

Cite this: *Analyst*, 2012, **137**, 5506

www.rsc.org/analyst

COMMUNICATION

A ligation-triggered highly sensitive fluorescent assay of adenosine triphosphate based on graphene oxide†

Wenping Zhu, Ziwei Zhao, Zhen Li, Jianhui Jiang,* Guoli Shen and Ruqin Yu*

Received 30th August 2012, Accepted 8th October 2012

DOI: 10.1039/c2an36223f

A simple, amplification-free and sensitive fluorescent biosensor for ATP detection was developed based on the ATP-dependent enzymatic reaction (ATP-DER) and the different adsorption affinity between graphene oxide (GO) and DNA structures. The proposed method was simple and convenient and also showed high sensitivity and selectivity to ATP.

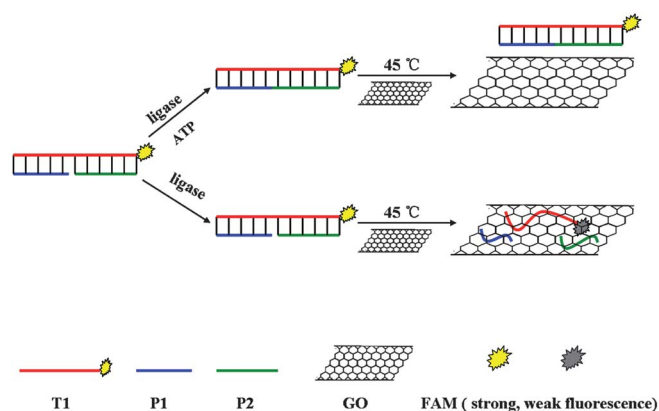
Adenosine 5'-triphosphate (ATP) is a multifunctional nucleoside triphosphate often used as a universal energy storage molecule in all living organisms.¹ It plays a critical role in the regulation of cellular metabolism and biochemical pathways in cell physiology.² ATP has also been widely used as an indicator of living organisms for cell viability and cell injury.³ Besides, ATP depletion is related to pathogenesis such as ischemia, Parkinson's disease, and hypoglycemia.^{4–6} Therefore, highly sensitive and selective detection of ATP in biochemical study and clinical diagnosis is of particular importance. The conventional method of ATP assay in homogeneous solution is luciferase (Luc)-mediated bioluminescence.^{7a–c} However, the method which involves costly bioluminescence reagents is tedious, time-consuming and of uncertain stability. Several methods have been reported for assaying ATP based on organic molecules, synthetic host–guest receptors, conjugated polymers and peptides.^{8–11} All these methods, however, either involve synthesis of complex compounds and multi-step chemical reactions, or suffer from insufficient selectivity limiting their applications. Because the anti-ATP DNA aptamer is a low-affinity species with a relatively low association constant with ATP, most of these anti-ATP DNA aptamer based methods rely on amplification techniques to improve the sensitivity. Furthermore, most of them cannot distinguish ATP from its analogues, such as adenosine, AMP and ADP. Nowadays, several novel strategies for ATP detection have been reported based on the ATP-dependent enzymatic reaction (ATP-DER).^{12,13} The ATP-DER based method is shown to be more selective than the aforementioned methods, which is attributed to the unique role of ATP in the enzymatic reaction. Although previous ATP assay strategies based on the ATP-DER are relatively sensitive and selective and have undergone

continued optimization, those methods usually employ multiple labels and necessitate amplification processes to improve the sensitivity, which could add cost and complexity for the assay. Therefore, developing new analytical methods for simple, convenient, amplification-free detection of ATP is of considerable interest.

Recently, graphene oxide (GO) has been used as a DNA-based optical platform for the detection of nucleic acids,^{14,15} proteins,^{16,17} metal ions,¹⁸ and small molecules^{19,20} as well as for drug delivery.²¹ Most GO-based DNA optical sensors rely on the preferential binding of GO to single-stranded DNA (ssDNA) over double-stranded ones (dsDNA), thereby quenching the fluorescence of dyes conjugated to ssDNA. Based on the unique property of GO, several robust biosensors for assay of ATP have been developed.^{22,23} However, most of them are involved with anti-ATP DNA aptamers. To the best of our knowledge, there has been no previous report of any optical ATP assay based on both the ATP-DER and GO platform.

Herein, we for the first time developed a simple, amplification-free and highly sensitive fluorescent biosensor for assay of ATP based on both the ATP-DER and GO platform. Schematic illustration of the fluorescent assay for ATP on the GO platform is shown in Scheme 1.

