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Label-free chemiluminescent ATP aptasensor based on graphene oxide and an instantaneous derivatization of guanine bases

Yinhuan Song, Xu Yang, Ziqi Li, Yanjun Zhao, Aiping Fan *

Tianjin Key Laboratory for Modern Drug Delivery and High Efficiency, School of Pharmaceutical Science and Technology, Tianjin University, Tianjin 300072, People's Republic of China

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ABSTRACT

In this work, a novel label-free chemiluminescent (CL) aptasensor has been developed for rapid and facile detection of adenosine triphosphate (ATP, as model analyte) using graphene oxide (GO) nano-platform. The strategy relies on the preferential binding of GO to single-stranded DNA (ssDNA) over rigid double-stranded DNA (dsDNA) or aptamer-target complexes, and the instantaneous derivative reaction between phenylglyoxal (PGO), a special CL reagent as the signaling molecule, and guanine nucleobases (G) of aptamer strands adsorbed on the surface of GO. In the absence of ATP, the aptamers adsorbed onto the surface of GO leading to a strong background CL signal. Conversely, in the presence of ATP, the aptamers formed the aptamer-ATP complexes which had weak binding ability to GO resulting in a significant CL signal decrease. The CL intensity was adversely related to the ATP concentration in the assay solution. The biosensor's signal decreased linearly with the logarithm of the concentration of ATP from 2 to 80 nmol with a detection limit of 1.4 nmol. The aptasensor also showed high selectivity against cytosine triphosphate (CTP), guanosine triphosphate (GTP), and uridine triphosphate (UTP). The method presented here holds the advantages of being label-free, cost effective, rapid, sensitive and selective, which would shows great promise for clinical application.

1. Introduction

DNA aptamers are synthetic single-strand oligonucleotides obtained through a strategy called "Systematic Evolution of Ligands by Exponential Enrichment" (SELEX); they have the advantage of high selectivity and affinity, excellent chemical stability, simple modifiability, and high flexibility in biosensor design for analyte detection (Tombelli et al., 2005; Willner and Zayats, 2007; Herr et al., 2006). In recent years, aptamers have been extensively applied as recognition elements for biosensor design, and analytical and diagnostic applications. For example, several aptamer-based sensors (aptasensor) for thrombin, adenosine triphosphate (ATP), cocaine, platelet-derived growth factor, and IgE have been developed using fluorescence resonance energy transfer (Wang et al., 2011; He et al., 2013), CL (Zhou et al., 2012; Wang et al., 2013), electrochemistry (Wen et al., 2011), and affinity probe capillary electrophoresis (Cheow and Han, 2011), respectively. However, there are still some limitations such as complicated labeling and purification processes, and time-consuming, which impede the wide application of these methods. Moreover, the modification or immobilization of aptamer to nanoparticles might affect the aptamer-target binding affinity (NHaveri et al., 2000; Ho and Leclerc, 2004). In order to overcome these shortcomings, many researchers focused on developing label-free methods (Jiang et al., 2004). For example, Yan et al. (2009) developed a label-free CL biosensing platform for the determination of adenosine based on a structure-switching aptamer.

Graphene and its derivative GO, a mono-layer carbon arranged in a honeycomb structure, has recently attracted intense interest due to its unique electrochemical properties, high thermal conductivity, excellent mechanical flexibility, large accessible surface area, as well as good biocompatibility (Geim and Novoselov, 2007; Cote et al., 2009). Recently, GO has been used for the construction of biosensors for detecting proteins (Yuan et al., 2013; Sharon et al., 2013), nucleic acids (Guo et al., 2013; Song et al., 2013), metal ions (Yan et al., 2013), and small molecules (He et al., 2011; Zhu et al., 2012), most of which relied on the preferential binding of GO to ssDNA over rigid dsDNA or aptamer-target complexes. For example, Pu et al. (2012) developed a fluorescent method for the detection of ATP based on the strong ssDNA adsorption ability of GO.

CL has been exploited for a wide range of applications in various fields owing to its extremely high sensitivity along with its other advantages, such as simple instrumentation, wide calibration ranges and suitability for miniaturization in analytical chemistry (Laura et al., 2009; Fan et al., 2009). In this work, we

* Corresponding author. Tel./fax: +86 22 87440581.
E-mail address: aipingfan1980@163.com (A. Fan).

