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PII: S0039-9140(15)00073-9
DOI: http://dx.doi.org/10.1016/j.talanta.2015.02.002
Reference: TAL15373

To appear in: *Talanta*

Received date: 9 December 2014
Revised date: 24 January 2015
Accepted date: 1 February 2015

Cite this article as: Qingai Chen, Qingquan Guo, Ying Chen, Jie Pang, Fengfu Fu, Liangqia Guo, An enzyme-free and label-free fluorescent biosensor for small molecules by G-quadruplex based hybridization chain reaction, *Talanta,* http://dx.doi.org/10.1016/j.talanta.2015.02.002

An Enzyme-free and label-free fluorescent biosensor for small molecules by G-quadruplex based hybridization chain reaction

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Abstract:

An enzyme-free and label-free fluorescent biosensor is developed by G-quadruplex-based hybridization chain reaction (HCR) for small molecules, using adenosine triphosphate (ATP) as the model. Aptamer probes for the recognition of small molecules are hybridized with blocking probes. The G-quadruplex sequences are incorporated into one of the two HCR hairpin probes. In the presence of small molecules (ATP), the formation of aptamer-ATP bioaffinity complexes induces the release of blocking probes, the released blocking probes initiate HCR and numerous G-quadruplexes along DNA nanowires are self-assembled after the HCR process. Using N-methyl mesoporphyrin IX (NMM) as the fluorophore, a "turn-on" fluorescence response can be achieved and detected as low as 15 μmol L^{-1} of ATP. This biosensor is applied to detect ATP in biologic samples with satisfactory results.

Keywords: Hybridization chain reaction; ATP; G-quadruplex; N-methyl mesoporphyrin IX

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1. Introduction

Signal amplification technologies based on DNA have been playing an important role in the development of biosensors. Polymerase chain reaction [1], ligase chain reaction [2], rolling circle amplification [3] have been extensively employed to improve the sensitivity and lower the detection limit. However, these amplified methods require protein enzymes, which are cost consuming and are sensitive to reaction conditions, such as temperature and pH. Recently, great attention has been focused on enzyme-free signal amplification strategies depending on the hybridization [4, 5] and strand-displacement reactions [6]. For example, isothermal hybridization chain reaction (HCR), which was first introduced by Dirk and Pierce [7], provides a fascinating strategy in DNA sensing applications. In HCR, two stable species of DNA hairpins coexist in solution until the introduction of initiator strands triggers a cascade of hybridization events that yield nicked double helices analogous to alternating copolymers. HCR not only provides an enzyme-free signal amplification method but also involves a unique assembly process, which works under mild conditions with no specific demand for ionic strength, pH or temperature. Nowadays, many studies combined the amplification capability of HCR with various sensing platforms and showed promising results for the detection of biomolecules such as DNA [8-22], microRNA [23,24], proteins [25-31], cells [32, 33]. However, to this day there are few reports for the detection of small molecules [34-35].

Herein, we developed a fluorescent biosensor for small molecule based on hybridization chain reaction as an enzyme-free signal amplification strategy and G-quadruplex as a label-free signaling platform. Adenosine triphosphate (ATP), a major energy resource involved in various biochemical pathways and metabolic processes and an important indicator in clinical diagnosis and biochemical study, served as a model target molecule. Although HCR has been applied for the detection of ATP [36-38], the involvement of labeling of hairpin probes brings about complexity and high cost which may limit its practical application. In this study we utilized N-methyl mesoporphyrin IX (NMM), a commercially available porphyrin derivative

which can selectively bind to the G-quadruplex structure instead of double-stranded DNA (dsDNA) and single-stranded DNA (ssDNA) with the result of fluorescence enhancement [39], as the fluorophore to construct a label-free G-quadruplex-based detection platform. In the presence of ATP, the formation of aptamer-ATP bioaffinity complexes triggers the release of blocking probes, which was originally partially complementary with the aptamer probes. At the same time, the released blocking probes trigger the HCR process to form DNA nanowires incorporated with numerous G-quadruplexes, resulting in the fluorescence enhancement. Therefore, by combining the amplification capability of HCR and the G-quadruplex-based signaling platform, the proposed method provided an enzyme-free and label-free fluorescent detection of small molecules.