The sensor system consists of two sensing elements: nicked DNA duplex and GO. The DNA sequences used in ligase reaction were carefully designed by our team, because the ligase reaction is DNA sequence-independent which only requires the DNA substrates are complementary. Therefore, the DNA sequences used in ligase reaction were carefully designed by our team (Table S1, ESI†). Firstly,



Scheme 1 Schematic illustration of the principal mechanism involved in the fluorescent assay of ATP on the GO platform.

State Key Laboratory for Chem/Biosensing and Chemometrics, College of Chemistry and Chemical Engineering, Hunan University, Changsha, 410082, P. R. China. E-mail: jianhuijiang@hnu.edu.cn; rquyu@hnu.edu.cn; Fax: +86-731-8822782; Tel: +86-731-8822577

† Electronic supplementary information (ESI) available: Experimental section. See DOI: 10.1039/c2an36223f

two half DNA probes P1 and P2 (Table S1, ESI†) were hybridized with dye-labeled template T1 (Table S1, ESI†) to form a DNA duplex substrate with a single-stranded nick. The nicked DNA duplex formed in this way could initiate ligation reaction using T4 DNA ligase in the presence of the cofactor ATP. The resulting unnicked DNA duplex was resistant to thermal denaturation, yielding a stable DNA duplex which had a weak affinity for GO.

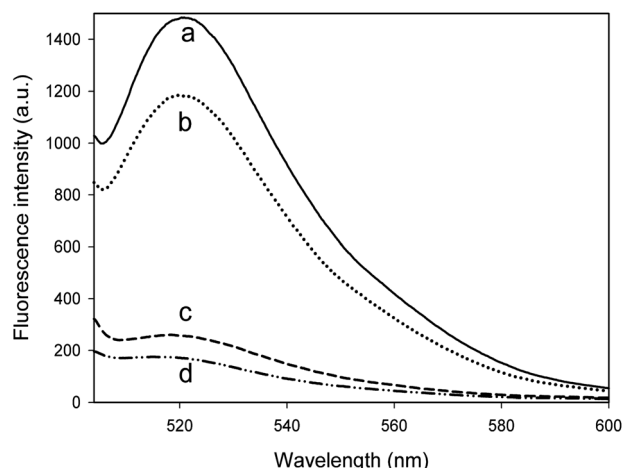


Fig. 1 Fluorescence emission spectra under different conditions: (a) 0.12 μM T1 + 0.12 μM P3; (b) 0.12 μM T1 + 0.12 μM P1 + 0.12 μM P2 + 200 nM ATP + ligase; (c) 0.12 μM T1 + 0.12 μM P1 + 0.12 μM P2 + ligase; and (d) 0.12 μM T1 + 0.12 μM P1 + 0.12 μM P2 + 200 nM ATP.

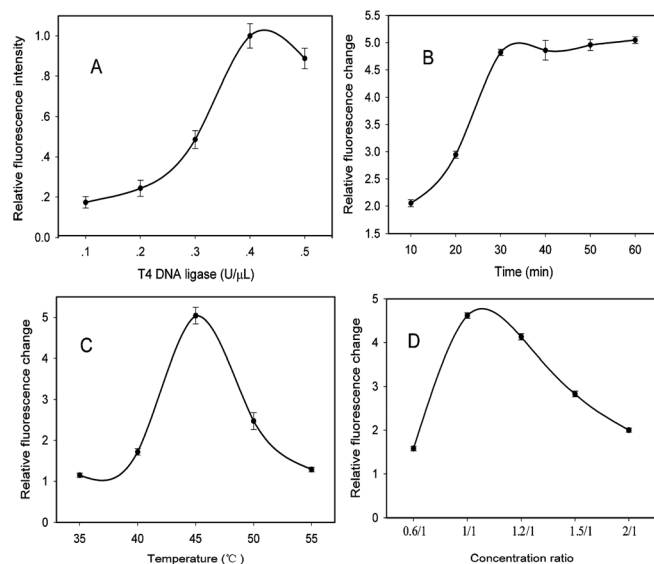


Fig. 2 (A) Dependency of relative fluorescence intensity of the ATP detection on the ligase concentration. (B) Dependency of relative fluorescence change of the ATP detection on the ligation time. (C) Dependency of relative fluorescence change of the ATP detection on denaturation temperature. (D) Dependency of relative fluorescence intensity of the ATP detection on the concentration ratio values. The concentration of ATP used here is 200 nM. The relative fluorescence intensity in (A) is defined as the ratio of fluorescence signal from other concentrations of ligase to that from 0.4 $\text{U } \mu\text{L}^{-1}$ of T4 DNA ligase. The relative fluorescence change is defined as the ratio of the fluorescence signal from 200 nM ATP to that from the absence of ATP.

