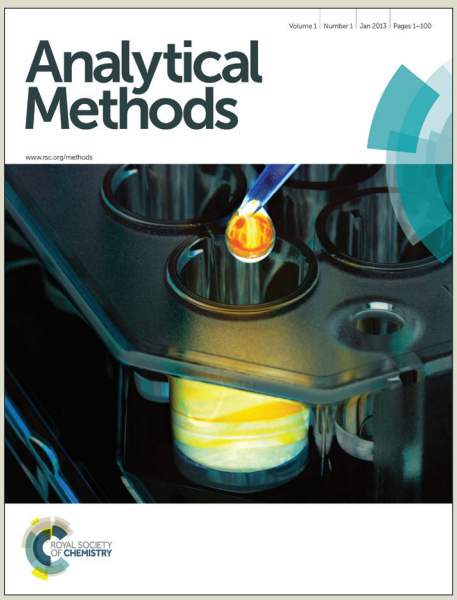
Analytical Methods

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/methods

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

Signal on fluorescence biosensor for Adenosine Triphosphate based on click chemistry

Ning Sun, [a] Qi Guo, [b] Jingwei Shao,[c*] Bin Qiu , [a,c*] Zhenyu Lin, [c] Wong, K.Y.[a] and Guonan Chen[c]

Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX

DOI: 10.1039/b000000x

Novel signal-on fluorescence biosensor for adenosine triphosphate (ATP) based on the target induced recombination of split aptamer fragments and click chemistry had been proposed. The CuSNPs modified on the Streptavidin Magnespheres Paramagnetic Particles (PMPs) can be destroyed by acid to form Cu(II). In the present of sodium ascorbate, Cu(II) can be reduced to Cu(I) and which in turn catalyzes the reaction between a weak-fluorescent 3-azido-7-hydroxycoumarin and propargyl alcohol to form a strong fluorescent 1,2,3-triazole compounds. The proposed sensor had been applied to detect ATP in the human serum samples with satisfied result.

1 Introduction

Adenosine triphosphate (ATP) is a universal energy carrier in biological systems which plays a crucial role in the regulation of cellular metabolism and maintaining life in biological tissues.[1] Abnormal level of ATP has been associated with several pathologies, such as cardiovascular disease, ischemia, and hypoglycemia. Therefore, highly sensitive and selective determination of ATP is not only of research interests but of clinical importance. Various approaches, such as bioluminescence,[2] chromatography,[3] electrochemical[4] and colorimetric[5] approaches have been developed for ATP detection. The luciferin–luciferase method for measuring ATP content is well known for its high sensitivity, but its accuracy is sometime questioned by quenching in matrix. Chromatography needs tedious sample separation and the accuracy is poor. While electrochemical methods can be affected by many other compounds that naturally exist in biological tissues such as ascorbate and glucose, so the specificity is not good enough. Though colorimetric method need not any equipments, but the sensitivity is not high enough. So it is necessary to develop a sensitive and selective method for ATP determination.

Aptamers are nucleic acid macromolecules of single-stranded DNA/RNA oligonucleotides which can bind to certain targets with high affinity and specificity. Due to its easy label, thermostability, easy synthesis and specificity, aptamer-based biosensors have attracted particular attentions. Many aptamer based biosensor for ATP had been developed. For examples, Fan et al. developed a target-responsive electrochemical aptamer switch for reagentless detection of nanomolar ATP.[6] Tang et al. developed a turn on fluorescence biosensor for ATP via the target induced structure-switching of molecular beacon with the detection limits of 25 nM,[7] the detection limit of this method is not low enough for serum samples. Zhu et al. developed an electrochemiluminescence (ECL) biosensor for ATP using quantum dot as ECL reagents with high sensitivity .[8] The aptamer can also be split into two fragments, and the two segments can recombine to form an intact aptamer in the present of target. Many biosensors had been developed based on this mechanism. For example, Kashefi-Kheyrabadi using silver nanoparticles as redox tag to construct an electrochemical aptasensor for the detection of ATP based on the recombination of the split aptamer.[9] Xu et al. reported a label-free and signal-on ECL aptasensor for ATP based on target-induced linkage of split aptamer fragments by using $[Ru(phen)_3]^{2+}$ intercalated into double-strand DNA as a probe.[10] We also established a signal-on ECL biosensor for ATP based on the recombination of aptamer fragments using $Ru(bpy)_3^{2+}$-doped silica nanoparticle as ECL reagent.[11] These electrochemical approaches own the character of high sensitivity, but the reproducibility is a problem since each measurement need freshly prepared modified electrode, this need well trained worker to achieve the target. It is necessary to develop some methods which can be operated easily and with high sensitivity for ATP determination.