2. Experimental

2.1. Chemical and materials

Tris(hydroxymethyl)aminomethane (Tris, BR), magnesium chloride ($MgCl_2$, AR) and sodium chloride (NaCl, AR) were bought from Sinopharm Group Co. Ltd. (Shanghai, China). Potassium chloride (KCl, AR) was obtained from Shanghai Experiment Reagent Co. Ltd. (Shanghai, China) N-methyl mesoporphyrin IX (NMM) was purchased from J&K Scientific Ltd. (Beijing, China) Adenosine triphosphate (ATP, reagent grade), cytidine triphosphate (CTP, molecular biology grade), guanosine triphosphate (GTP, molecular biology grade), uridine triphosphate (UTP, molecular biology grade) and HPLC-purified DNA (Table 1) were obtained from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China) All other chemicals were of analytical grade. The water used was purified by Millipore Milli-Q (18 MΩ·cm).

The DNA solutions were prepared in 20 mmol L^{-1} Tris-HCl buffer (pH 8.2, 300 mmol L^{-1} NaCl, 5 mmol L^{-1} $MgCl_2$) and quantified by measuring the absorbance at 260 nm. The stock solution of NMM (1.56 mmol L^{-1}) was prepared in dimethyl sulfoxide (DMSO), stored in darkness at -20 °C. Before use, NMM solution was diluted with

water and the concentration was measured on a Lambda 750 spectrophotometer (PE, USA) at 379 nm assuming an extinction coefficient of 1.45×10^5 mol^{-1} L cm^{-1} [39].

2.2. Detection of ATP by fluorescence measurements

Hairpin probes H1 (5.0 μmol L^{-1}) and H2 (5.0 μmol L^{-1}) were respectively heated to 95 °C for 2 min and cooled down slowly to room temperature for at least 1 h to form the hairpin structure before use. Aptamer probe 4 was annealed with blocking probe in a similar manner to form double-stranded DNA.

30 μL of 1 μmol L^{-1} annealed product of aptamer probe 4 and blocking probe, and 10 μL of ATP with different concentrations were added to 113 μL Tris-HCl buffer (20 mmol L^{-1}, pH 8.2, 300 mmol L^{-1} NaCl, 5 mmol L^{-1} $MgCl_2$), the mixture was incubated at 37 °C for 30 min. Then, 10 μL H1 (5 μmol L^{-1}), 10 μL H2 (5 μmol L^{-1}) and 12 μL KCl (1 mol L^{-1}) were added to the above mixture. After incubation at 37 °C for 60 min, 15 μL NMM (13.5 μmol L^{-1}) was added and the resulting mixture was incubate for another 5 min. Fluorescence measurements (excited at 399 nm) were carried out on a Cary Eclipse fluorospectrophotometer (Varian, USA).

2.3. Gel electrophoresis

Hairpin probes H1 (10 μmol L^{-1}) and H2 (10 μmol L^{-1}) were respectively heated to 95 °C for 2 min and cooled down slowly to room temperature for at least 1 h to form the hairpin structure before use. Aptamer probe 4 (10 μmol L^{-1}) was annealed with the blocking probes (10 μmol L^{-1}) in a similar manner to form double-stranded DNA.

10 μL different concentrations of ATP and 10 μL 5 μmol L^{-1} annealed product of aptamer probe 4 and blocking probe were incubated in 17 μL Tris-HCl buffer (20 mmol L^{-1}, pH 8.2, 300 mmol L^{-1} NaCl, 5 mmol L^{-1} $MgCl_2$) at 37 °C for 1 h. Then, 5 μL H1 (10 μmol L^{-1}), 5 μL H2 (10 μmol L^{-1}) and 3μL KCl (1 mol L^{-1}) were added to the above mixture and incubated at 37 °C for 5 h. The SYBR Green II was used as DNA stain and mixed with the DNA samples. A 7.5% polyacrylamide gel was prepared with 1×TBE buffer (pH 8.0). 8.0 μL of each DNA sample was loaded into the lane and performed at

a constant potential of 50 V for 150 min with 1×TBE (pH 8.0) as the running buffer. Gel electrophoresis was performed on a DYY-6C electrophoresis system (Beijing Liuyi instrument factory, China).

