Accepted Manuscript

A label-free fluorescent biosensor for the detection of protein kinase activity based on gold nanoclusters/graphene oxide hybrid materials

Qing Liu, Ning Li, Mengke Wang, Lei Wang, Xingguang Su

PII: S0003-2670(18)30158-2

DOI: 10.1016/j.aca.2018.01.053

Reference: ACA 235700

To appear in: *Analytica Chimica Acta*

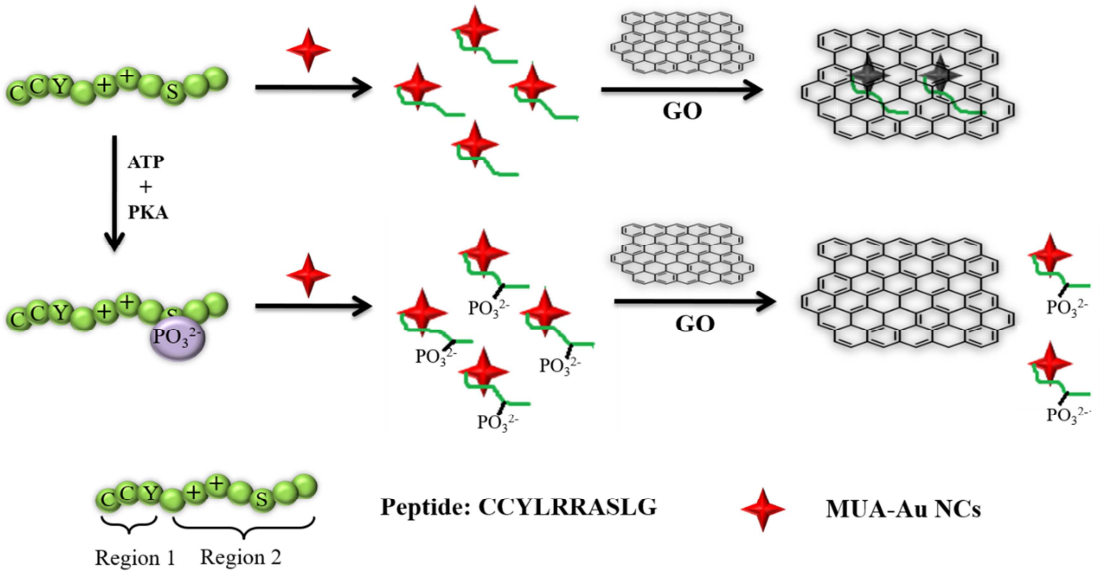
Received Date: 16 September 2017

Revised Date: 20 December 2017

Accepted Date: 22 January 2018

Please cite this article as: Q. Liu, N. Li, M. Wang, L. Wang, X. Su, A label-free fluorescent biosensor for the detection of protein kinase activity based on gold nanoclusters/graphene oxide hybrid materials, *Analytica Chimica Acta* (2018), doi: 10.1016/j.aca.2018.01.053.

Graphic Abstract



A label-free fluorescent biosensor for the detection of protein kinase activity based on gold nanoclusters/graphene oxide hybrid materials

Qing Liu[a], Ning Li[a,b], Mengke Wang[a], Lei Wang[a], Xingguang Su[a*]

a. Department of Analytical Chemistry, College of Chemistry, Jilin University, Changchun 130012, China

b. Department of Respiratory, China-Japan Union Hospital of Jilin University, Changchun 130031, Jilin, China

*Corresponding author

Tel.: +86-431-85168352

E-mail address: suxg@jlu.edu.cn

ABSTRACT

Protein kinase (PKA) can regulate many cellular biological processes by phosphorylation substrate peptide or protein. A new fluorescent biosensing method for the detection of PKA activity was developed by using 11-mercaptoundecanoic acid-capped gold nanoclusters (MUA-Au NCs) and graphene oxide (GO) with low background noise. In this strategy, the special designed peptide could be anchored on the surface of MUA-Au NCs by the Au-S bond and also adsorbed on the surface of GO owing to the electrostatic interaction. As a result, the fluorescence of MUA-Au NCs was quenched leading to low background fluorescence due to the forster resonance energy transfer (FRET) between MUA-Au NCs and GO via peptide as a bridge. However, when the substrate peptide was phosphorylated by PKA, the FRET between GO and MUA-Au NCs was disrupted because of the weakened interaction between the phosphorylated peptide and the GO, resulting in recovery of the fluorescence intensity. The developed label-free fluorescence "turn-off-on" method can detect protein kinase activity in the range of 0.6-2.0 U mL^{-1} with a detection limit of 0.17 U mL^{-1} (3σ). The feasibility of this present method for kinase inhibitor screening was also studied by assessment of H-89 kinase inhibition with an IC_{50} value of 0.049 μmol L^{-1}.

Keywords: MUA-Au NCs; Graphene oxide; Fluorescence; Protein kinase activity

1. Introduction

PKA (protein kinase A), a kind of protein kinases, can catalyze phosphorylation of serine, threonine or tyrosine in a peptide or protein substrate by transferring the γ-phosphoryl from adenosine-5'-triphosphate (ATP) [1,2]. Intracellular phosphorylation by protein kinase is a process that could regulate many cellular biological processes, including cellular signal communications, cell cycle progression and differentiation, gene expression and apoptosis, which plays a pivotal role in metabolic pathways [3,4]. However, it is also found that the over-expression and aberrant activity of protein kinases may be an indication or a cause of multiple complex diseases, such as carcinogenesis, cardiovascular diseases, diabetes, inflammation and Alzheimer's disease [5,6]. For this reason, protein kinases have become the main targets of the cancer drug development for pharmaceutical companies [7]. Therefore, the establishment of accurate and sensitive kinase assays for monitoring kinases activity and screening potential inhibitors is of great significance on the clinical pharmacology and drug discovery [8,9].

Recently, many methods have been applied for protein kinase detection, including electrogenerated chemiluminescence (ECL) [10], electrochemistry [11,12], colorimetry [13], fluorimetry [14,15]. For example, carboxypeptidase Y (CPY) digestion strategy has been designed by Song et al. [16] by using peptide-Au NCs as effective optical probe for kinase activity monitoring. Although the use of enzyme digestion could make detection with high sensitivity, the reaction time of carboxypeptidase Y for digesting the peptide is relatively long and the price of carboxypeptidase Y is relatively high. Yang et al. [17] report a new sensing strategy for portable and sensitive detection of protein kinases (PK) activity by use of a commercial personal glucose meter (PGM) for signal readout with the assistance of Zr^{4+}-functionalized magnetic beads (ZrMBs). The idea of using very convenient device personal glucose meter for detection of PK activity is very interesting and novel. However, the testing procedure is very tedious and time-consuming.

