1 Feasibility of the CL biosensor for ATP detection

Firstly, the feasibility of the CL biosensor was verified by agarose gel electrophoresis. The electrophoresis experiment was performed by 1.0 % agarose gels and run in 1×TAE buffer (40 mM Tris, 20 mM HAc, 10 mM EDTA, pH 8.0) at room temperature under a constant voltage of 100 V for 50 min with loading of 10 μL of each sample into the lanes. As shown in the inset of Fig. 1, a long DNA product of ligation-RCA triggered by 5 nM ATP was observed in lane B1, in which the RCA product showed extremely low mobility, meaning that the length of the RCA product was more than 10000 bp. In addition, a blurry band, suggesting the length of DNA is about 1000 bp, was also seen in Fig. 1B1. The above results indicated that the length of RCA products ranged from ~1000 to > 10000 bp, and most of the products were longer than 10000 bp. In contrast, there is no band in the absence of ATP (data not shown). We further reduced the electrophoresis time to 20 min. As shown in the inset of Fig. 1, a band between 100 and 250 bp, which is consistent with the total length of cDNA and padlock probe, was observed in lane A1, indicating no RCA product was generated in the absence of ATP. Secondly, we investigated the corresponding CL intensities of the sensing strategy in the absence and presence of ATP, respectively. As shown in Fig.1, the control experiment without ATP only exhibited a low CL intensity, while 5 nM ATP showed a significant CL intensity enhancement. These results indicated that ATP acted as a trigger of the ligation-RCA reaction. In the presence of ATP, the two ends of the padlock probe could be ligated and the subsequent RCA reaction could be initiated to produce lots of complementary copies of the circular template. Therefore, more gold probes could be assembled on the surface of 96-well plate resulting in an enhanced CL signal. In contrast, in the absence of ATP, the DNA ligase was inactive and could not catalyze the ligation reaction of padlock probe and initiate the subsequent RCA reaction.

3.2 Optimization of assay conditions for the determination of ATP

In order to achieve the best sensing performance, several experimental parameters were investigated systematically. Firstly, ATP dependent ligation reaction, a crucial step for ATP sensing, has a significant effect on RCA process. Experimental conditions which affect ligation reaction, such as the amount of T4 DNA ligase and cDNA, and the molar ratio of cDNA to padlock probe were optimized firstly. As shown in Fig. 2A, the relative CL intensity (CL_1 deducted CL_0, where CL_1 and CL_0

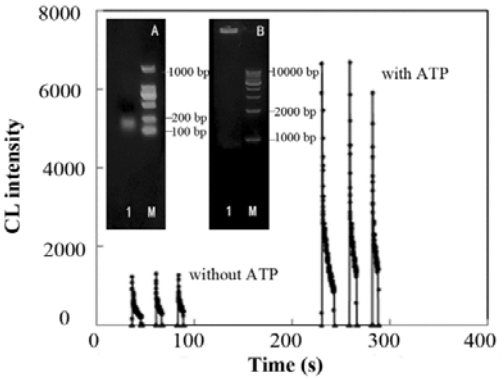


Fig. 1. CL intensity in the absence (A) and presence (B) of ATP; the corresponding agarose gel electrophoretic experiments were depicted in the inset. The marker was indicated by M.

were the CL intensity in the presence and absence of ATP, respectively) increased rapidly with increase of T4 DNA ligase from 0.1 to 0.5 U, and then reached a plateau between 0.5 – 1 U. Hence, 0.5 U of T4 DNA ligase was selected for the following studies. The full hybridization equilibrium between the cDNA and padlock probe is a key factor for ensuring efficient ligation reaction. It is essential to optimize the molar ratio of cDNA to padlock probe. Using a fixed amount of cDNA (1.25 pmol), the molar ratio of cDNA to padlock probe was investigated. As shown in Fig. 2B, when the molar ratio increased to 5:1, the maximum relative CL intensity was achieved. Higher molar ratio brought a gradual decrease in relative CL intensity. This phenomenon may be attributed to that a large excess of padlock probe may disturb its hybridization with cDNA in a head-to-tail fashion and subsequent RCA reaction. Therefore, the optimum molar ratio of cDNA to padlock probe was 5:1. Fig. 2C showed the effect of cDNA amount on the relative CL intensity. The strongest relative CL intensity was obtained by using 1.25 pmol of cDNA. Hence, 1.25 pmol cDNA was selected for the further study.