3. Results and discussion

3.1. Biosensor Design.

The principle of the enzyme-free and label-free fluorescent biosensor is shown in Figure 1. This biosensor concludes a fluorophore (NMM), an aptamer probe, a blocking probe and two fuel hairpin probes (H1, H2) with an additional 6 nucleotide (nt) sticky end in both. Complementary sequences between H1 and H2 are marked by the same color. A G-quadruplex sequence (brick red color, Figure 1) is caged in the hairpin probe H1. Two-thirds of the G-quadruplex sequence is locked in the loop and one-third is concealed in the stem which is connected to the end of the blocking probe recognition region with a mismatch base pair in between. 3'-terminal of the aptamer probes is designed to be hybridized with 5'-terminal of the blocking probes. The annealed product of aptamer probes and blocking probes, H1, and H2 are stable in solution, and NMM is weakly fluorescent in the absence of ATP. The introduction of ATP results in the formation of aptamer-ATP bioaffinity complexes and triggers the release of blocking probes. At the same time, the released blocking probes are hybridized with the sticky end of H1 and simultaneously trigger H1 to be opened. The G-quadruplex sequence in the opened H1 can form a G-quadruplex structure stabilized by K^+ ions. Then, the opened H1 is hybridized with the sticky end of H2 and triggers H2 to be opened at the same time. The opened H2 can be used as a new initiator to hybridize with a new H1 and trigger the new H1 to be opened. Thus, H1 and H2 are cross-opened successively and self-assembled into long DNA nanowires incorporated with numerous G-quadruplexes [21, 22, 30, 39], resulting in fluorescence enhancement after binding with NMM.

To characterize the occurrence of hybridization chain reaction, gel electrophoresis was performed in the presence and absence of ATP. As shown in Figure S1, there were

only low molecular weight bands in lane 2 (only H1) and lane 3 (H1/H2), indicating no HCR occurred. Once the annealed product of aptamer probe 4 and blocking probe, and ATP were introduced (lane 4-7), smears of high molecular weight bands were observed, which indicated that ATP triggered the release of blocking probe and initiated the HCR process between H1 and H2 to form long dsDNA nanowires. Moreover, the molecular weight of the resulting dsDNA nanowires was inversely related to ATP concentration due to the fact that the more ATP was present the more blocking probe was released, which was similar to the result reported by Dirk and Pierce [7].

3.2.Optimization of sensing conditions

To achieve the best sensing performance, the sequences of aptamer probes, concentrations of aptamer probes, K^+ and NMM, and reaction time were optimized. 3'-terminal of blocking probes, which can initiate HCR, is designed to be hybridized with part of aptamer probes at the 3'-terminal in the absence of ATP. Aptamer probes consist of an ATP aptamer domain [40] for the recognition of ATP molecules and an extension domain at the 3'-terminal. The number of nucleotide in the extension domain can be adjusted to regulate the number of base pairs complementary to blocking probes. The stability of the annealed product of aptamer probe and blocking probe would be enhanced from aptamer probe 1 to 7 (see Table 1) due to the increase of the number of base pairs complementary to the blocking probes. The sequence of aptamer probes should be designed to inhibit the release of blocking probes in the absence of ATP and trigger the release of blocking probes in the presence of ATP. As shown in Figure 2, fluorescent signal ratio (I_F/I_0) was plotted as a function of aptamer probes, where I_F and I_0 were the fluorescent intensity in the presence and absence of ATP, respectively. As the number of nucleotide in the extension domain of aptamer probes (aptamer probe 1, 2, 3, 4) increased, the stability of the annealed product of aptamer probes and blocking probes increased, which would decrease the background signal. However, excessive stability (in the case of aptamer probe 5, 6, 7) would impede the release of blocking probes at the presence of ATP. Aptamer probe 4 with 15 base pairs complementary to

blocking probes at the 3'-terminal, offered the best performance. Thus, aptamer probe 4 was chosen for further investigation.

K^+ ions were reported as coordination cations to stabilize G-quadruplexes. The influence of K^+ ions concentration on fluorescence enhancement was shown in Figure S2. The fluorescent response was increased with the increase of K^+ ions concentration and reached a plateau at 60 ~ 80 mmol L^{-1} of K^+ ions. The optimal concentration of K^+ ions was chosen at 60 mmol L^{-1}. As shown in Figure S3, the fluorescent signal ratio (I_F/I_0) was plotted as a function of concentration of aptamer probe 4 while keeping aptamer probe 4 and blocking probes with equal proportion. The fluorescent response almost reached its maximum when the concentration of aptamer probe 4 increased to 150 nmol L^{-1}. The optimal concentration of aptamer probe 4 was chosen at 150 nmol L^{-1}. The concentration of NMM was investigated and the result was shown in Figure S4. The fluorescent response enhanced with the increase of NMM concentration and reached a maximum at 1.01 μmol L^{-1} of NMM. The optimal concentration of NMM was chosen at 1.01 μmol L^{-1}. On addition of ATP, the ATP aptamer domain in aptamer probe 4 bound ATP molecules to produce aptamer-ATP bioaffinity complexes and released blocking probes. The reaction time for binding ATP was studied with an optimal time of 30 min (Figure S5).