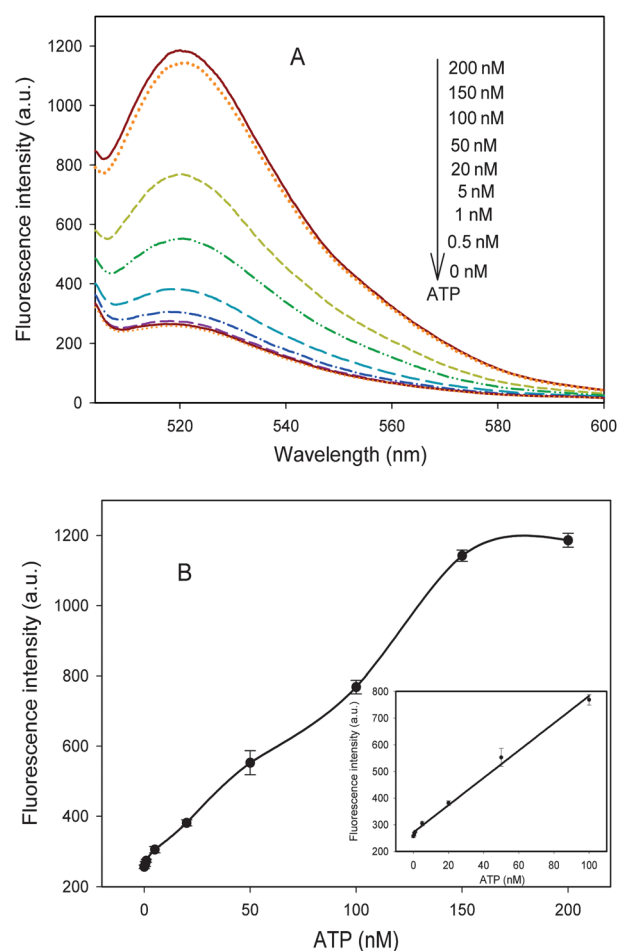


Fig. 3 (A) Fluorescence spectra response of the fluorescence assay for ATP of varying concentrations. Up to bottom: 200, 150, 100, 50, 20, 5, 1, 0.5, and 0 nM. Experiments were conducted based on the optimal experimental conditions. (B) Fluorescence spectra response at 520 nm versus ATP concentration (inset: dependence of fluorescence intensity on ATP concentration). The error bars represented for standard deviation (SD) across three repetitive experiments.

Obvious fluorescence quenching did not occur to the unnicked DNA duplex after addition of GO and a strong fluorescence signal was obtained. However, the ligation reaction would not occur in the absence of ATP and the nicked DNA duplex was subject to thermal denaturation, separating two half DNA probes and dye-labeled template. Upon addition of GO, the three separated single-stranded DNA would immediately be adsorbed onto the GO surface, resulting in efficient quenching of the fluorescence intensity. The fluorescence intensity was directly proportional to the concentration of ATP. By monitoring the fluorescence changes of the sensing system, highly sensitive ATP detection could be realized.

To verify the feasibility of the proposed strategy, an experiment for proof of principle was carried out. Another DNA oligonucleotide named as P3 (the sequence of P3 which contains P1 and P2 synthesized as one sequence is fully complementary to T1, Table S1, ESI†) was used as the control. Fig. 1 displays the fluorescence emission spectra under different conditions. As shown in Fig. 1, in the absence of ATP or T4 DNA ligase, the ligation reaction could not be initiated, resulting in the separation of two half DNA probes and dye-labeled template

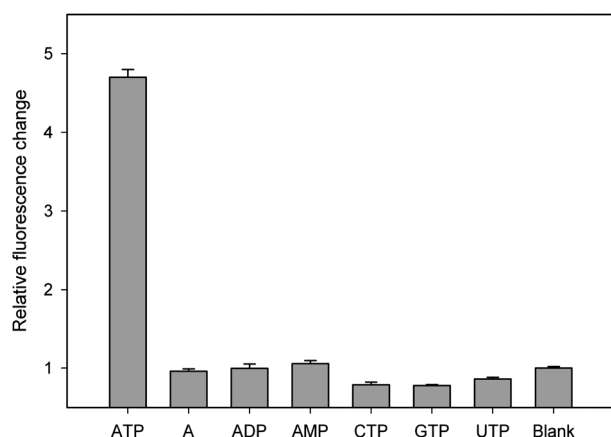


Fig. 4 Selectivity of the proposed biosensor for ATP compared to other nucleosides and ATP analogues. ATP and the six interfering species are all at the same concentration of 200 nM. The definition of the relative fluorescence change is defined as the ratio of the fluorescence signal from 200 nM ATP or other nucleosides and ATP analogues to that from the absence of ATP. The “Blank” stands for the fluorescence background signal from the absence of ATP.