In principle, the more complementary copies of the circular template are produced, the stronger CL intensity will be generated. So the effect of phi29 DNA polymerase concentration and RCA reaction time were examined. As shown in Fig. 3A, the relative CL intensity increased with the increase of phi29 DNA polymerase from 0.25 to 1 U and then reached a plateau between 1-2 U. Thus, 1 U of phi29 DNA polymerase was selected for the following experiments. To obtain a maximum response using a minimal concentration of gold probe, we then optimized the molar ratio of the biotinylated reporter probe to streptavidin-gold and the concentration of gold probe. The molar ratio of the

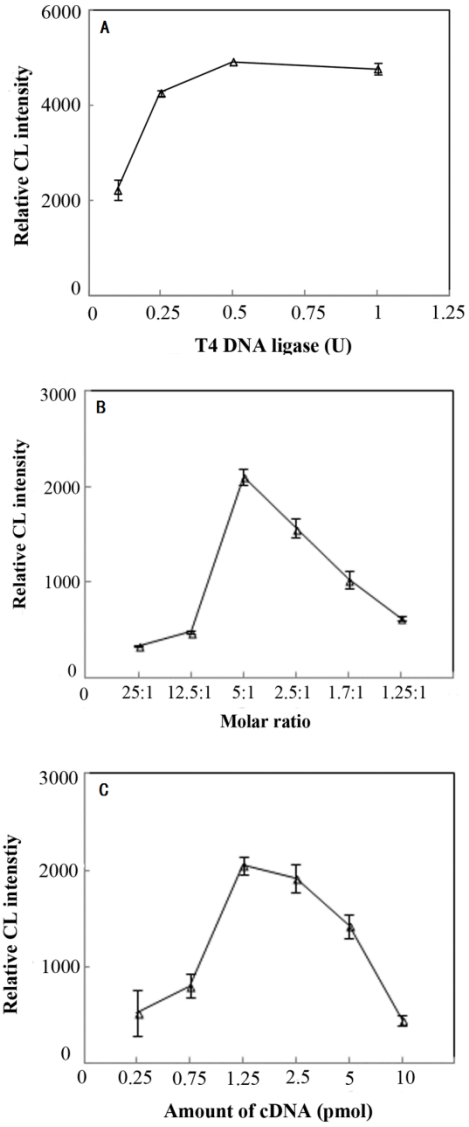


Fig. 2. (A) The effect of T4 DNA ligase amount on the relative CL intensity of ATP sensing system, experimental conditions: cDNA, 1.25 pmol; padlock probe, 80 fmol; ATP, 50 nM; dNTPs, 1 mM; phi29 DNA polymerase, 0.5 U; RCA time, 2 h; gold probe, 0.928 nM; $HAuCl_4$, 0.05 mM; NH_2OH, 0.5 mM; T_R, 4 min. (B) The effect of molar ratio of cDNA to padlock probe on the relative CL intensity of ATP sensing system, experimental conditions: ATP, 5 nM; T4 DNA ligase, 0.5 U; other experimental conditions were the same as (A). (C) The effect of cDNA amount on the relative CL intensity of ATP sensing system. Experimental conditions: molar ratio of cDNA to padlock probe, 5:1; other experimental conditions were the same as (B). The error bars show the standard deviations for three replicate determinations.

biotinylated reporter probe to streptavidin-gold was evaluated using a fixed concentration of streptavidin-gold (0.928 nM). As shown in Fig. 3B, the relative CL intensity reached maximu by using 2.5 pmol of biotinylated reporter DNA. Finally, we optimized the concentration of gold probe by investigating the relative CL intensity of the CL biosensor with different concentration of gold probe (Fig. 3C). The relative CL intensity increased rapidly by raising the concentration of gold probe concentration at the beginning, leveled off between 0.928 and 2.32 nM, and then decreased, due to the greatly enhanced