Cu(I) catalyzed azide-alkyne cycloaddition (CuAAC) is one of the most famous click reactions, in the presence of Cu(I), azide can react rapidly with terminal alkyne to form 1,2,3-triazole species. [12] This reaction owns the characters of excellent selectivity, high efficiency and mild reaction conditions. Many different sensors, such as colorimetric,[13, 14] electrochemical[15-18] and fluorescent sensors[19, 20] had been developed based on this reaction, but the targets are limited, such as Cu(II), [14-16,21] ascorbic acid,[17,18] protein,[22,23] organophosphate pesticides.[24] It is necessary to find out some way to expand the detection targets.

Early study shows that nonfluorescent 3-azidocoumarins can react with terminal alkynes to form intense fluorescent 1,2,3-triazole products through CuAAC.[25] In this study, a simple, sensitive and selective signal-on (signal enhanced after the addition of target) fluorescence sensor for ATP detection based on ATP-induced recombination of split aptamer fragments and

CuAAC reaction had been developed. The sensor combined the advantages of high selectivity of aptamer and CuAAC reaction and high sensitivity of fluorescent biosensor. The proposed biosensor has been applied to detect ATP in serum samples with satisfying results.

2 Experimental Section

2.1Chemicals

Sodium ascorbate, propargyl alcohol, imidazole and other reagents were obtained from Alfa Aesar China Co. Ltd. (Tianjin). The synthesis of 3-azido-7-hydroxycoumarin has been described elsewhere.[26] Streptavidin Magnespheres Paramagnetic Particles (PMPs) was purchased from Promega Corporation (Madison,USA). ATP, together with its analogues, cytidine triphosphate (CTP), guanosine triphosphate (GTP), uridine triphosphate (UTP) were purchased from Sigma (St. Louis, MO) and used without further purification. All other chemicals were of analytical reagent grade. Double-distilled water was used throughout the whole process. Phosphate buffer solution (PBS, pH 7.4) was used as the DNA buffer solution.

The two aptamer fragments with some modifications for ATP were synthesized by Sangon Inc. (Shanghai, China). And their sequences were shown as follows.

5'-biotion modified ssDNA1:

Biotin-**AAAAA ACCTGGGGGAGTAT-3'**

3'-amino group modified ssDNA2 for CuS particles conjugation (NH_2-DNA):

5'-TGCGGAGGAAGGT-NH_2-3'

2.2 Modification of ssDNA2 with CuS nanoparticles

20 nm diameter CuS nanoparticles with carboxyl groups were prepared according to the published method firstly (Inset of Fig.1 shows its TEM image) [27]. Then 200 μL of 0.1 M imidazole solution (pH 6.8) was added into NH_2-DNA (1.24 μM). After gentle shaking for 30 min, 100 μL of 0.1 M EDC solution (cross-linking agent) and 2.0 mL of CuS colloid were added to the mixture and reacted at room temperature for 24 h. Under these conditions, condensation reaction between amino groups and carboxy groups has been proceeded, therefore NH_2-DNA tagged with CuS NPs was formed, and then it was separated with other reagents by centrifugation at a rotate speed of 10000 rpm for 30 min. The precipitate was washed with water for 3 times and then resuspended in water. The solution of CuS NPs modified DNA (CuS NPs-ssDNA2) was stored at -5 °C for later using.

2.3 Procedures for ATP detection

A portion of 0.6 mL of 1 mg/mL PMPs was washed by buffer solution once and then dispersed in 0.6 mL of buffer solution. ssDNA1 was added into the solution to achieve a final concentration of 0.5 μM, and the mixture was mixed on a shaker for 30 min at room temperature. The specific combination of streptavidin with biotion[28] allowed the combination of PMPs with ssDNA1. After that, PMPs with ssDNA1 were separated from the mixture by a magnet for the reason that PMPs possessed paramagnetic were able to attract by a magnet. And then, they were further washed by buffer solution once and then dispersed in 0.6 mL of buffer solution, consequently, PMPs with ssDNA1 (PMPs-ssDNA1) was formed.