Among these methods, fluorimetry have attracted widespread attention in the majority of researchers owing to good properties of high sensitivity, simplicity. Metal nanoclusters (NCs), as a new type of luminescent nanomaterials, have attracted a great deal of interest. It is composed of a few to roughly a hundred atoms with diameters below 2 nm [18]. The size of metal NCs is comparable to the Fermi wavelength, resulting in molecule-like properties including discrete

energy levels and size-dependent fluorescence [19,20]. Up to now, among mental NCs, gold nanoclusters (Au NCs) have been widely used as fluorescent sensors for detection of metal ions [21,22], various biomolecules [23,24] and in vitro or in vivo imaging [25,26] owing to their colloidal stability, good biocompatibility and strong photoluminescence.

Graphene oxide (GO), a single-atomic-layered two-dimensional carbon nanomaterial, is a promising material for future high-tech applications due to the high charge carrier mobilities, the electrical and thermal conductivity, combined with transparency and mechanical strength [27]. Because GO can act as a "nanoquencher" on the photoluminance of the fluorophore via FRET or non-radiative dipole-dipole coupling, GO has attracted increasing attention in fluorescence-based biosensing [28,29]. Many papers have reported that there is a strong interaction between GO and amino acids such as Arg, His, and Lys [30-34].

Herein, we constructed a fluorescence MUA-Au NCs/peptide/GO platform to detect protein kinase activity. The special designed peptide could be anchored on the surface of MUA-Au NCs by Au-S bond. Meanwhile, the peptide can be adsorbed on the surface of GO due to the electrostatic interaction of positive Arg with negative GO. When peptide was introduced into MUA-Au NCs/GO system, the MUA-Au NCs was adsorbed onto the GO surface via peptide as a bridge, and the fluorescence of MUA-Au NCs was quenched by GO via FRET. Furthermore, when the substrate peptide was phosphorylated, the phosphorylated peptide showed weakened interaction with the GO. So, the FRET between GO and MUA-Au NCs was disrupted, leading to the recovery of the fluorescence intensity. Compared with turn-off assays, this label-free fluorescent method could reduce background noise. And there is no need to use extra enzymes, which can reduce the reaction time and cost.

2. Experiment

2.1 Materials and apparatus

Protein kinase A (PKA, catalytic subunit from bovine heart), adenosine-5'-triphosphate (ATP) disodium salt hydrate, urease, glucose oxidase (GOx), horseradish peroxidase (HRP) and N-[2[[[3-(4-Brompophenyl)-2-propenyl]amino]ethyl]-5-isoquinolinesulfonamide (H-89) were purchased from Sigmae-Aldrich. Cysteine-terminated peptide (NH_2-CCYLRRASLG-COOH) was purchased from GL Biochem Ltd. (Shanghai, China). 11-Mercaptoundecanoic acid (MUA) was purchased from J&K technology Co., Ltd. (Beijing, China). Hydrogen tetrachloroaurate (III)

tetrahydrate ($HAuCl_4 \cdot 4H_2O$) was from Sinopharm Group Chemical Regent Co., Ltd. (Shanghai, China). Graphite powder (100 mesh) was purchased from HuaCheng Biological Co., Ltd (ChangChun). Other inorganic reagents (analytically pure) were purchased from Beijing Chemical Co. and were used without further purification. The water used in all experiments had a resistivity higher than18 $M\Omega\ cm^{-1}$.

Transmission electron microscopy (TEM) was recorded through a Hitachi H-800 electron microscope at an acceleration voltage of 200 kV with a CCD camera. FT-IR spectra were obtained with a Bruker IFS66V FT-IR spectrometer equipped with a DGTS detector. Fluorescence spectra were recorded by using a RF-5301 PC spectrofluorophotometer (Shimadzu, Japan) equipped with a xenon lamp using right-angle geometry. UV-Vis spectrum was recorded on an UV-Vis spectrometer (Shimadzu Co., Ltd., Japan). All pH measurements were made with a PHS-3C pH meter (Tuopu Co., Hangzhou, China). All temperature measurements were accomplished using a water bath.

2.2 The preparation of GO by the modified hummers method

Graphene oxide (GO) was prepared from graphite powder by the modified Hummers method [35]. Typically, 1.0 g of graphite powder and 25 mL H_2SO_4 (98%) were respectively added to 250 mL beaker and stirred 30 min. Afterwards, 3.5 g $KMnO_4$ was slowly introduced to the system and the mixture was stirred for 30 min in an ice bath. Next, after stirred at 35 °C for another 1 h, 90 mL deionized water was dropwise added to the mixture and the temperature was increased to 90 °C stirred for another 30 min. Subsequently, 90 mL diluted water and 10 mL of 30% H_2O_2 solution were added to end the reaction. The color of the mixture changed to bright yellow quickly. The resulting mixture was washed with distilled water several times and further purified in a dialysis bag with a molecular weight cutoff of 10000 Da against deionized water until the pH of the solution became neutral. Finally, the concentration of GO stock solution is 4.33 mg mL^{-1}.

2.3 Synthesis of MUA-Au NCs

The fluorescent Au NCs were synthesized according to a modified protocol described previously [36,37]. In a typical experiment, 11-MUA (6.6 mg) and 75 μL NaOH (1.0 mol L^{-1}) were dissolved in water (9 mL). An aqueous solution of $HAuCl_4$ (0.75 mL, 10 mmol L^{-1}) was slowly added to the mixture. Then the thorough mixture was left to stand for overnight at room temperature without stirring, and the solution remained colorless. The prepared Au NCs were

dialyzed with a 3000 Da cutoff dialysis membrane to remove all small molecule impurities. The obtained Au NCs solution was filtered by passing through 0.45 μm filters. The purified MUA-Au NCs solution was stored at 4°C before use.

2.4 Fluorescence measurement of PKA activity and its inhibitor

For PKA activity detection, 40 μL 1.0 mmol L^{-1} peptide, 15 μL 1.0 mmol L^{-1} ATP and 20 μL different concentrations of PKA were mixed with 25 μL PBS solution (1 mmol L^{-1}, pH=7.4). After incubated at 37 °C for 1h, 20 μL MUA-Au NCs, 250 μL PBS solution (1 mmol L^{-1}, pH=6.2) and 30 μL GO were added to the resulting solution to give final volumes of 400 μL. After 1 min, the fluorescence spectra were recorded from 580 nm to 700 nm with an excitation wavelength of 280 nm. All measurements were performed at room temperature (298 K).

For assay of PKA inhibitor, 40 μL 1.0 mmol L^{-1} peptide, 10 μL 20 U mL^{-1} PKA, 15 μL 1.0 mmol L^{-1} ATP and 40 μL PBS buffer solution (1 mmol L^{-1}, pH=7.4) were mixed with 10 μL different concentrations of inhibitor H-89. After incubated at 37 °C for 1h, 20 μL MUA-Au NCs, 250 μL PBS solution (1 mmol L^{-1}, pH=6.2) and 30 μL GO were added to the resulting solution to give final volumes of 400 μL. The following procedures were the same to above-mentioned PKA activity detection.