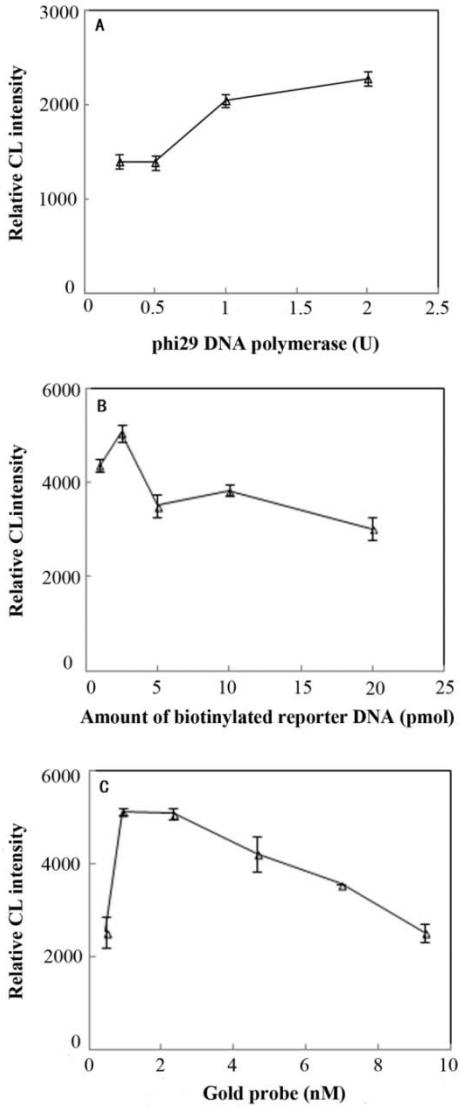


Fig. 3. (A) The effect of phi29 DNA polymerase amount on the relative CL intensity of ATP sensing system, experimental conditions: cDNA, 1.25 pmol; other experimental conditions were the same as Fig. 2.(C). (B) The effect of biotinylated reporter DNA on the relative CL intensity of ATP sensing system, experimental conditions: phi29 DNA polymerase 1 U; RCA time, 1.5 h; other experimental conditions were the same as (A). (C) The effect of gold probe on the relative CL intensity of ATP sensing system, experimental conditions: biotinylated reporter DNA, 2.5 pmol; other experimental conditions were the same as (B). The error bars show the standard deviations for three replicate determinations.

background signal. Hence, 0.928 nM gold probe was selected for the following experiments.

3.3 Assay performance of the ATP-DER CL sensing system for ATP detection

The quantitative behavior of the CL assay was assessed with different concentrations of ATP under the optimized conditions. Fig.4 shows the relative CL intensity of the sensing system upon addition of different concentrations of ATP. The relative CL intensity increased remarkably with the increasing concentration of ATP from 0.1 to 5 nM, indicating that the formation of RCA products (or the capture of gold probes on the surface of 96-well plate) was highly dependent on the concentration of target ATP.

A calibration graph in the concentration range of 0.1-5 nM showed a linear correlation (R^2=0.9808) between the concentration of ATP (nM) and the CL intensity, represented by I= 1009.1C + 281.4. Based on the 3σ rule, as low as 100 pM of target ATP could be sensitively detected using this method which exhibited 70 000 times enhancement of sensitivity over previous label-free CL detection of ATP[30] and 1 850 times over the CRET format[31]. In addition, the amplified CL signal was coupled to a relatively good reproducibility. A series of eight repetitive measurements of ATP solution (5 nM) yielded reproducible signals with a relative standard deviation of 4.6 %.

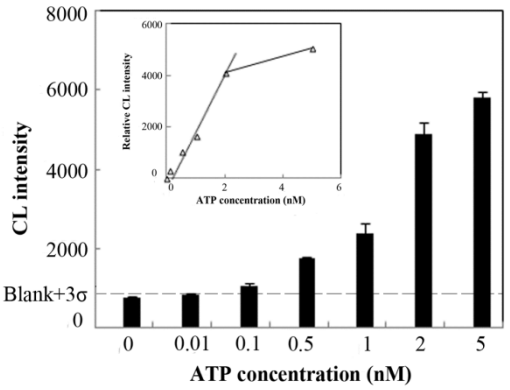


Fig. 4. CL intensity vs. different concentrations of ATP. The inset displays the linear relationship between relative CL intensity and ATP concentration in the range from 0.1 to 5 nM. The error bars show the standard deviations for three replicate determinations.