PMPs-ssDNA1 solution (2.0 μM), ssDNA2-CuS particles solution (2.0μM) and various concentrations of ATP were mixed and kept for 2 h. Then, CuS NPs modified on PMPs can be separated easily from that in the solution by the magnet and then washed by the buffer solution for three times and then redispersed in 0.2 mL of buffer solution. Then HNO_3 (50 μM) was added to the mixtures to make CuS dissolved to produce Cu(II). 2 min later, propargyl alcohol (25 μM), 3-azido-7-hydroxycoumarin (25 μM) and sodium ascorbate (3 mM) were added into the above mixed solution. Cu(II) can react with sodium ascorbate to produce Cu(I). The reaction mixture was held for 3.0 h to make the CuAAC reaction between the azide and alkyne groups occurred efficiently in the presence of the Cu (I) catalyst at room temperature. Fluorescence spectra of the mixtures were recorded on a Varian Cary Eclipse at the excitation wavelength of 365 nm.

3 Results and discussion

3.1 The principle of the fluorescent biosensor

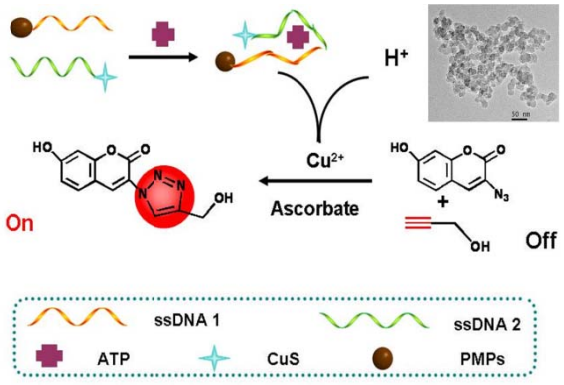


Fig.1 The principle of the proposed fluorescence biosensor. The inset is the TEM image of CuS nanoparticles.

The principle of the fluorescent sensor was shown in Fig.1. A single aptamer for ATP is split into two fragments (ssDNA1 and ssDNA2), early study proved that the cutting site doesn't hinder target combination by circular permutation[11]. ssDNA1 and ssDNA2 had been modified with PMPs and CuS nanoparticles, respectively. In the absence of ATP, there has inferior interaction between the two fragments, and little CuS particles can be modified on PMPs. In the presence of ATP, owing to the formation of stable complex between the PMPs-ssDNA1 and CuS NPs modified ssDNA2, CuS NPs can be immobilized onto the PMPs and be separated by magnet easily. CuS can be destroyed by acid to produce Cu(II), at present of sodium ascorbate, Cu(II) will be reduced to Cu(I) and which in turn initiates the CuAAC reaction between weak-fluorescent 3-azido-7-hydroxycoumarin and propargyl alcohol to form strong fluorescent 1,2,3-triazole compound. The enhanced fluorescent signal has a relationship with the ATP concentration, based on which, a sensitive signal on fluorescent biosensor for ATP can be developed.

A simple experiment has been performed to verify our protocol. 3-azido-7-hydroxycoumarin give off weak fluorescence signal (curve a in Fig.2(A)). If ATP had been

added into the mixed solution of PMPs-ssDNA1 and CuS NPs-ssDNA2, almostly no signal enhancement had been detected(curve b in Fig. 2(A)). But if ATP had been added into the mixed solution, enhanced fluorescence intensity had been detected (curve c in Fig.2(A)).This indicates that the fluorescent enhancement has some relationship with ATP, which maybe applied to develop a sensor for ATP.