2.5 Detection of PKA in HepG-2 cell lysates

HepG-2 Cells (1×10^6 cells) were cultured in Dulbecco's modified Eagle's medium (DMEM)/F-12 (1:1) supplemented with 10% fetal bovine serum and were incubated at 37°C under a humidified atmosphere containing 5% CO_2 for 3-4 days. After serum free medium replacing the culture medium, the cultured cells were centrifuged to remove the nutrient solution and then broken with an ultrasonic liquid processor for 2 min. The cell lysates were centrifuged again and ready for use. Then, three different concentrations of PKA were added to the cell lysates to prepare the spiked samples. For kinase activity assays in HepG-2 cell lysate, the same experiments were carried out except that the equal volume of above-prepared cell lysate samples in stead of pure PKA were added to the reaction system for investigation.

3. Results and Discussion

3.1 The characterization of MUA-Au NCs and GO

The MUA-Au NCs used in this work was characterized by using TEM, FT-IR, UV-vis and fluorescence spectra. The TEM image of MUA-Au NCs (Fig. 1a) shows that the MUA-Au NCs

are near spherical with well-dispersion, and have an average diameter of 1.72 nm. Fig. 1b displays the FTIR spectra of MUA and MUA-Au NCs. It can be seen that the characteristic S-H stretching band (2560 cm^{-1}) of MUA disappears in the FTIR spectra of MUA-Au NCs, indicating the formation of the covalent bonds (Au-S-R) between MUA and Au NCs. As shown in Fig 1c, the prepared MUA-Au NCs solution appeared almost colorless in visible light and emitted intense orange-red fluorescence under UV light irradiation. The UV-vis absorption spectrum of MUA-Au NCs exhibits main absorption bands at 229 nm, and there is no apparent localized surface plasmon resonance band of Au NPs in the visible region [38]. When the MUA-Au NCs were excited at varying wavelength from 260 to 350 nm, the fluorescence spectra were always centered at 602 nm (Fig. S1). Meanwhile, when the excitation wavelength increases or decreases than 280 nm, the maximum fluorescence intensity gradually decreased. The fluorescence spectrum of the MUA-Au NCs exhibited a peak at 602 nm (red line) with the excitation peak at 280 nm (black line) (Fig. 1c). While there is no emission peak in the fluorescence spectrum of MUA or $HAuCl_4$ (Fig. S2). FT-IR spectrometry was obtained to study the existence of surface functional groups on the GO (Fig. 1d). Peak 3400 cm^{-1} and 1400 cm^{-1} were attributed to characteristic intense stretching vibration and deformation vibration of C-OH. Peak 1730 cm^{-1} combined peak arising from the skeletal vibrations of oxidized graphite sheets 1620 cm^{-1} indicated the existence of C=O. Meanwhile, the stretching vibration peak of carbonyl appeared at 1050 cm^{-1}. And a small peak at 850 cm^{-1} was corresponding to bending vibrations of C-O [39].

Figure 1

3.2 The strategy for the detection of PKA activity

The designed strategy for fluorescent detection of protein kinase (PKA) is illustrated in Scheme 1. The special designed peptide H_2N-CCYLRRASLG-COOH containing two regions was used in this work. Region 1 is CCY, which the cysteine at N terminal could be anchored on the surface of MUA-Au NCs by Au-S bond [13]. Another region is LRRASLG. On the one hand, it is the specific peptide substrate for cAMP-dependent protein kinase A (PKA), which can be phosphorylated at the serine residue (S) in the presence of PKA and ATP. On the other hand, region 2 containing two positively charged Arg residues (RR) could led to adsorption between the

peptide and GO, which was attributed to the electrostatic interaction of positive Arg with negative GO [40]. So, when the peptide was introduced to the MUA-Au NCs/GO system, peptide can be adsorbed onto the GO due to electrostatic interaction. As peptide was anchored on the surface of MUA-Au NCs by Au-S bond [32,40,41], which would lead to MUA-Au NCs adsorbed onto the GO surface via peptide as a bridge, resulting in FRET and fluorescence quenching of MUA-Au NCs. However, after the substrate peptide was phosphorylated when ATP and PKA existed at the same time, a phosphate group containing two negatively charged was introduced to peptide, and caused net charge of the peptide being zero. The electrostatic interaction between phosphorylated peptide and GO was weaker than that between peptide without phosphorylation and GO. So, the FRET between GO and MUA-Au NCs was disrupted, leading to the gradual recovery of fluorescence signal. Thus, the activity of protein kinase could be monitored based on this fluorescence "turn-off-on" assay.

In order to verify the feasibility of this method, some experiments were carried out. As shown in Fig. 2, when the special designed peptide was added to the MUA-Au NCs/GO system, the fluorescence intensity of the system would decrease. However, the same concentration of peptides exhibited little effect on the fluorescence of MUA-Au NCs (Fig. S3). Moreover, there is no significant change of the fluorescence intensity of the MUA-Au NCs/GO/peptide system after mixed with ATP or PKA separately. When peptide was phosphorylated by PKA, phosphorylation peptide did not quench the fluorescence intensity of the MUA-Au NCs/GO system. Furthermore, with the increase of PKA concentration, the amount of phosphorylated peptide also increased leading to the fluorescence intensity of the MUA-Au NCs/GO system gradually increased. All these results indicating that the PKA activity could be detected by this fluorescent assay.

Scheme 1

Figure 2

3.3 Optimization of experimental conditions

In order to achieve better sensing performance for PKA, experimental parameters, including pH, the concentration of GO, peptide and ATP were optimized. The effect of pH was firstly studied over the range of pH 6.2-8.6. It can be observed from Fig. 3a that the fluorescence

intensity of MUA-Au NCs/peptide/GO system was increased with the increase of pH in the range of 6.2-8.6, while the fluorescence intensity of MUA-Au NCs/peptide/GO/PKA/ATP system displayed higher fluorescence signals with smaller increase. This may be attributed to the positive charge of peptide was decreased after peptide was phosphorylated, and resulting in the decrease of interaction between peptide and GO. Because the difference of fluorescence intensity between the two systems was decreased with the increase of pH, so pH 6.2 was selected for the further experiment. Then, the effect of the GO concentration in the range of 0-0.24 mg mL^{-1} was investigated. As shown in Fig. 3b, the fluorescence intensity ratio I/I_0 of MUA-Au NCs (I_0 and I are the fluorescence intensity of MUA-Au NCs in the absence and presence of GO) can be slightly quenched by the GO in the range of 0-0.16 mg mL^{-1}. When the concentration of GO exceeds 0.16 mg mL^{-1}, it could cause a great influence on the fluorescence intensity of MUA-Au NCs. Therefore, 0.15 mg mL^{-1} GO was chosen in the further experiments. The effect of the peptide concentration was shown in Fig. 3c. It can be seen that the fluorescence intensity ratio I/I_0 of MUA-Au NCs/peptide/GO system (I_0 and I are the fluorescence intensity of MUA-Au NCs/GO system in the absence and presence of peptide) decreased with the increasing of peptide concentration until 100 μmol L^{-1} and then increased. The higher concentration of peptide above 100 uM is not beneficial to the interactions between MUA-Au NCs/peptide and GO, this is due to the extra peptide would be adsorbed on the surface of GO, which could prevent the peptide connected to MUA-Au NCs interacting with GO. So, a final concentration of 100 μmol L^{-1} peptide was adopted for the following experiments. Fig. 3d illustrated the effect of ATP concentration on the fluorescence intensity ratio of MUA-Au NCs/peptide/GO/PKA/ATP system (I_0 and I are the fluorescence intensity of MUA-Au NCs/peptide/GO/PKA system in the absence and presence of ATP). As shown in Fig. 3d, the fluorescence intensity of MUA-Au NCs/GO/peptide/PKA/ATP system reached the maximum when the concentration of ATP was increased to be 37.5 μmol L^{-1}. Thus, 37.5 μmol L^{-1} ATP was chosen in the further experiments.