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found that GO-aptamer complex showed strong CL intensity instead of CL quenching based on the instantaneous derivative reaction between PGO, a special CL reagent as the signaling molecule, and guanine nucleobases of aptamer strands adsorbed on the surface of GO. Then we designed a label-free CL aptasensor for the simple and rapid detection of small molecules using ATP as a model analyte. It is well known that ATP plays an important role in many biological processes and is often regarded as a universal energy storage molecule in all living organisms (Frundzhyan and Ugarova, 2007; Wang et al., 2005). The intracellular ATP level has been used as an indicator for cell viability and cell injury. The detection of ATP therefore is of great value (Eguchi et al., 1997). To the best of our knowledge, label-free CL aptasensor for proteins or small molecules by taking advantage of the different adsorption affinities of GO towards ssDNA and dsDNA has not been reported yet.

In this paper, we developed a rapid and simple CL aptasensor for ATP based on GO nano-platform and a structure-switching aptamer without any label (Scheme 1). In the absence of target, the DNA aptamer adsorbs on GO via a π–π stacking interaction between the ring structure in the nucleobases and nucleosides, and the hexagonal cells of GO. GO-aptamer complexes were then washed and separated by centrifugation for direct CL analysis. The CL signal was obtained via the derivatization reaction between PGO that reacted specially and instantaneously with guanine (G) nucleobases of the aptamers (Kai et al., 1994). A strong background of CL signal was observed in the absence of ATP. In contrast, in the presence of target, the aptamer forms the ligand-binding structure; the weak binding between aptamer-ATP complexes and GO surface makes less aptamers being adsorbed on the surface of GO, and thus a significantly decreased CL emission was observed. The CL emission intensity will be negatively related to the concentration of the ATP added in the assay solution. This GO-based CL aptasensor is simple and cost-effective because the aptamers used here do not need any label.

2. Experimental

2.1. Materials and chemicals

Graphite powder (99%) was purchased from Heowns Biochem. Technologies Co., Ltd. (Tianjin, China). PGO was purchased from Tokyo chemical industry Co., Ltd. (Tokyo, Japan). Tetrabutylammonium hydroxide, N,N-dimethylformamide (DMF), Tween 20 and other reagents were obtained from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). ATP, CTP, GTP, UTP, ATP binding aptamer (5′-ACC TGG GGG AGT ATT GCG GAG GAA GGT-3′), and a random DNA oligonucleotide (5′-TGT CCG TGC TAG AAG GAA ACA GTT ACC A-3′) were acquired from Shanghai Sangon Biotechnology Co., Ltd. (Shanghai, China). 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.

Ten milli molar phosphate buffer solution (PBS, pH 8) containing 2.5 mM $MgCl_2$ was used as a binding buffer, and PBS containing 0.05% (w/v) Tween 20 was used as a washing buffer. ATP stock solution (0.02 M) was prepared by dissolving 0.012 g ATP in 1 mL of binding buffer.

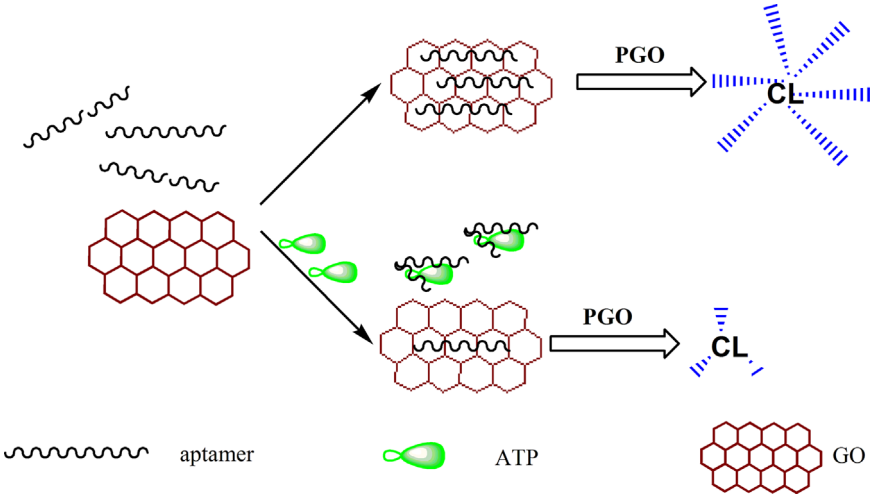
2.2. Apparatus

CL measurements were performed with a BPCL chemiluminescence analyzer (Beijing, China).