3.3. Sensitivity and selectivity

The fluorescent signals toward different concentrations of ATP were measured under the optimal conditions. Fluorescent spectra of this biosensor at the presence of different concentrations of ATP were shown in Figure 3(A). Fluorescent intensity enhanced with the increase of ATP concentration due to the release of blocking probes and the trigger of HCR process, resulting in the formation of DNA nanowires incorporated with numerous G-quadruplexes. Figure 3(B) showed the fluorescent intensity at 610 nm increased over ATP concentration of 0 ~ 2000 μmol L^{-1}. The calibration curve (inset of Figure 3(B)) indicated that there was a good linear relationship between fluorescent intensity and the concentration of ATP over the range

of 30 - 800 μmol L^{-1} (R^2=0.9989). The detection limit was about 15 μmol L^{-1} (3σ/K, in which σ is the standard deviation for the blank solution, and K is the slope of the calibration curve).

CTP, GTP, UTP and ATP belong to the nucleoside triphosphate family, they usually coexist in real biological samples. Differentiation of ATP from the other nucleoside triphosphate is of significant importance. The specificity of this biosensor was tested by comparing the response of ATP with those of three ATP analogues, CTP, GTP, and UTP at the same concentration. As shown in Figure 4, only ATP caused an obvious fluorescent increase, while none of the ATP analogues induced any appreciable fluorescent change compared to the blank sample, indicating the excellent selectivity of this biosensor.

3.4. Application

To assess the application of this biosensor in complex biological systems, the analysis of ATP in urine was carried out. Before measurements, urine samples were prepared by centrifugation at 1000 rpm for 5 min and the supernatant was collected and stored in the refrigerator at 4 °C. Different concentrations of ATP were added to the supernatant and measured according to the detection method. The results were listed in Table 2, indicating this biosensor was feasible to analyze ATP in biological samples.

4. Conclusions

In summary, a new enzyme-free and label-free fluorescent biosensor for small molecules by G-quadruplex-based HCR was developed. HCR was triggered by the released blocking probes at the present of target small molecules and numerous G-quadruplexes along DNA nanowires were self-assembled, resulting in fluorescent enhancement of NMM. Although the sensitivity for the detection of ATP (15 μmol L^{-1}) was inferior than that of other HCR-based biosensors [36-38], this “turn-on” biosensor allowed to specific detection of ATP with no need of any kind of enzyme, label, or sophisticated equipment. More importantly, this strategy can generally be applied to

different targets by simply altering the aptamer probes according to the sequences of different aptamers.

Acknowledgements

The authors gratefully acknowledge NSFC (21177023) and Science Foundation of Fujian Province of China (2013J01044) for financial support.

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Captions

Figure 1. Schematic representation of fluorescent detection of ATP by G-quadruplex based HCR.

Figure 2. Fluorescent signal ratio (I_F/I_0) with different aptamer probes.
(Condition: [H1]=[H2]=0.25 μmol L^{-1}, [ATP]=800 μmol L^{-1}, [KCl]=60 mmol L^{-1}, [blocking probe]= [aptamer probe]=150 nmol L^{-1}, [NMM]=1.01 μmol L^{-1})

Figure 3. (A) Fluorescence spectra in the presence of various concentration of ATP. (B) Plot of ATP concentration vs I_F. Inset is the calibration curve. The error bars indicated the standard deviations of three measurements.

Figure 4. Fluorescent response of this biosensor to ATP, GTP, CTP, and UTP (each 800

μmol L^{-1}).

Table 1. Sequences of DNA used in this work

Name	Sequence (5'→3')
Aptamer probe 1	ACC TGG GGG AGT ATT GCG GAG GAA GGT CT
Aptamer probe 2	ACC TGG GGG AGT ATT GCG GAG GAA GGT CT**T**
Aptamer probe 3	ACC TGG GGG AGT ATT GCG GAG GAA GGT CT**T A**
Aptamer probe 4	ACC TGG GGG AGT ATT GCG GAG GAA GGT CT**T AG**
Aptamer probe 5	ACC TGG GGG AGT ATT GCG GAG GAA GGT CT**T AGT**
Aptamer probe 6	ACC TGG GGG AGT ATT GCG GAG GAA GGT CT**T AGT T**
Aptamer probe 7	ACC TGG GGG AGT ATT GCG GAG GAA GGT CT**T AGT TCA**
Blocking probe	CGT GCT GAA CTA AGA CCT TCC TCC
Hairpin probe 1 (H1)	GGA GGA AGG TCT TAG TTC AGC AC**G** TGG GTA GGG CGG GTT GGG ATA TAC CCA **G**GT GCT GAA CTA AGA CCT
Hairpin probe 2 (H2)	**C**GT GCT GAA CTA AGA CCT TCC TCC AGG TCT TAG TTC AGC AC**C** TGG GTA