Figure 3

3.4 Detection of the PKA activity and inhibitor

Under the optimum conditions, the quantitative behavior of the proposed fluorescence

method for the determination of PKA was studied. As shown in Fig. 4a, it can be observed that the fluorescence intensity of the MUA-Au NCs/peptide/GO/PKA/ATP system was gradually increased with the increase concentration of PKA in the range of 0-2.0 U mL^{-1}. Inset showed the linear relationship between the fluorescence intensity and concentration of PKA in the range of 0.60-2.0 U mL^{-1}. The linear regression equation was $I/I_0=0.972+0.310C_{PKA}$, U mL^{-1} (I and I_0 are the fluorescence intensity of MUA-Au NCs/GO/peptide/ATP system in the presence and absence of PKA), and the corresponding regression coefficient $R^2=0.993$. The detection limit (LOD) for PKA is 0.17 U mL^{-1}. A comparison between this method and some other reported methods for PKA determination in linear range and detection limit was listed in Table S1. Lu's group [9] presents a novel fluorometric method for probing the activity and inhibition of protein kinase based on positively charged gold nanoparticles and substrate peptide. The quantification range is 0.5-1.0 U mL^{-1}. The detection limit is 0.5 U mL^{-1}. Zhou and coworkers [13] report a colorimetric gold nanoparticles (AuNPs)/peptide platform for monitoring the activity and inhibition of protein kinases. The detection limit is 0.232 U mL^{-1}. So, it could be found that our method showed a comparable quantification range and detection limit.

Enzyme inhibitors could recognize specific enzyme and reduce its catalytic activity considerably, the screening of potential inhibitors is of great significance in drug discovery [42]. To further investigate whether this method could be potential applied for protein kinase inhibitor screening, the experiments were performed in the presence of a potential and cell-permeable PKA inhibitor H-89 with different concentrations [43]. The fixed PKA concentration (2.0 U mL^{-1}), peptide and ATP were preincubated with various concentrations of H-89, after which the reaction solution was mixed with MUA-Au NCs/GO. As shown in Fig. 4b, the decrease of fluorescence intensity ratio I/I_0 of MUA-Au NCs/peptide/GO/PKA/ATP system (I_0 and I are the fluorescence intensity of MUA-Au NCs/GO/peptide/ATP/PKA system in the absence and presence of H-89) was accompanied by the increasing of the concentrations of H-89. When the concentration of H-89 reached 0.1 μmol L^{-1}, the fluorescence intensity ratio of the system reached a platform, indicating the activity of PKA was completely inhibited. From the sigmoidal profile, the IC_{50} value (the half-maximal inhibitory concentration) was found to be 0.049 μmol L^{-1}. These results clearly illustrated that the proposed method shows adequate capacity for assaying protein kinase inhibitors and protein kinase inhibitors screening.

Figure 4

3.5 Selectivity for PKA detection

The selectivity is one of the significant parameters of developed method to evaluate its performance for detection a specific species in a complex biomedical system. So, the selectivity experiment for our proposed biosensor was carried out with various enzymes added. Fig. 5 illustrated that 4.0 U mL^{-1} urease, glucose oxidase (GOx) and horseradish peroxidase (HRP) did not cause any significant change on the fluorescence intensity of MUA-Au NCs/GO/peptide/ATP system in comparison with the addition of 2.0 U mL^{-1} PKA. These results demonstrated that the novel fluorescence sensor displays good selectivity for the determination PKA activity.

Figure 5

3.6 Detection of PKA in Cell Lysates

In order to further investigate feasibility of this fluorescent sensor in real sample detection, the detection of PKA in HepG-2 cell lysates was carried out. The results obtained by standard addition method were listed in Table 1. It could be seen that the average recoveries of PKA in the real samples were in the range of 97.5-102.5% and the RSD was lower than 2.0%, which indicates that the accuracy and precision of the proposed method were satisfactory.

Table 1

4. Conclusions

In summary, we established a label-free fluorescence “turn-off-on” method for protein kinase activity detection and inhibitor screening based on MUA-Au NCs and GO. The special designed peptide could be anchored on the surface of MUA-Au NCs by the Au-S bond, and the MUA-Au NCs was adsorbed onto the GO surface via peptide as a bridge, leading to the fluorescence quenching of MUA-Au NCs via FRET. Due to the decrease of electrostatic interaction between phosphorylated peptide and GO, the FRET between GO and MUA-Au NCs was destroyed, which

induced the fluorescence recovery of MUA-Au NCs. The proposed MUA-Au NCs/GO kinase sensing strategy could avoided laborious and time-consuming steps and reduced background noise. Furthermore, this sensing platform shows great potential in fundamental research of protein kinase-related biochemistry and high-throughput screening in kinase-targeted drug discovery.

Acknowledgments

This work was financially supported by the National Natural Science Foundation of China (No. 21275063 and No. 21575048), the Science and Technology Development project of Jilin province, China (No. 20150204010GX) and the Graduate Innovation Fund of Jilin University (No. 2017077).

References

[1] R.S. Agnes, F. Jernigan, J.R. Shell, V. Sharma, D.S. Lawrence, Suborganelle Sensing of Mitochondrial cAMP-Dependent Protein Kinase Activity, J. Am. Chem. Soc. 132 (2010) 6075-6080.

[2] C.L. Wang, L.Y. Wei, C.J. Yuan, K.C. Hwang, Reusable Amperometric Biosensor for Measuring Protein Tyrosine Kinase Activity, Analytical Chemistry 84 (2012) 971-977.

[3] D.M. Rothman, M.D. Shults, B. Imperiali, Chemical approaches for investigating phosphorylation in signal transduction networks, Trends in Cell Biology, 15 (2005) 502-510.