2.3. Preparation of GO

Graphene oxide was synthesized from graphite powder by a modified Hummers method (Hummers and Offeman, 1958). In the pretreatment step, 25 mL of concentrated H_2SO_4 was heated to 90 °C in a 100 mL round bottomed flask containing 5 g $K_2S_2O_8$; 5 g P_2O_5 was added with stirring until all of the reactants were completely dissolved. The mixture was then cooled to 80 °C. 6 g of graphite powder was added into the mixture and then kept at 80 °C for 4.5 h. The mixture was finally diluted with 1 L of deionized water and purified using a 0.2 μm Nylon Millipore filter. The solid was transferred to a drying dish and then vaccum-dried.

In the oxidation step, 23 mL H_2SO_4 was placed into a 100 mL beaker and chilled to 0 °C using an ice bath. 0.5 g of pretreated graphite was then added to the acid and stirred. Three grams $KMnO_4$ was added slowly to the mixture that was then allowed to react for 2 h at 0 °C and another 2 h at 35 °C. The mixture was diluted with 46 mL deionized water and stirred for 2 h. After 140 mL of deionized water added in the solution, 10 mL of 30% H_2O_2 was added to the mixture resulting in a brilliant yellow color along with bubbling. The mixture was allowed to settle for at least a day, and then the clear supernatant was decanted. The soft sediment was centrifuged and washed with a total of 1 L of 10% HCl solution followed by 1 L of deionized water to remove the acid. The resulting solid was dried via lyophilization and diluted to make a 2% (w/w) dispersion that was put through dialysis for 2 weeks to remove any remaining metal.



Scheme 1. Schematic illustration of the label-free CL aptasensor for ATP based on GO and an instantaneous derivatization of guanine bases.

2.4. Procedure of CL detection of GO-aptamer complexes

Different amounts of ATP-binding aptamer (diluted with binding buffer) were mixed with 24 μg of GO solution in centrifuge tubes, respectively. The mixtures were incubated at room temperature for 4 min. The aptamer-GO complexes were then isolated by centrifugation (10,000 rpm for 1 min), re-suspended in a 100 μL of washing buffer, and re-centrifuged (10,000 rpm for 1 min). The precipitates were re-suspended in 10 μL of tetrabutylammonium hydroxide-phosphate buffer (pH 8.5) and transferred into 14×40 mm^2 glass tubes which were then placed in the luminescence reader. Finally, 100 μL of 30 mM PGO in DMF was injected and the CL signal was displayed and then integrated 10 s for each signal.

2.5. Procedure of the CL aptasensor for sensing ATP

Different amounts of ATP (0–120 nmol) were mixed with 120 pmol of ATP-binding aptamer (diluted with binding buffer solution) in centrifuge tubes respectively. The mixtures were equilibrated under gentle shaking at 37 °C for 30 min. Then, 24 μg of GO was added to the mixtures and placed for another 4 min at room temperature. The aptamer-GO complexes were then isolated by centrifugation (10,000 rpm for 1 min), re-suspended with 100 μL of washing buffer, and re-centrifuged (10,000 rpm for 1 min). The GO-aptamer complexes were transferred into 14×40 mm^2 glass tubes and detected as described above.