Compared to previously reported anti-ATP DNA aptamer-based sensing systems, the ATP-DER based CL sensing system exhibits highly specificity for ATP detection, because the ligation-RCA cannot occur in the absence of the cofactor ATP.The specificity of the CL sensing strategy was examined by challenging it with several possible interfering targets, including UTP, CTP, GTP, AMP, BSA and calmodulin. Fig. 5 illustrated the histograms of the relative CL intensity in the presence of ATP and other possible interfering targets. It could be observed that negligible signals were obtained in the presence of 5 nM of UTP, CTP, GTP and AMP, and 500 nM of BSA and calmodulin. The experimental results demonstrated that the ATP-DER based CL assay developed here showed high selectively toward ATP detection, which makes it promising for practical application.

3.4 Analysis of ATP in serum samples

To demonstrate the feasibility of the approach in complex biological matrices, we applied this CL sensing system to detect the ATP spiked human serum. The addition of serum would lead to the matrix effect, so the dilution factor of serum was optimized in the preliminary experiment. And a better performance in terms of higher relative CL intensity was obtained when 100-fould diluted serum was employed, so 100-fold diluted serum was applied in the following experiment. Firstly, the dose-response curve generated from a representative serum sample was investigaed. We noticed that the CL signals obtained in serum samples were similar to that in buffer solutions. As shown in Fig. 6, the relative CL intensity increased upon increasing the concentration of the spiked ATP, with linearity (R^2 = 0.9866) existing between the relative CL intensity and the concentration of ATP over the range from 0.1 to 2 nM. Secondly, the ability of the ATP-DER-based CL assay to detect ATP in real sample was tested by using a standard addition method. Different amounts of ATP were added into 100-fold diluted serum samples and determined under the optimal experimental conditions. The data for the quantization of ATP were shown in Table 2, which indicated recoveries ranging from 93.0% to 113.1%. The result indicated the very little interference of complex matrices in the designed strategy, and the proposed method could be used in the real sample analysis.

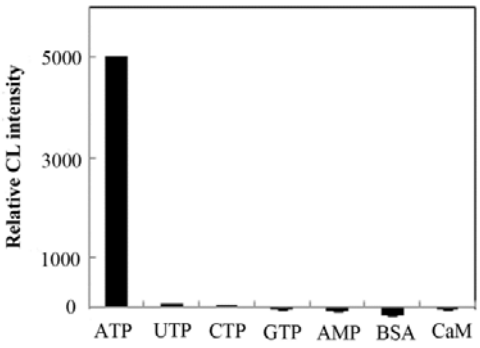


Fig. 5. Selectivity of the CL sensing system for ATP compared to other possible interfering targets. The concentration of ATP, UTP, CTP, GTP and AMP are 5 nM. The concentration of BSA and CaM are 500 nM. Other experimental conditions were the same as Fig. 4. The error bars show the standard deviations for three replicate determinations.

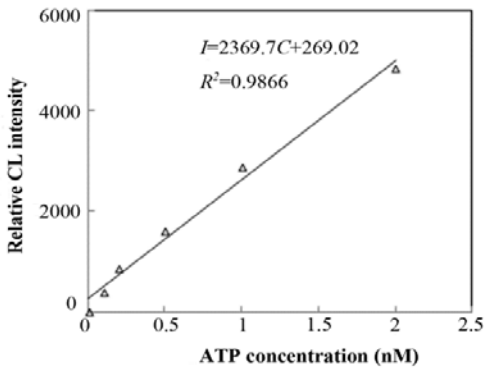


Fig. 6. Correlation between the results measured by proposed method for ATP detection. It displays the linear relationship between relative CL intensity and ATP concentration in the range from 0.1 to 2 nM.

Table 2. Recovery of ATP spiked into human serum samples.

Amount added (nM)	Recovery (%)
0.5	113.1±8.6
1	93.0±9.6
2	111.4±19.8

4. Conclusion

In summary, a novel CL sensing strategy for ATP detection was developed by taking advantage of ATP-DER for the increased specificity, and the powerful signal amplification capability of RCA and hydroxylamine-amplified Au NPs. The proposed