[4] Y.L. Zhou, H.S. Yin, X. Li, Z. Li, S.Y Ai, H. Lin, Electrochemical biosensor for protein kinase A activity assay based on gold nanoparticles-carbon nanospheres, phos-tag-biotin and β-galactosidase, Biosensors and Bioelectronics 86 (2016) 508-515.

[5] G.B Manoharan, E. Enkvist, M. Kasari, K. Viht, M. Zenn, A. Prinz, O. Filhol, F.W. Herberg, A. Uri, FRET-based screening assay using small-molecule photoluminescent probes in lysate of cells overexpressing RFP-fused protein kinases, Analytical Biochemistry 481 (2015) 10-17.

[6] J. Schlessinger, Cell Signaling by Receptor Tyrosine Kinases, Cell, 103 (2000) 211-225.

[7] G.B. Manoharan, E. Enkvist, A. Uri, Combining chemical and genetic approaches for development of responsive FRET-based sensor systems for protein kinases, Biophysical Chemistry 211 (2016) 39-48.

[8] J. Bai, C.H. Liu, T. Yang, F.F. Wang, Z.P. Li, A versatile platform for highly sensitive detection of kinase activity based on metal ion-mediated FRET using an anionic conjugated polymer, Chem.

Commun. 49 (2013) 3887-3889.

[9] G.Y. Lu, P.L. Tan, C.Y. Lei, Z. Nie, Y. Huang, S.Z. Yao, Fluorescent detection of protein kinase based on positively charged gold nanoparticles, Talanta 128 (2014) 360-365.

[10] S.J. Xu, Y. Liu, T.H Wang, J.H. Li, Highly Sensitive Electrogenerated Chemiluminescence Biosensor in Profiling Protein Kinase Activity and Inhibition Using Gold Nanoparticle as Signal Transduction Probes, Anal. Chem. 82 (2010) 9566-9572.

[11] Z.H. Wang, N. Sun, Y. He, Y. Liu, J.H. Li, DNA Assembled Gold Nanoparticles Polymeric Network Blocks Modular Highly Sensitive Electrochemical Biosensors for Protein Kinase Activity Analysis and Inhibition, Anal. Chem. 86 (2014) 6153-6159.

[12] X.M. Miao, Z.B. Li, A.H. Zhu, Z.Z. Feng, J.Tian, X. Peng, Ultrasensitive electrochemical detection of protein tyrosine kinase-7 by gold nanoparticles and methylene blue assisted signal amplification, Biosensors and Bioelectronics 83 (2016) 39-44.

[13] J. Zhou, X.H. Xu, X. Liu, H. Li, Z. Nie, M. Qing, Y. Huang, S.Z. Yao, A gold nanoparticles colorimetric assay for label-free detection of protein kinase activity based on phosphorylation protection against exopeptidase cleavage, Biosensors and Bioelectronics 53 (2014) 295-300.

[14] R. Freeman, T. Finder, R. Gill, I. Willner, Probing Protein Kinase (CK2) and Alkaline Phosphatase with CdSe/ZnS Quantum Dots, Nano Lett., 10 (2010) 2192-2196.

[15] Y. Wang, L. Zhang, R.P. Liang, J.M. Bai, J.D. Qiu, Using Graphene Quantum Dots as Photoluminescent Probes for Protein Kinase Sensing, Anal. Chem. 85 (2013) 9148-9155.

[16] W. Song, R.P. Liang, Y. Wang, L. Zhang, J.D. Qiu, Green synthesis of peptide-templated gold nanoclusters as novel fluorescence probes for detecting protein kinase activity, Chem. Commun. 51 (2015) 10006-10009.

[17] W. Yanga, X. Lu, Y. Wang, S. Sun, C. Liu, Z. Lia, Portable and sensitive detection of protein kinase activity by usingcommercial personal glucose meter, Sensors and Actuators B 210 (2015) 508-512.

[18] L. Shang, S.J. Dong, G. U. Nienhaus, Ultra-small fluorescent metal nanoclusters: Synthesis and biological applications, Nano Today 6 (2011) 401-418.

[19] L.Z. Hu, L. Deng, S. Alsaiari, D.Y. Zhang, N.M. Khashab, “Light-on” Sensing of Antioxidants Using Gold Nanoclusters, Anal. Chem. 86 (2014) 4989-4994.

[20] L.B. Zhang, E. Wang, Metal nanoclusters: New fluorescent probes for sensors and

bioimaging, Nano Today 9 (2014) 132-157.

[21] Y. Zhang, J.J. Jiang, M. Li, P.F. Gao, L.H. Shi, G.M. Zhang, C. Dong, S.M. Shuang, Bright far-red/near-infrared gold nanoclusters for highly selective and ultra-sensitive detection of Hg^{2+}, Sensors and Actuators B 238 (2017) 683-692.

[22] Q. Zhao, H. Yan, P. Liu, Y.Y. Yao, Y.D. Wu, J. Zhang, H.X. Li, X.Q. Gong, J. Chang, An ultra-sensitive and colorimetric sensor for copper and iron based on glutathione-functionalized gold nanoclusters, Analytica Chimica Acta 948 (2016) 73-79.

[23] X.L. Hu, X.M. Wu, X. Fang, Z.J. Li, G.L. Wang, Switchable fluorescence of gold nanoclusters for probing the activity of alkaline phosphatase and its application in immunoassay, Biosensors and Bioelectronics 77 (2016) 666-672.

[24] J. Lan, H.Y. Zou, Q. Wang, P. Zeng, Y.F. Li, C.Z. Huang, Sensitive and selective turn off-on fluorescence detection of heparin based on the energy transfer platform using the BSA-stabilized Au nanoclusters/amino-functionalized graphene oxide hybrids, Talanta 161 (2016) 482-488.

[25] P. Zhang, X.X. Yang, Y. Wang, N.W. Zhao, Z.H. Xiong, C.Z. Huang, Rapid synthesis of highly luminescent and stable Au_{20} nanoclusters for active tumor-targeted imaging in vitro and in vivo, Nanoscale 6 (2014) 2261-2269.

[26] J. Qiao, X.Y. Mu, L. Qi, J.J. Deng, L.Q Mao, Folic acid-functionalized fluorescent gold nanoclusters with polymers as linkers for cancer cell imaging, Chem. Commun. 49 (2013) 8030-8032.

[27] S. Eigler, A. Hirsch, Chemistry with Graphene and Graphene Oxide-Challenges for Synthetic Chemists, Angew. Chem. Int. Ed. 53 (2014) 7720-7738.

[28] M. Zhang, B.C. Yin, W.H. Tan, B.C. Ye, A versatile graphene-based fluorescence “on/off” switch for multiplex detection of various targets, Biosensors and Bioelectronics 26 (2011) 3260-3265.

[29] Z.P. Liu, X.G. Su, One-pot synthesis of strongly fluorescent DNA-CuInS2 quantum dots for label-free and ultrasensitive detection of anthrax lethal factor DNA, Analytica Chimica Acta 942 (2016) 86-95.