after thermal denaturation. Upon addition of GO, the fluorescence of the dye-labeled template was greatly quenched, so we observed two rather weak fluorescence signals (curves c or d). Whereas in the presence of T4 DNA ligase and ATP (200 nM), the formed stable unnicked DNA duplex after ligation reaction was resistant to thermal denaturation, and as a result the fluorescence would not be efficiently quenched by GO. Therefore, a strong fluorescence signal (curve b) was observed. When P3 was used instead of P1 and P2 to hybridize with T1 to form a stable DNA duplex, which had the largest capacity of resistance to thermal denaturation, an obvious fluorescence signal with the highest fluorescence intensity was obtained (curve a). These experimental results clearly demonstrate the feasibility of the proposed strategy for sensitive ATP detection.

Fluorescence responses of the ATP detection were greatly dependent on the experimental conditions, such as the concentration of T4 DNA ligase, denaturation temperature, ligation time and the concentration ratio of P1 and P2 to the template T1 (fixed at 0.12 μM). In order to achieve the best assay performance, a series of systematic optimization experiments aiming at these conditions were carried out to obtain the optimal experimental conditions. Fig. 2 depicts typical fluorescence responses in correlation to these experimental conditions.

Experimental results showed that the following conditions could provide a maximum S/N ratio for the sensing system: 0.4 $\text{U } \mu\text{L}^{-1}$ of T4 DNA ligase, ligation time of 30 min, denaturation temperature of 45 $^{\circ}\text{C}$ and the concentration ratio of 1 : 1 for P1 and P2 to the template T1 (fixed at 0.12 μM). The optimal experimental conditions were chosen to be the above conditions and used throughout subsequent experiments. In addition, since the fluorescence signal is highly sensitive to the denaturation temperature, carefully controlling the temperature is of great importance to the experiment.

We chose the fluorescence emission from carboxyfluorescein (FAM) at 520 nm to evaluate the performance of the fluorescent biosensor. The ATP concentration was quantified based on the optimal experimental conditions. Fig. 3A depicts typical fluorescence spectral responses of the fluorescent biosensor to ATP of varying

concentrations. One could observe dynamically increased fluorescence peaks with increasing ATP concentration ranging from 0.5 to 200 nM. There was a good linear correlation between the peak intensity and the ATP concentration in the range from 0.5 to 100 nM, as shown in Fig. 3B (inset). The linear calibration equation was $F = 5.065C + 274.9$ (F is the fluorescence intensity and C is the concentration of ATP) and the correlation coefficient $R^2 = 0.995$. The detection limit of ATP was 0.3 nM according to the 3σ rule, which was two orders of magnitude lower than the previously reported amplified aptamer-based assay²³ and comparable to the ligation-triggered DNAzyme cascade for amplified fluorescence detection.¹² These results demonstrate that the proposed strategy can be used conveniently for highly sensitive ATP detection.

We further investigated the selectivity of the proposed strategy by examining the fluorescence responses of the biosensor to ATP over other nucleosides and ATP analogues. As shown in Fig. 4 the fluorescent biosensor exhibited very weak fluorescence responses to other interfering species such as A, ADP, AMP, CTP, GTP and UTP, which contrasted clearly with the strong fluorescence response to ATP. The results obviously indicate that the proposed strategy has high selectivity to ATP and can distinguish ATP from other nucleosides and ATP analogues.

In conclusion, we have developed a simple, amplification-free and highly sensitive fluorescent biosensor for ATP detection. The strategy relies on the ATP-dependent enzymatic reaction and the different adsorption affinity between GO and DNA structures. This proposed strategy offered three prominent advantages: firstly, the ligation-triggered method is fast and convenient to detect. Secondly, the method is amplification-free but much more sensitive than most of the other anti-ATP DNA aptamer-based analytical methods and comparable to those amplification-assisted ATP-DER based methods. Finally, it exhibits superior selectivity to ATP over other nucleosides and ATP analogues. Given the simplicity, sensitivity, and selectivity of this approach, the proposed strategy may become a new method of choice for simple and convenient ATP detection.

This work was supported by NSFC (21025521, 21035001, 21190041), National Key Basic Research Program (2011CB911000), CSIRT Program and NSF of Hunan Province (10JJ7002).