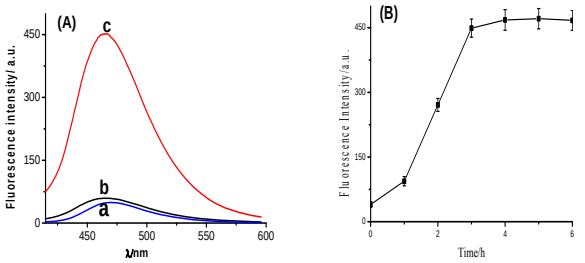


Fig. 2 (A) The fluorescence o 3-azido-7-hydroxycoumarin (a) and the system in the absence (b) and presence (c) of ATP. Excitation wavelength: 365nm, C_{azido}=25μM , C_{alkyne} =25 μM, C_{SA}=3 mM. (B) The relationship between the fluorescence intensities of the system and the click reaction times.

3.2 Optimization of the click reaction times

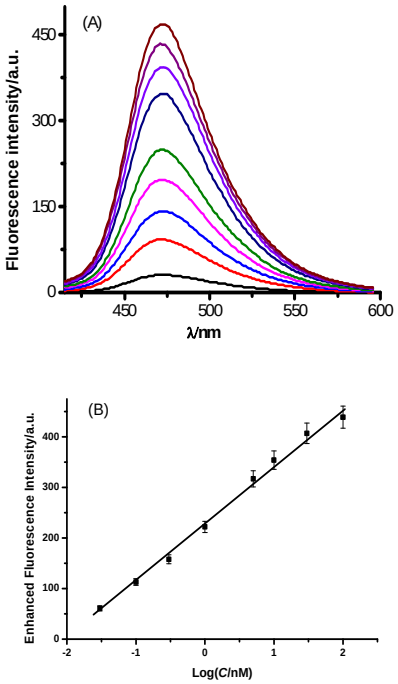


Fig. 3 (A) The fluorescence spectra at different concentrations of ATP. a to k: 0 nM, 0.03 nM, 0.1 nM, 0.3 nM, 1 nM, 5 nM, 10 nM, 30 nM, 100 nM. (B) The calibration curve between the fluorescence intensity and logarithm of ATP concentrations.

In the presence of excess amount of sodium ascorbate, the fluorescence intensities of system had been affected by the reaction time between 3-azido-7-hydroxycoumarin and propargyl alcohol. As shown in Fig.2(B), the fluorescence intensities of the system increased sharply with the addition the sodium ascorbate, then increased slowly with the prolong of the reaction and then reached a saturation plateau after 3 h. This means the system had been reacted thorough and no further fluorescent 1,2,3-triazole produced after 3 h. Thus, 3 hours had been chosen as the best reaction time in the subsequent studies.

3.3 Calibration curve for ATP

Fig. 3(A) shows the fluorescence intensities at different ATP concentrations. If the solution contains no ATP, very low fluorescence signals had been detected. The fluorescence intensities increase with the concentrations of ATP, and the enhanced fluorescence intensity has a good linear relationship with the logarithm of ATP concentrations in the range of 0.03-100 nM(Fig.3(B)). The regression equation can be expressed as

$$\Delta I=226.9+115.6\log(C/nM) \quad R= 0.9928$$

Where ΔI is the enhanced fluorescence intensity after the addition of ATP, C is ATP concentration in the cell. The regression coefficient R is 0.9928. The detection limit (LOD) is calculated to be 0.02 nM (S/N=3). The linear range is biologically significant since the normally ATP concentration in serum samples is lower than 10^{-8} M. The detection limit is four orders lower than the early reported fluorescence method [7] and one order lower than the ECL method.[11] The reason of the lower LOD maybe lie in that CuS nanoparticles are released and functioned as a catalyst for the CuAAC reaction, which amplifies the variation of fluorescence responses.

Seven different freshly prepared biosensors had been applied to detect 0.5 nM ATP, the relative standard deviation (RSD) of the detected fluorescence signal is 3.91%, this indicates that the proposed method has good reproducibility in the fabrication of the biosensor. PMPs-ssDNA1 and CuS NPs-ssDNA2 had been prepared and kept in -5 °C, then each week some solution had been taken out to test the same sample(0.5 nM), the RSD is 4.22%(n=7), which is nearly the same with the freshly prepared one. This indicated the prepared solution can be well preserved without changing its characters. So compared with electrochemical approachs[8-11], the proposed method can be operated easily by mixing the prepared reagents with the target, seprating by the magnet and then detected.