Table 3. Comparison of sensitivity for different ATP assay methods

Analytical method	Label	Detection limit	Linearity range	Method	Reference
Electrochemistry	$[Fe(CN)_6]^{3-/4-}$	0.05 nM	0.1-1000 nM	ATP-DER	24
Fluorescence	QDs	24 μM	0.1-1 mM	Aptamer-based	16
Fluorescence	FAM	2 μM	5-2500 μM	Aptamer-based	14
Fluorescence	QDs	3.7 μM	10-350 μM	Aptamer-based	21
Fluorescence	Cy3	1000 nM	10-100 μM	ATP-DER	27
Fluorescence	Label free	0.5 pM	0.001-100 nM	ATP-DER	28
Fluorescence	NMM	50 nM	0.05-0.6 μM	ATP-DER	26
Fluorescence	FAM	0.1 nM	0.1-300 nM	ATP-DER	22
CL	Label free	70 μM	0.1-4 mM	Aptamer-based	30
CL	QDs	185 nM	50-231 μM	Aptamer-based	31
ECL	$Ru(phen)_3^{2+}$	0.2 nM	0.5-1000 nM	Aptamer-based	10
CL	Au NPs	100 pM	0.1-5 nM	ATP-DER	This work

ATP-DER-based CL sensing system demonstrated high sensitivity that can detect the presence of ATP at a concentration as low as 100 pM (corresponding to 5 fmol in a 50 μL volume), which compares favorably with those of other aptamer-based ATP detection techniques (Table 3). In addition, this ATP-DER based CL biosensor demonstrated extraordinary specificity towards ATP and could distinguish the target molecule ATP from its analogues. ATP has also been determined in diluted serum, indicating the applicability of this assay. In addition, it is worthwhile to point out that no sophisticated equipment is required. The proposed method provide a new and universal platform for design of novel DNA ligation reaction-based CL sensing system for other cofactors, monitoring activity of DNA ligase, and may find wide application in clinical diagnosis of ATP relative diseases as well as environmental and biomedical fields.

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Notes and references

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1 M. Erecinska and D.F. Wilson, *J. Membr. Biol.*, 1982, **70**, 1-14.
2 L. Kong, J. Xu, Y.Y. Xu, Y. Xiang, R. Yuan and Y.Q. Chai, *Biosens. Bioelectron.*, 2013, **42**, 193-197.
3 Y. Eguchi, S. Shimizu and Y. Tsujimoto, *Cancer Res.*, 1997, **57**, 1835-1840.
4 E.H. Abraham, K.M. Sterling, R.J. Kim, A.Y. Salikhova, H.B. Huffman, M.A. Crockett, N. Johnston, H.W. Parker, W.E. Boyle, A. Hartov, E. Demidenko, J. Efird, J. Kahn, S.A. Grubman, D.M. Jefferson, S.C. Robson, J.H. Thakar, A. Lorico, G. Rappa, A.C. Sartorelli and P. Okunieff, *Blood Cell Mol. Dis.*, 2001, **27**, 165-180.
5 J.A. Cruz-Aguado, Y. Chen, Z. Zhang, N.H. Elowe, M.A. Brook and J.D. Brennan, *J. Am. Chem. Soc.*, 2004, **126**, 6878-6879.
6 K.T. Bush, S.H. Keller and S.K. Nigam, *J. Clin. Invest.*, 2000, **106**, 621-626.
7 S. Przedborski and M. Vila, Clin. *Neurosci. Res.*, 2001, **1**, 407-418.
8 R.A. Harkness and O.D. Saugstad, *Scand. J. Clin. Lab. Invest.*, 1997, **57**, 655-672.
9 J.J. Lu, M. Yan, L. Ge, S.G. Ge, S.W. Wang, J.X. Yan and J.H. Yu, *Biosens. Bioelectron.*, 2013, **47**, 271-277.
10 N.N. Bu, A. Gao, X.W. He and X.B. Yin, *Biosens. Bioelectron.*, 2013, **43**, 200-204.
11 G.F. Jie, J.X. Yuan and J. Zhang, *Biosens. Bioelectron.*, 2012, **31**, 69-76.
12 B.C. Yin, Y.M. Guan and B.C. Ye, *Chem. Commun.*, 2012, **48**, 4208-4210.
13 B.Q. Liu, Y.L. Cui, D.P. Tang, H.H. Yang and G.N. Chen, *Chem. Commun.*, 2012, **48**, 2624-2626.
14 Y. He, Z.G. Wang, H.W. Tang and D.W. Pang, *Biosens. Bioelectron.*, 2011, **29**, 76-81.
15 J.M. Liu and X.P. Yan, *Biosens. Bioelectron.*, 2012, **36**, 135-141.
16 Z. Chen, G. Li, L. Zhang, J.F. Jiang, Z. Li, Z.H. Peng and L. Deng, *Anal. Bioanal. Chem.*, 2008, **392**, 1185-1188.
17 Y.Y. Xu, J. Xu, Y. Xiang, R. Yuan and Y.Q. Chai, *Biosens. Bioelectron.*, 2014, **51**, 293-296.
18 M. Zhang, S.M. Guo, Y.R. Li, P. Zuo and B.C. Ye, *Chem. Commun.*, 2012, **48**, 5488-5490.
19 A.H. Liang, H.X. Ouyang and Z.L. Jiang, *Analyst*, 2011, **136**, 4514-4519.
20 F. Liu, J. Zhang, R. Chen, L.L. Chen and L. Deng, *Chem. Biodivers.*, 2011, **8**, 311-316.
21 J.N. Tian, Y. Wang, S. Chen, Y.X. Jiang, Y.C. Zhao and S.L. Zhao, *Microchim Acta*, 2013, **180**, 203-209.
22 L.M. Lu, X.B. Zhang, R.M. Kong, B. Yang and W.H. Tan, *J. Am. Chem. Soc.*, 2011, **133**, 11686-11691.
23 C.B. Ma, Z.W. Tang, K.M. Wang, X.H. Yang and W.H. Tan, *Analyst*, 2013, **138**, 3013-3017.
24 Y.H. Wang, X.X. He, K.M. Wang and X.Q. Ni, *Biosens. Bioelectron.*, 2010, **25**, 2101-2106.
25 W.P. Zhu, Z.W. Zhao, Z. Li, J.H. Jiang, G.L. Shen and R.Q. Yu, *Analyst*, 2012, **137**, 5506-5509.
26 J.T. Ren, J.H. Wang, J. Wang and E. Wang, *Biosens. Bioelectron.*, 2014, **51**, 336-342.
27 S.L. Daubendiek, K. Ryan and E.T. Kool, *J. Am. Chem. Soc.*, 1995, **117**, 7818-7819.
28 Q.W. Xue, L. Wang and W. Jiang, *Chem. Commun.*, 2013, **49**, 2640-2642.
29 E.J. Cho, L.T. Yang, M. Levy and A.D. Ellington, *J. Am. Chem. Soc.*, 2005, **127**, 2022-2023.
30 Y.H. Song, X. Yang, Z.Q. Li, Y.J. Zhao and A.P. Fan, *Biosens. Bioelectron.*, 2014, **51**, 232-237.
31 Z.M. Zhou, Y. Yu and Y.D. Zhao, *Analyst*, 2012, **137**, 4262-4266.