[30] J. Zhou, X.H. Xu. W. Liu, X. Liu, Z. Nie, M. Qing, L.H. Nie, S.Z. Yao, Graphene Oxide-Peptide Nanocomplex as a Versatile Fluorescence Probe of Protein Kinase Activity Based on Phosphorylation Protection against Carboxypeptidase Digestion, Anal. Chem. 85 (2013)

5746-5754

[31] M. Zhang, B.C. Yin, X.F. Wang, B.C. Ye, Interaction of peptides with graphene oxide and its application for real-time monitoring of protease activity, Chem. Commun. 47 (2011) 2399-2401.

[32] P.D. Nguyen, V.T. Cong, C. Baek, J. Min, Fabrication of peptide stabilized fluorescent gold nanocluster/graphene oxide nanocomplex and its application in turn-on detection of metalloproteinase-9, Biosensors and Bioelectronics 89 (2017) 666-672.

[33] S.R. Russell1, S.A. Claridge, Peptide interfaces with graphene: an emerging intersection of analytical chemistry, theory, and materials, Anal. Bioanal. Chem. 408 (2016) 2649-2658.

[34] E. Song, D. Cheng, Y. Song, M.D. Jiang, J.F. Yu, Y.Y. Wang, A graphene oxide-based FRET sensor for rapid and sensitive detection of matrix metalloproteinase 2 in human serum sample, Biosensors and Bioelectronics 47 (2013) 445-450.

[35] W.D. Na, T.Y. Hu, X.G. Su, Sensitive detection of acid phosphatase based on graphene quantum dots nanoassembly, Analyst 141 (2016) 4926-4932.

[36] J. Sun, F. Yang, D. Zhao, C.X. Chen, X.R. Yang, Integrated Logic Gate for Fluorescence Turn-on Detection of Histidine and Cysteine Based on Ag/Au Bimetallic Nanoclusters-Cu^{2+} Ensemble, ACS Appl. Mater. Interfaces 7 (2015) 6860-6866.

[37] J. Sun, F. Yang, D. Zhao, X.R. Yang, Highly Sensitive Real-Time Assay of Inorganic Pyrophosphatase Activity Based on the Fluorescent Gold Nanoclusters, Anal. Chem. 86 (2014) 7883-7889.

[38] J. Sun, X.Yang, Gold nanoclusters-Cu^{2+} ensemble-based fluorescence turn-on and real-time assay for acetylcholinesterase activity and inhibitor screening, Biosensors and Bioelectronics 74 (2015) 177-182.

[39] K.V. Ragavana, N.K. Rastogi, Graphene-copper oxide nanocomposite with intrinsic peroxidase activity for enhancement of chemiluminescence signals and its application for detection of Bisphenol-A, Sensors and Actuators B 229 (2016) 570-580.

[40] S.Q. Li, Y.W. Fu, X.J. Ma, Y.D. Zhang, Label-free fluorometric detection of chymotrypsin activity using graphene oxide/nucleic-acid-stabilized silver nanoclusters hybrid materials, Biosensors and Bioelectronics 88 (2017) 210-216.

[41] L. Hu, S. Han, S. Parveen, Y. Yuan, L. Zhang, G. Xu, Highly sensitive fluorescent detection of trypsin based on BSA-stabilized gold nanoclusters, Biosensors and Bioelectronics 32 (2012)

297-299.

[42] W. Song, R.P. Liang, Y. Wang, L. Zhang, J.D. Qiu, Gold nanoclusters-based dual-emission ratiometric fluorescence probe for monitoring protein kinase, Sensors and Actuators B 226 (2016) 144-150.

[43] L. Wang, X. Yan, X.G. Su, A label-free and sensitive fluorescent assay for one step detection of protein kinase activity and inhibition, Analytica Chimica Acta 935 (2016) 224-230.

Captions:

Fig. 1 (a) The TEM image of MUA-Au NCs. (b) FT-IR spectrum of MUA (black line) and MUA-Au NCs (red line). (c) The UV-vis absorption spectrum (blue line) and fluorescence excitation (black line) and emission (red line) spectra of MUA-Au NCs. The inset shows the corresponding photographs of MUA-Au NCs under the visible and UV light, respectively. (d) FT-IR spectrum of GO

Scheme 1 The schematic illustration of fluorescent assay for monitoring protein kinase activity based on MUA-gold nanoclusters (Au NCs) and graphene oxide (GO).

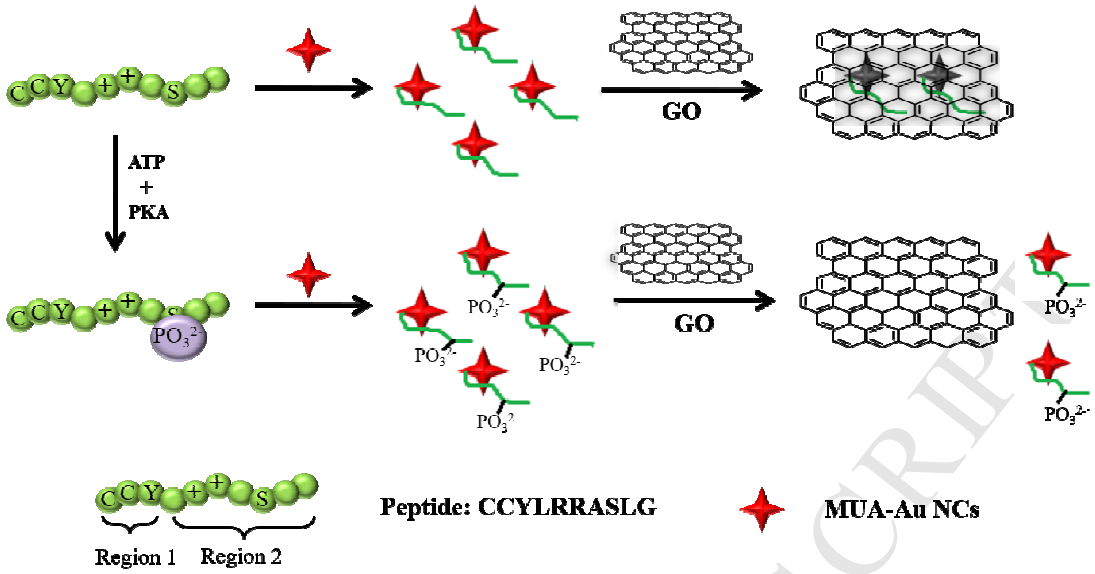
Fig.2 The fluorescence spectra of MUA-Au NCs+GO (black line), MUA-Au NCs+GO+peptide (red line), MUA-Au NCs+GO+peptide+ATP (blue line), MUA-Au NCs+GO+peptide+PKA (pink line), MUA-Au NCs+GO+peptide+ATP+PKA (green line) system, respectively.