3.4 Specificity and practicality of the biosensor

Three ATP analogues of cytidine triphosphate (CTP), guanosine triphosphate (GTP) and uridine triphosphate (UTP) (all concentrations are 100 nM) are employed to examine the selectivity of the sensor to ATP (0.1 nM). As illustrated in Fig.4, the fluorescence intensities from the analogues are nearly the same with that of the background, but a strong fluorescence increasing had been detected form ATP. The comparative results indicated that the proposed biosensing system has an excellent ability to identify ATP, which can be ascribed to the high selectivity of the aptamer.

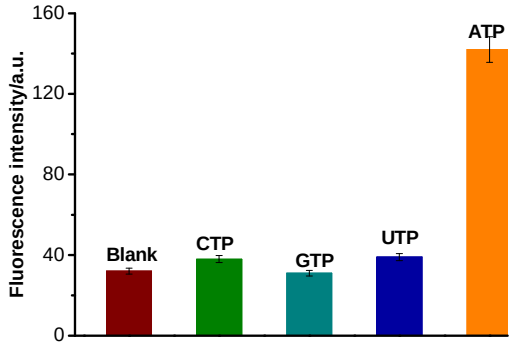


Fig. 4 Selectivity of the sensor towards ATP. ATP: 0.1 nM; The concentration of the interference is 100 nM. C_{azido}=25μM , C_{alkyne} =25 μM, C_{SA}=3 mM.

The proposed biosensor had been applied to detect the ATP in human serum samples (provided by the Affiliated Hospital of Fuzhou University). As shown in Table 1, the average of ATP concentration in the serum samples is 18 nM. The recoveries by the standard addition of ATP into the real samples had been detected to evaluate the veracity of the proposed method, the recoveries are between 95.2% and 105.5%, the RSDs are between 3.89% and 4.32%. These results indicate that the proposed biosensor is successfully applied to detect ATP in the complex samples.

Table 1 Detection of ATP concentration in the human serum samples (n=7)

Sample No.	Detected (mol/L)	Added (mol/L)	Found (mol/L)	Recovery (%)	RSD (%)
1	1.83×10^{-10}	1.00×10^{-10}	0.98×10^{-10}	98.0	4.32
2	1.76×10^{-10}	2.00×10^{-10}	2.11×10^{-10}	105.5	4.13
3	1.82×10^{-10}	5.00×10^{-10}	4.76×10^{-10}	95.2	3.89

4 Conclusion

In summary, a novel signal on fluorescence biosensor for ATP has been proposed by taking advantages of CuAAC reaction and ATP-induced recombination of split aptamer fragments. The Cu(II) comes from target-dependent binding of DNA- CuS NPs, and can be reduced to Cu(I) by ascorbate, which in turn induces the CuAAC reaction between weak-fluorescent 3-azido-7-hydroxycoumarin and propargyl alcohol to form a fluorescent 1,2,3-triazole compounds. It is found that the quantification of ATP is relevant to the fluorescent intensity of the system. The sensor can be applied to detect the ATP concentration in blood or serum samples easiy with satisfied results. In addition, the proposed biosensor shows high sensitivity and good selectivity, this fluorescence method may be helpful to expand the utility of click chemistry in fluorescence detection for bioassays.

Acknowledgement

This project was financially supported by the National Basic Research Program of China (No.2010CB732403), Key Project of Fujian Provincial Science and Technology program (2011Y0048，2011N5008).

Notes and references

[a]*Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Hunghom, Kowloon, Hong Kong, China.*
[b]*Shenzhen Zhijun Pharmaceutical Co., LTD., Shenzhen, Guangdong, China.*
[c]*Department of Chemistry, Fuzhou University, Fuzhou, Fujian, 350002, China*