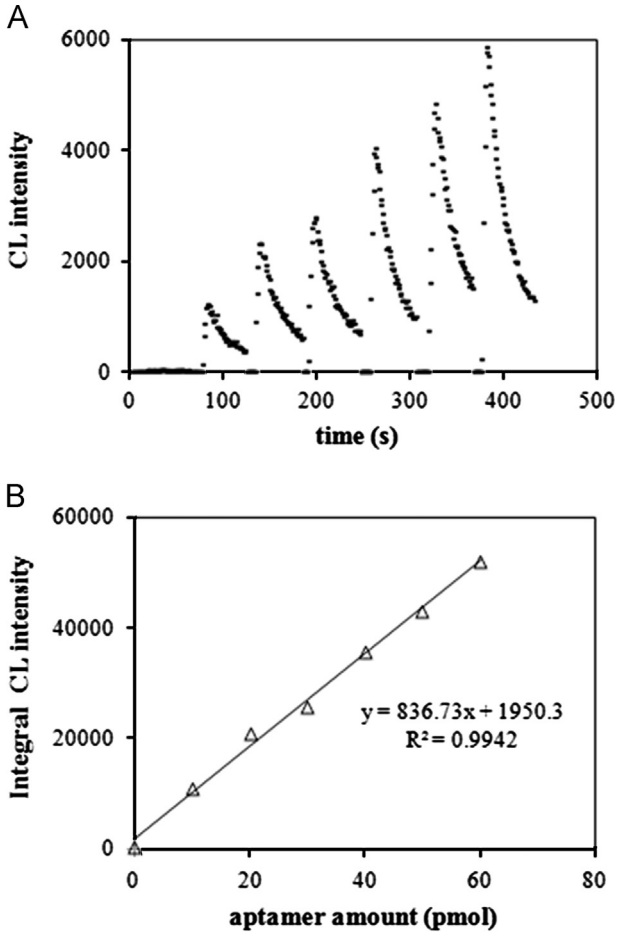


Fig. 1. CL kinetic curve (A) and integral CL intensity (B) of GO absorbed with different amount of aptamer. Experimental conditions: pH 8; $MgCl_2$, 2.5 mM; GO, 24 μg; T_{GA}, 4 min; aptamer amount in (A) from left to right: 0, 10, 20, 30, 40, 50, and 60 pmol, respectively.

3. Results and discussion

3.1. Characterization of GO

GO was characterized using transmission electron microscopy (TEM, Hitachi H-800 microscope, Japan), UV–vis spectrophotometer (Cary 60 UV–vis from Agilent, United States), and X-ray diffraction (XRD, D/MAX-2500 from Rigaku Corporation). After sonication for 4 h in water, GO remained as a single layer or occasionally multi-layers with few drapes according to TEM analysis (Fig. S1A). The UV–vis spectrum of aqueous GO dispersion was presented in Fig. S1B. A strong absorption band at 230 nm and a shoulder at 310 nm were observed in the spectrum of GO, which corresponded to the π–π^* and n–π^* plasmon peak, respectively. The XRD pattern of GO revealed a sharp 002 reflection at $2\theta = 11.0^\circ$ (Fig. S1C). The typical peak for graphite is at $2\theta = 26.4^\circ$ according to previous work (Lu et al., 2011). The interlay space of GO was larger than that of the graphite, which was attributed to the introduction of oxygenated functional group on the carbon sheets.

3.2. CL responses of GO-aptamer complex

We have firstly investigated the CL responses of GO-aptamer complexes. Different amounts of ATP aptamers were mixed with 24 μg of GO solution in centrifuge tube respectively, and the mixtures were incubated at room temperature for 4 min. After centrifugation, CL responses of GO-aptamer complexes were measured by the BPCL chemiluminescence analyzer. As shown in Fig. 1A, after the addition of PGO in DMF, a maximum CL signal was observed within 10 s and then diminished. The integral CL intensity increased dramatically with increasing aptamer amount, and a good linear relationship between the integral CL intensity and the aptamer amount was produced with a correlation coefficient of 0.994 (Fig. 1B).