32 C.M. Niemeyer, Angew. Chem. Int. Ed., 2001, **40**, 4128-4158.
33 A. Merkoci, M. Aldavert, S. Marin and S. Alegret, *Trends Anal. Chem*., 2005, **24**, 341-349.
34 S. Bi, Y.M. Yan, X.Y. Yang and S.S. Zhang, *Chem. Eur. J*., 2009, **15**, 4704-4709.
35 L.M. Zanoli, R. D'Agata and G. Spoto, *Anal Bioanal Chem*., 2012, **402**, 1759–1771.
36 D.B. Zhu, X.M. Zhou and D. Xing, *Anal. Chim. Acta*., 2012, **25**, 39-43.
37 D.J. Lin, J. Wu, M. Wang, F. Yan and H.X. Ju, *Anal. Chem*., 2012, **84**, 3662-3668.
38 Y.J. Niu, P. Wang, Y.J. Zhao and A.P. Fan, *Analyst*, 2013, **138**, 1475–1482.
39 A.P. Fan, C.W. Lau and J.Z Lu, *Analyst*, 2009, **134**, 497-503.
40 P. Wang, Y.H. Song, Y.J. Zhao and A.P. Fan, *Talanta*, 2013, **103**, 392-397.
41 G. Frens, *Nat. Phys. Sci*., 1973, **241**, 20–22.
42 C. Guarise, L. Pasquato, V. De Filippis and P. Scrimin, *Proc. Natl. Acad. Sci U. S. A*., 2006, **103**, 3978-3982.
43 Y.J. Niu, Y.J. Zhao and A.P. Fan, *Anal. Chem*., 2011, **83**, 7500-7506.