Fig. 3 (a) Effect of pH on the fluorescence intensity of the MUA-Au NCs/peptide/GO system (black curve) and MUA-Au NCs/peptide/GO/PKA/ATP system (red curve). GO, 0.15 mg mL^{-1}; Peptide, 100 μmol L^{-1}; PKA, 2.0 U mL^{-1}; ATP, 37.5 μmol L^{-1}; (b) Effect of GO concentration on the fluorescence intensity ratio of MUA-Au NCs. I_0 and I are the fluorescence intensity of MUA-Au NCs in the absence and presence of GO (c) Effect of peptide concentration on the fluorescence intensity ratio of MUA-Au NCs/GO system. I_0 and I are the fluorescence intensity of MUA-Au NCs /GO in the absence and presence of peptide (d) Effect of ATP concentration on the fluorescence intensity ratio of MUA-Au NCs/peptide/GO/PKA/ATP system. I_0 and I are the fluorescence intensity of MUA-Au NCs/peptide/GO/PKA/ATP system in the absence and presence of ATP. GO, 0.15 mg mL^{-1}; Peptide, 100 μmol L^{-1}; PKA, 2.0 U mL^{-1}; The error bars were obtained from three parallel test results.

Fig. 4 (a) The fluorescence spectra of the MUA-Au NCs/peptide/GO/ATP mixed with various amounts of PKA. The concentrations of PKA from bottom to up were 0, 0.60, 0.80, 1.0, 1.20, 1.40, 1.60, 1.80, 2.0 U mL^{-1}. Inset: The linearity of the relative fluorescence intensity (I/I_0) versus and PKA concentration. I_0 and I are the fluorescence intensity of MUA-Au NCs/peptide/GO/PKA/ATP system in the absence and presence of PKA, respectively. (b) The relationship between the fluorescence intensity ratio I/I_0 of MUA-Au NCs/peptide/GO/ATP/PKA system and H-89 concentration. I and I_0 are the fluorescence intensity of MUA-Au NCs/peptide/GO/ATP/PKA system in the absence and presence of H-89, respectively. The error bars were obtained from three parallel test results.

Fig. 5 The selectivity of developed method for PKA assay. The PKA concentration was 2.0 U mL^{-1} while GOx, urease, HRP were 4.0 U mL^{-1}. I_0 and I are the fluorescence intensity of MUA-Au NCs/peptide/GO/ATP system in the absence and presence of enzyme, respectively. The error bars were obtained from three parallel test results.