3.3. Feasibility for label-free CL detection of ATP

Based on the above phenomenon, we designed the label-free CL aptasensor for ATP. In the absence of ATP, the aptamers adsorb onto the surface of GO leading to a strong background CL signal. Conversely, in the presence of ATP, the aptamers form the aptamer-ATP complexes which have weak binding ability to GO resulting in a significant CL signal decrease. We then conducted a proof-of-concept experiment for the label-free CL detection of ATP. A random DNA oligonucleotide was used as negative control. Fig. 2

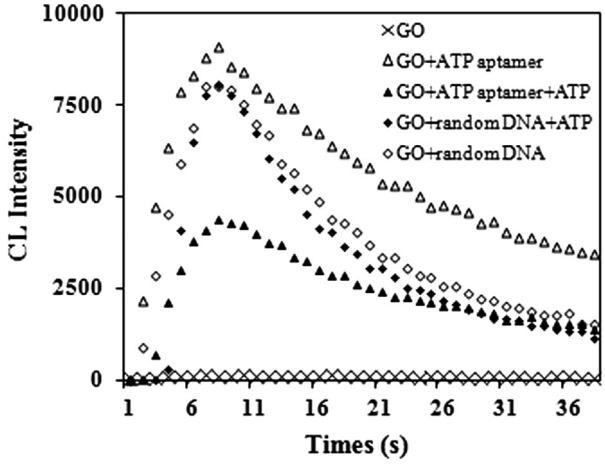


Fig. 2. CL kinetic curve under different conditions: (△) 120 pmol aptamer+24 μg GO; (◇) 120 pmol random DNA+24 μg GO; (▲) 120 pmol aptamer+24 μg GO +40 nmol ATP; (◆) 120 pmol random DNA+24 μg GO+40 nmol ATP and (×) 24 μg GO.

displayed the CL kinetic curve under different conditions. The aptamer-GO conjugate had a strong background CL intensity that was decreased by about 52% in the presence of 40 nmol ATP suggesting weaker adsorption affinity of GO for aptamer-ATP complexes. In terms of random DNA group, the CL intensities maintain almost the same both in the absence and presence of ATP.

3.4. Optimization of assay conditions

The assay performance of the aptasensor was greatly dependent on the experimental conditions, such as aptamer amount, GO concentration, pH, and incubation time. Hence, a series of systematic optimization experiments were carried out to obtain the optimal assay conditions. Fig. 3 illustrated typical CL intensities in correlation to these experimental conditions. Firstly, the effect of the aptamer amount on the assay performance was investigated. As shown in Fig. 3A, the integral CL intensity increased rapidly with increased aptamer amount in the absence of ATP, which revealed the increased levels of DNA aptamer adsorbed onto the GO surface. However, in the presence of ATP, the extent of the integral CL intensity increase was much lower than that in the presence of ATP. This indicated a weaker binding of GO to aptamer-ATP complexes against single-stranded DNA aptamers, which was consistent with previous investigations. The ΔCL ($\Delta CL = CL_0 - CL$, where CL_0 and CL are the integral CL intensity in the absence and presence of ATP, respectively) signal increased by raising the amount of ATP aptamer at the beginning, and then almost levelled off between 120 and 150 pmol. Hence, subsequent work employed 120 pmol of aptamer. The effect of GO amount was shown in Fig. 3B. Irrespective of presence of ATP, the integral CL intensities increased when the amount of GO ranged from 8 to 24 μg and reached a maximum at 24 μg. The integral CL intensities decreased gradually for the GO amount above 24 μg. Thus 24 μg of GO was selected for the further study. pH is another important factor because it can affect the formation of ATP-aptamer complexes and the ssDNA adsorption onto GO. According to previous references, the electrostatic repulsion between DNA and GO can be increased by raising pH. We investigated five buffers at pH 6, 7, 8, 9, and 10 (Fig. 3C). The strongest ΔCL signal was observed at pH 8. Therefore, pH 8 was selected for the following experiments. The effect of incubation time of DNA aptamer and GO (named as T_{GA}) were also investigated. As shown in Fig. 3D, the integral CL intensity increased rapidly at beginning and then got steady after 4 min with or without ATP. Thus 4 min was employed as the optimal incubation time of aptamer and GO for the subsequent experiments.