Complementary bases are underlined

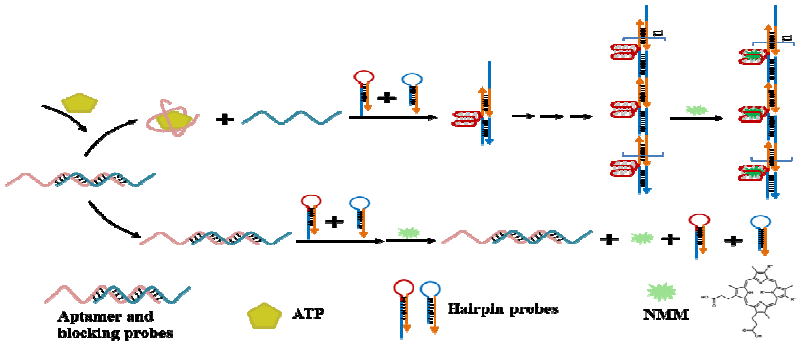
Table 2. Analysis of ATP in urine samples

Urine samples	Added (μmol L^{-1})	Found (μmol L^{-1}) Mean ± SD[a]	Recovery (%)
Sample 1	60.0	62.1 ± 2.2	103.4
Sample 2	300.0	304.8 ± 6.8	101.6
Sample 3	600.0	591.3 ± 9.8	98.6
Sample 4	800.0	791.2 ±1.5	98.9

a: SD was the standard derivation of three measurements

Highlights

- An enzyme-free and label-free biosensor for small molecules was developed.
- DNA nanowires were self-assembled through HCR upon the recognition of targets.
- G-quadruplexes bound with NMM were used as a versatile signaling reporter.



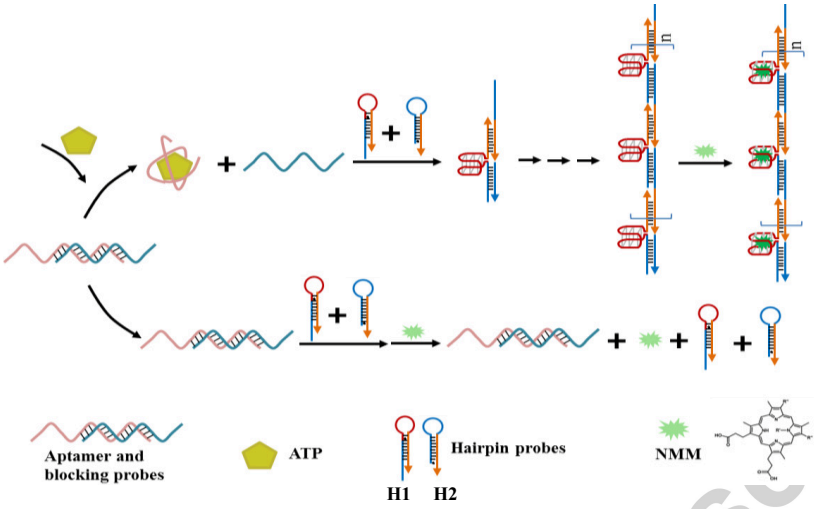


Figure 1.

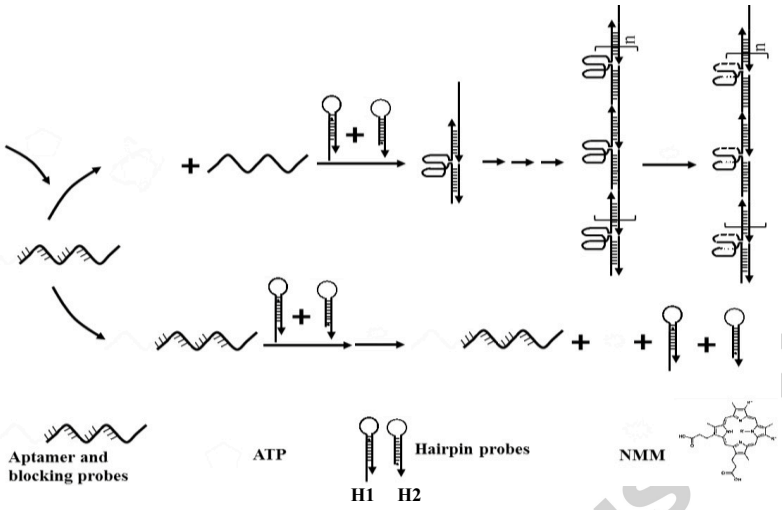


Figure 1.