Table 1 Determination of PKA in HepG-2 cell lysates



Scheme 1

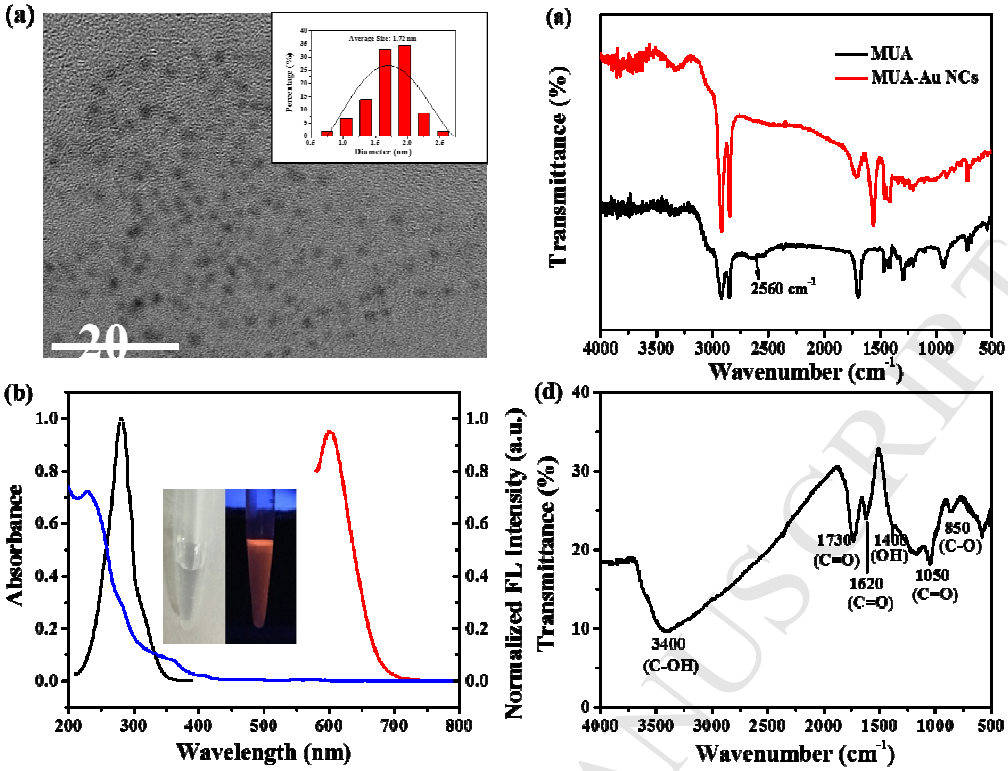


Fig. 1

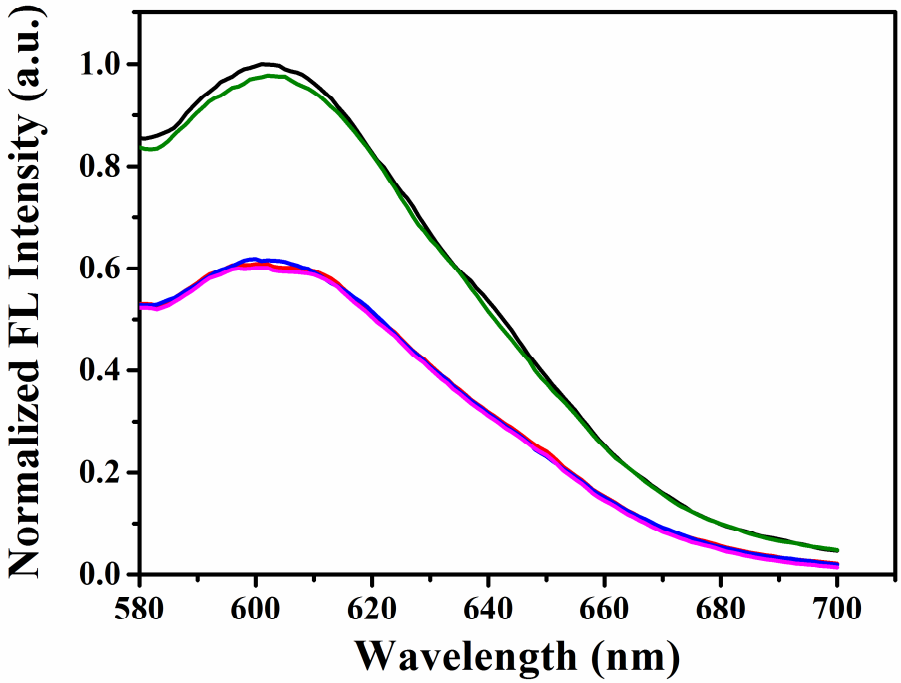


Fig. 2

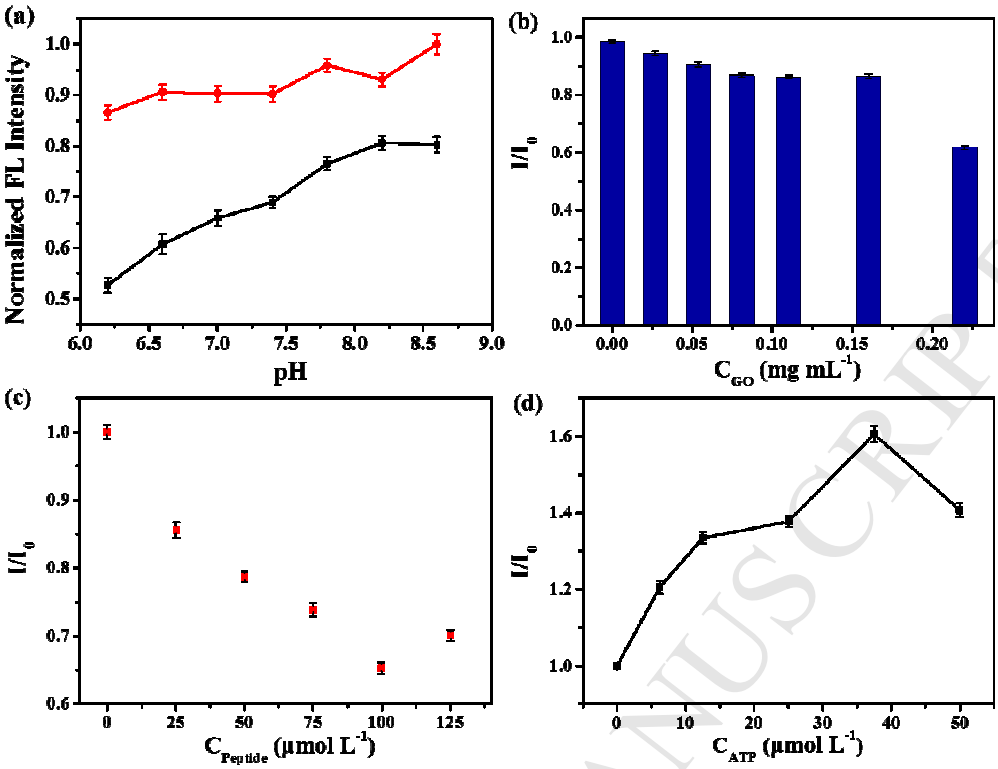


Fig. 3

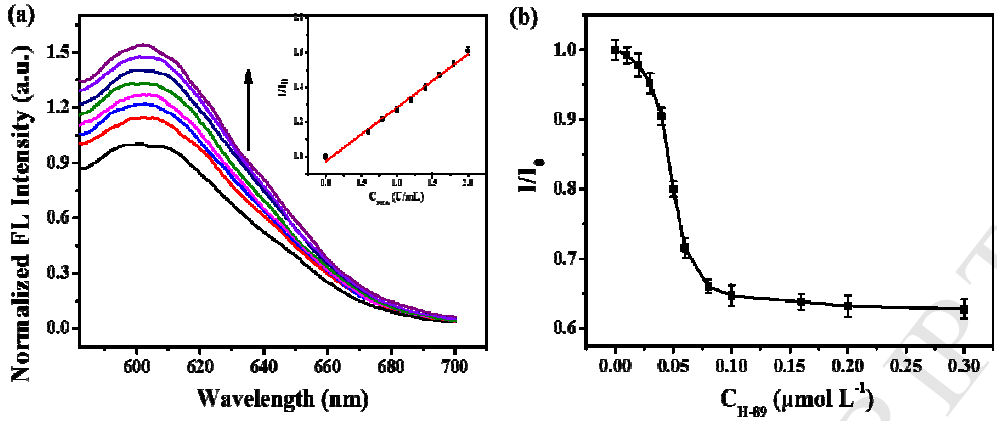


Fig. 4

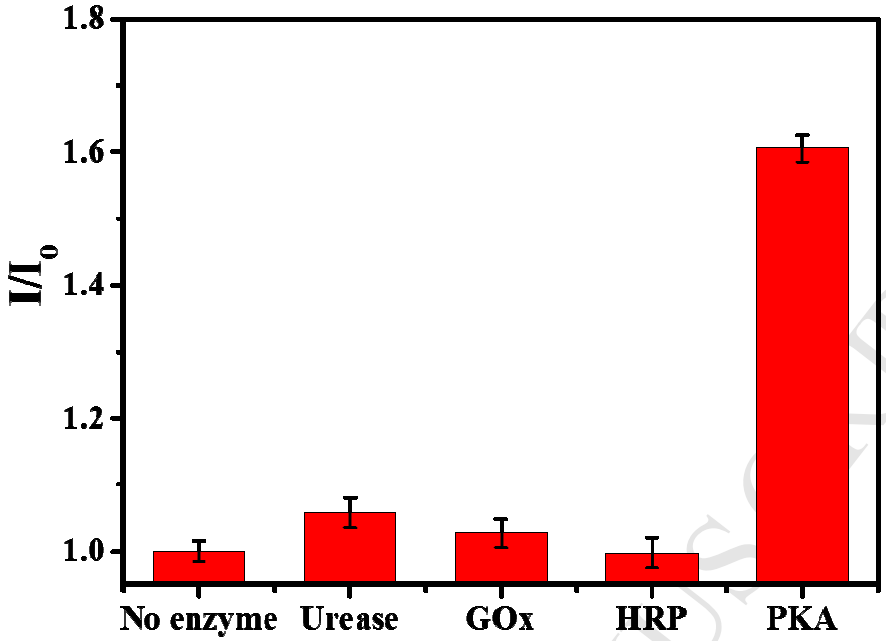


Fig. 5

1

Table 1

Sample	PKA added/U mL^{-1}	PKA found/U mL^{-1}	Recovery (%)	RSD (n=3, %)
HepG-2 cell lysates	0	-	-	-
	0.80	0.82	102.5	0.75
	1.20	1.19	99.2	1.65
	1.60	1.56	97.5	1.37

2

► A simple and sensitive fluorescent “turn-off-on” assay for label-free detection of protein kinase (PKA) activity has been developed.

► Based on substrate peptide binds to GO with higher affinity than phosphorylated peptide, the protein kinase activity could be facilely detection with high sensitivity

► The feasibility of this proposed method for kinase inhibitor screening was also studied.

► The sensing platform was also applied to monitoring PKA in cell lysates with satisfactory results