1 S. Zadran, D. Sanchez, H. Zadran, A. Amighi, E. Otiniano and K. Wong, *Biotechnol. Lett.*, 2013, **35**, 175-180.
2 G. Carrea, R. Bovara, G. Mazzola, S. Girotti, A. Roda and S. Ghini, *Anal. Chem.*, 1986, **58**, 331-333.
3 M. R. Stratford and M. F. Dennis, *J Chromatogr. B.*, 1994, **662**, 15-20.
4 A. Kueng, C. Kranz and B. Mizaikoff, *Biosens. Bioelectron.*, 2004, **19**, 1301-1307.
5 D. Deng, N. Xia, S. Li, C. Xu, T. Sun, H. Pang and L. Liu, *Sensors (Basel)*, 2012, **12**, 15078-15087.
6 X. Zuo, S. Song, J. Zhang, D. Pan, L. Wang and C. Fan, *J. Am. Chem. Soc.*, 2007, **129**, 1042-1043.
7 F. Li, Z. Du, L. Yang and B. Tang, *Biosens. Bioelectron.*, 2013, **41**, 907-910.
8 H. Huang, Y. Tan, J. Shi, G. Liang and J. J. Zhu, *Nanoscale*, 2010, **2**, 606-612.
9 L. Kashefi-Kheyrabadi and M. A. Mehrgardi, *Biosens. Bioelectron.*, 2012, **37**, 94-98.
10 Z. Liu, W. Zhang, L. Hu, H. Li, S. Zhu and G. Xu, *Chemistry*, 2010, **16**, 13356-13359.
11 Z. Lin, F. Luo, Q. Liu, L. Chen, B. Qiu, Z. Cai and G. Chen, *Chem. Commun .(Camb)*, 2011, **47**, 8064-8066.
12 H. C. Kolb, M. G. Finn and K. B. Sharpless, *Angew. Chem. Int. Ed.* ., 2001, **40**, 2004-2021.
13 W. Qu, Y. Liu, D. Liu, Z. Wang and X. Jiang, *Angew. Chem. Int. Ed.*, 2011, **50**, 3442-3445.
14 Y. Zhou, S. Wang, K. Zhang and X. Jiang, *Angew. Chem. Int. Ed.*, 2008, **47**, 7454-7456.
15 S. Qiu, L. Xie, S. Gao, Q. Liu, Z. Lin, B. Qiu and G. Chen, *Anal. Chim. Acta*, 2011, **707**, 57-61.
16 S. Qiu, S. Gao, X. Zhu, Z. Lin, B. Qiu and G. Chen, *Analyst*, 2011, **136**, 1580-1585.
17 S. Qiu, S. Gao, L. Xie, H. Chen, Q. Liu, Z. Lin, B. Qiu and G. Chen, *Analyst*, 2011, **136**, 3962-3966.
18 S. Qiu, S. Gao, Q. Liu, Z. Lin, B. Qiu and G. Chen, *Biosens. Bioelectron.*, 2011, **26**, 4326-4330.
19 S. Qiu, M. Miao, T. Wang, Z. Lin, L. Guo, B. Qiu and G. Chen, *Biosens. Bioelectron.*, 2013, **42**, 332-336.
20 S. Qiu, X. Li, W. Xiong, L. Xie, L. Guo, Z. Lin, B. Qiu and G. Chen, *Biosens. Bioelectron.*, 2013, **41**, 403-408.
21 Q. Shen, S. Tang, W. Li, Z. Nie, Z. Liu, Y. Huang and S. Yao, *Chem. Commun. (Camb)*, 2012, **48**, 281-283.
22 K. Zhu, Y. Zhang, S. He, W. Chen, J. Shen, Z. Wang and X. Jiang, *Anal. Chem.*, 2012, **84**, 4267-4270.
23 M. A. Kamaruddin, P. Ung, M. I. Hossain, B. Jarasrassamee, W. O'Malley, P. Thompson, D. Scanlon, H. C. Cheng and B. Graham, *Bioorg. Med. Chem. Lett.*, 2011, **21**, 329-331.
24 G. Fu, W. Chen, X. Yue and X. Jiang, *Talanta*, 2013, **103**, 110-115.
25 K. Sivakumar, F. Xie, B. M. Cash, S. Long, H. N. Barnhill and Q. Wang, *Org. Lett.*, 2004, **6**, 4603-4606.
26 J. P. Collman, N. K. Devaraj and C. E. Chidsey, *Langmuir*, 2004, **20**, 1051-1053.
27 S. Zhang, H. Zhong and C. Ding, *Anal. Chem.*, 2008, **80**, 7206-7212.
28 P. C. Weber, D. H. Ohlendorf, J. J. Wendoloski and F. R. Salemme, *Science*, 1989, **243**, 85-88