3.5. Sensitivity for ATP detection

To evaluate the sensitivity of the designed label-free CL aptasensor, the assay was practiced at different amount of ATP (as model analyte). As shown in Fig. 4, in the absence of ATP, the aptasensor exhibited a strong background CL intensity. The CL intensity of the aptasensor significantly decreased with the increasing amount of ATP from 2 to 20 nmol, and then exhibited gradual decrease with the increasing amount of ATP from 20 to 80 nmol. The CL signals decreased linearly with the logarithm of the amount of ATP. And we speculated that the semi-logarithmic relationship between CL signal and the amount of ATP was derived from the binding equilibrium between aptamer and target ATP (Jing and Bowser, 2011). The linear relationship between CL

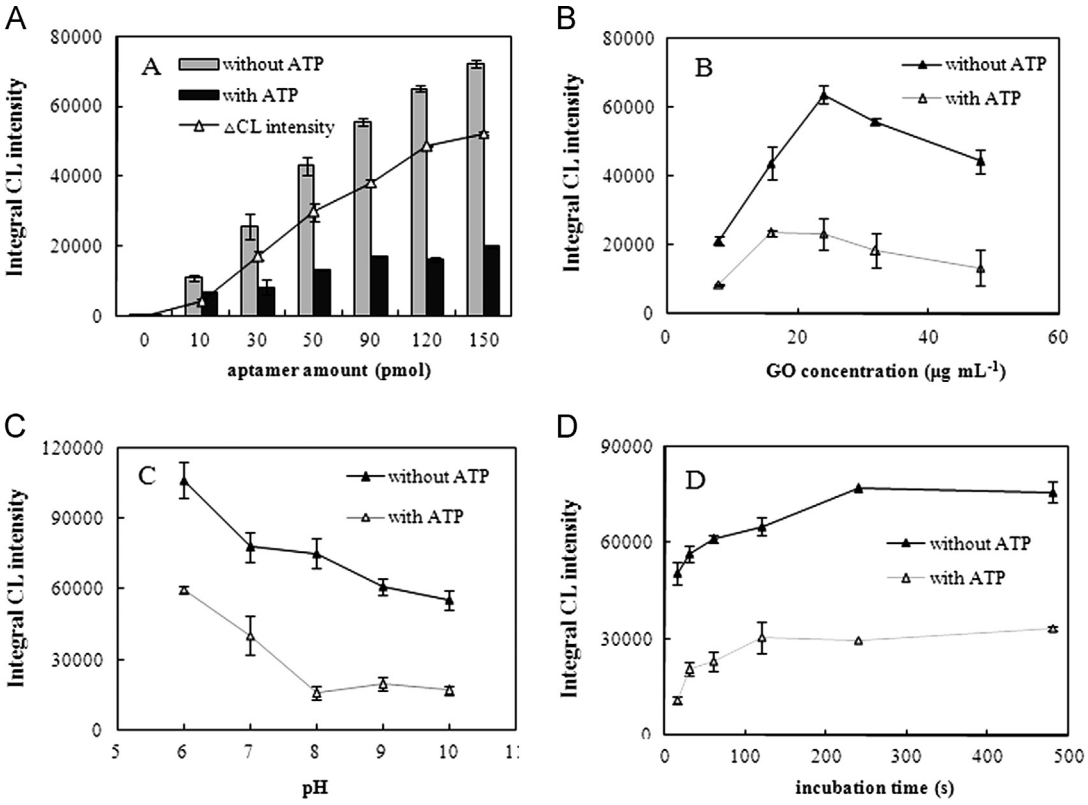


Fig. 3. Optimization of experimental conditions. (A) the effect of aptamer amount on ΔCL intensity (triangle) and CL intensity (histogram) in the presence and absence of ATP. Experimental conditions: pH 8; $MgCl_2$, 2.5 mM; GO, 24 μg; T_{GA}, 30 s; ATP, 40 nmol. (B) The effect of GO amount on CL intensity in the presence and absence of ATP. Experimental conditions: aptamer, 120 pmol; other experimental conditions were the same as (A). (C) The effect of pH of binding buffer on CL intensity in the presence and absence of ATP. Experimental conditions were the same as (B). (D) The effect of T_{GA} on CL intensity in the presence and absence of ATP. Experimental conditions were the same as (B). The error bars show the standard deviations for three replicate determinations.

intensity and the logarithm of ATP concentration in the range of 2–80 nmol was shown in the inset. The regression equation is $I=-28063\log c+82879$, in which I is CL intensity and c is the amount of ATP (nmol) with a correlation coefficient of 0.9923. The detection limit (defined as 3σ/slope) was determined to be 1.4 nmol (where σ is the standard deviation of a blank solution, $n=5$) under optimal conditions.