Notes and references

- 1 V. Frundzhyan and N. Ugarova, *Luminescence*, 2007, **22**, 241.
- 2 J. Wang, Y. X. Jiang, C. S. Zhou and X. H. Fang, *Anal. Chem.*, 2005, **77**, 3542–3546.
- 3 Y. Eguchi, S. Shimizu and Y. Tsujimoto, *Cancer Res.*, 1997, **57**, 1835–1840.
- 4 K. T. Bush, S. H. Keller and S. K. Nigam, *J. Clin. Invest.*, 2000, **106**, 621.
- 5 S. Przedborski and M. Vila, *Clin. Neurosci. Res.*, 2001, **1**, 407.
- 6 R. A. Harkness and O. D. Saugstad, *Scand. J. Clin. Lab. Invest.*, 1997, **57**, 655.
- 7 (a) T. Wilson and J. W. Hastings, *Annu. Rev. Cell Dev. Biol.*, 1998, **14**, 197; (b) H. J. Kennedy, A. E. Pouli, E. K. Ainscow, L. S. Jouaville, R. Rizzuto and G. A. Rutter, *J. Biol. Chem.*, 1999, **274**, 13281; (c) C. J. Bell, G. Manfredi, E. J. Griffiths and G. A. Rutter, *Methods Cell Biol.*, 2007, **80**, 341.
- 8 A. Ojida, Y. Mito-oka, K. Sada and I. Hamachi, *J. Am. Chem. Soc.*, 2004, **126**, 2454–2463.
- 9 S. Mizukami, T. Nagano, Y. Urano, A. Odani and K. Kikuchi, *J. Am. Chem. Soc.*, 2002, **124**, 3920–3925.
- 10 C. Li, M. Numata, M. Takeuchi and S. Shinkai, *Angew. Chem., Int. Ed.*, 2005, **44**, 6371–6374.
- 11 S. C. McCleskey, M. J. Griffin, S. E. Schneider and J. T. McDevitt, *J. Am. Chem. Soc.*, 2003, **125**, 1114–1115.

- 12 L. M. Lu, X. B. Zhang, R. M. Kong, B. Yang and W. H. Tan, *J. Am. Chem. Soc.*, 2011, **133**, 11686–11691.
- 13 Y. H. Wang, X. X. He, K. M. Wang and X. Q. Ni, *Biosens. Bioelectron.*, 2010, **25**, 2101–2106.
- 14 C. H. Lu, H. H. Yang, C. L. Zhu, X. Chen and G. N. Chen, *Angew. Chem., Int. Ed.*, 2009, **48**, 4785–4787.
- 15 S. J. He, B. Song, D. Li, C. F. Zhu, W. P. Qi, Y. Q. Wen, L. H. Wang, S. P. Song, H. P. Fang and C. H. Fan, *Adv. Funct. Mater.*, 2010, **20**, 453–459.
- 16 H. X. Chang, L. H. Tang, Y. Wang, J. H. Jiang and J. H. Li, *Anal. Chem.*, 2010, **82**, 2341–2346.
- 17 H. Jang, Y. K. Kim, H. M. Kwon, W. S. Yeo, D. E. Kim and D. H. Min, *Angew. Chem., Int. Ed.*, 2010, **49**, 5703–5707.
- 18 X. H. Zhao, R. M. Kong, X. B. Zhang, H. M. Meng, W. N. Liu, W. H. Tan, G. L. Shen and R. Q. Yu, *Anal. Chem.*, 2011, **83**, 5062–5066.
- 19 Y. Wang, Z. H. Li, D. H. Hu, C. T. Lin, J. H. Li and Y. H. Lin, *J. Am. Chem. Soc.*, 2010, **132**, 9274–9276.
- 20 C. H. Lu, J. Li, M. H. Lin, Y. W. Wang, H. H. Yang, X. Chen and G. N. Chen, *Angew. Chem., Int. Ed.*, 2010, **49**, 8454–8457.
- 21 C. H. Lu, C. L. Zhu, J. Li, J. J. Liu, X. Chen and H. H. Yang, *Chem. Commun.*, 2010, **46**, 3116–3118.
- 22 Y. He, Z. G. Wang, H. W. Tang and D. W. Pang, *Biosens. Bioelectron.*, 2011, **29**, 76–81.
- 23 C. H. Lu, J. Li, M. H. Lin, Y. W. Wang, H. H. Yang, X. Chen and G. N. Chen, *Angew. Chem., Int. Ed.*, 2010, **122**, 8632–8635.