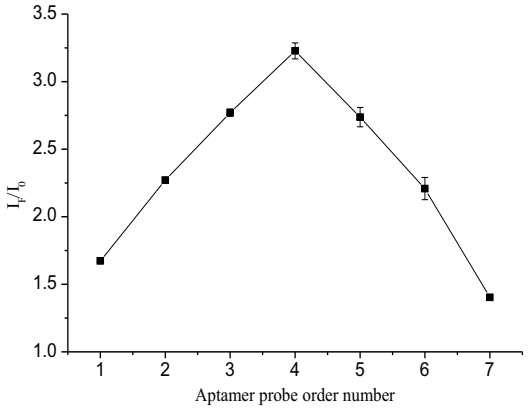
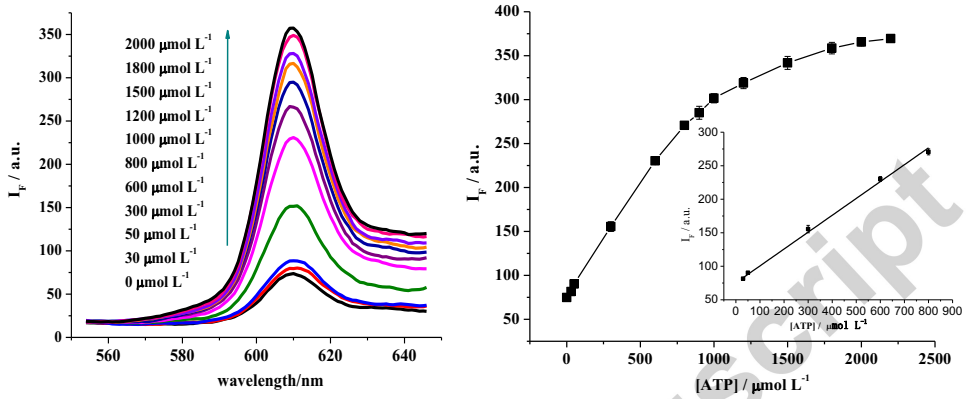


Figure 2.



A B

Figure 3.

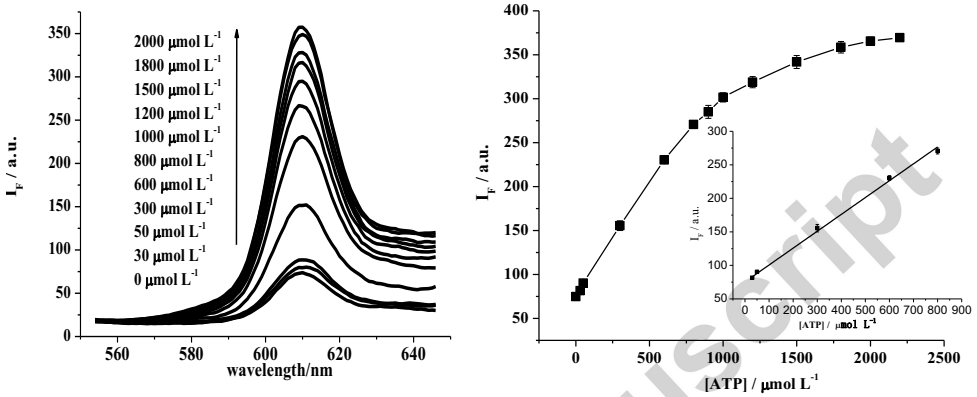


Figure 3.

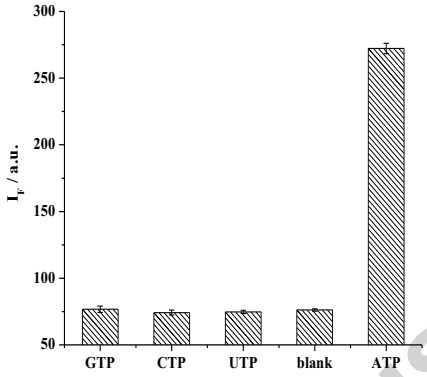


Figure 4.