3.6. Specificity and reproducibility of the aptasensor

We further investigated the selectivity of the proposed strategy by examing the CL responses of the aptasensor to ATP over ATP analogs, such as GTP, CTP and UTP under the optimized conditions. As shown in Fig. 5, the ΔCL intensity in the presence of ATP was much stronger than that in the presence of GTP, CTP, and UTP. The results revealed that the proposed label-free CL aptasensor had high selectivity to ATP, which was due to the specific recognition between ATP aptamer and ATP. The reproducibility of the aptasensor was evaluated using the following method: a series of nine aptasensor were incubated with 20 nmol of ATP, the nine aptasensors exhibited similar CL intensities and the relative standard deviation (RSD) was 8.4%.

3.7. Application to sample analysis

Finally, the ability of the label-free CL aptasensor to detect the ATP in matrices 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 (Table S1). It was found that the recoveries for ATP were 99–105% with a RSD of 5–9%, which were acceptable for quantitative assays performed in biological samples.

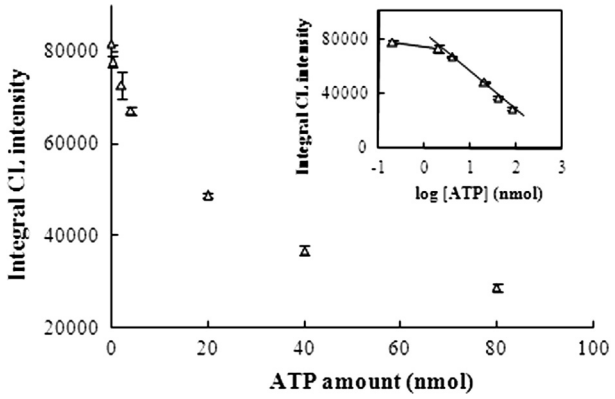


Fig. 4. CL responses of label-free aptamersensor for ATP detection at different amounts. Inset: The calibration plot of $\log c_{ATP}$ vs. integral CL intensity in the range from 2 nmol to 80 nmol. The error bars show the standard deviations for three replicate determinations.

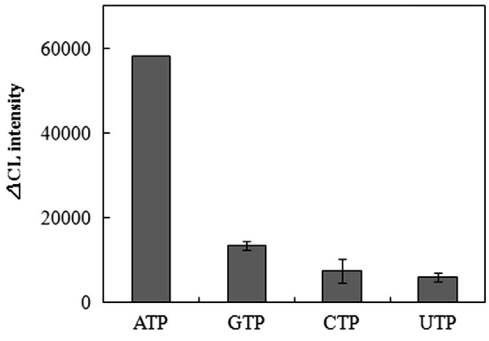


Fig. 5. Selectivity of the label-free CL aptasensor to ATP compared to GTP, CTP, and UTP. The amounts of ATP, GTP, CTP, and UTP were 40 nmol. The error bars show the standard deviations for three replicate determinations.

4. Conclusion

In summary, we have developed a novel label-free CL aptasensor for ATP detection by using an instantaneous derivatization of guanine bases and GO as a nano-platform. The strategy relies on the excellent specificity of aptamer towards ATP, as well as the different adsorption affinity between GO and DNA structures. In the absence of target ATP, the aptamer showed strong adsorption ability to GO and the aptamer-GO complex exhibits strong CL background signal. Conversely, the presence of target ATP facilitated the formation of aptamer-ATP complex leading to a significant decrease of CL intensity. The proposed method offered three prominent advantages: firstly, aptamers used in this method do not need any label, which would avoid the time-consuming labeling process and multistep experimental procedure as reported in other fluorescence/luminescence assays. Secondly, Compared with other methods for ATP detection (Wang et al., 2008; Wang and Liu, 2008; Cai et al., 2011), the proposed assay was fast and convenient to detect. The assay process can be completed within 40 min. Finally, given that a similar system can be constructed with other molecules detection, including cocaine, thrombin, IgE, and so on. We expected that the novel label-free strategy would find universal use in the areas of small molecule detection and protein analysis.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2013.07.039.

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