Electronic Supplementary Material (ESI) for Chemical Communications

Supporting Information

Phosphorylation-induced formation of cytochrome c-peptide complex: a novel fluorescent sensing platform for protein kinase assay

Shuang Wu, Xiang-Juan Kong, Yao Cen, Ru-Qin Yu and Xia Chu*

State Key Laboratory of Chemo/Bio-Sensing and Chemometrics, College of Chemistry and Chemical Engineering, Hunan University, Changsha 410082, P. R. China

E-mail: xiachu@hnu.edu.cn

Experimental Section

Materials and Measurements

Casein kinase II (CK2), cyclin-dependent kinase (CDK1) and cAMP-dependent protein kinase (PKA, catalytic subunit) were purchased from New England Biolabs (Beverly, MA, USA). Carboxypeptidase Y (CPY), thrombin and lysozyme was purchased from Sigma-Aldrich (St. Louis, MO, USA). Peptides, FITC-DEDADISDEEDYDLGL (peptide S), FITC-DEDADI$_p$SDEEDYDLGL (Peptide pS), FITC-DDDDPKTPKKAKKLRRRLL (peptide T) and FITC-LRRASLG (kemptide) were synthesized by GL Biochem Ltd. (Shanghai, China). ATP, cytochrome c (Cyt c) and BSA were obtained from Sangon (Shanghai, China). Hemoglobin was purchased from Worthington Biochemical Corporation (Lakewood, USA). Human recombinant G9a histone methyltransferase was purchased from Cayman Chemical (Ann Arbor, MI, USA). TBCA was purchased from Calbiochem (Merck, Darmstadt, Germany). Tris−HCl buffer (100 mM Tris−HCl, pH 8.5) was used to study the interaction between Cyt c and peptide. All solutions were prepared using ultrapure water, which was obtained through a Millipore Milli-Q water purification system (Billerica, MA, USA) and had an electric resistance >18.2 MΩ. The fluorescence spectra were measured at room temperature in a 100 μL quartz cuvette on an F-7000 spectrofluorometer (Hitachi, Japan). The excitation wavelength was 480 nm.

FL quenching assay

To assess the FL quenching of peptide S and free FITC on Cyt c, 10 μL Cyt c solution with various concentrations were mixed with 70 μL Tris−HCl buffer (100 mM, pH 8.5), then 20 μL peptide S (4 μM) and 20 μL free FITC (4 μM), respectively, were added for 5 min and the emission spectra was measured.

Hydrolyzation of peptide S by CPY

The peptide S (4 μM) was incubated with CPY (2.42 U/mL) for 20 min at 25℃. 70 μL of Tris−HCl buffer (100 mM, pH 8.5) and 5 μL of Cyt c (3 mM) was then

added in 25 μL of the reaction mixture for 5 min to measure the FL spectrum. A range of concentrations (0−4.84 U/mL) of CPY were exploited to optimize the amount of CPY. To optimize the incubation time, the same experiments were carried out with 2.42 U/mL of CPY at different incubation times (0−50 min).

Detection of CK2 activity and inhibition.

The phosphorylation reaction was performed at 30℃ for 60 min in 20 μL of assay solution containing peptide S (4 μM), CK2 (0−800 U/mL), ATP (50 μM), Tris−HCl buffer (20 mM, pH 7.5), and $MgCl_2$ (10 mM). After phosphorylation, the products were incubated with CPY (2.42 U/mL) for 20 min at 25℃. Then 70 μL of Tris−HCl buffer (100 mM, pH 8.5) and 5 μL of Cyt c (3 mM) was added in 25 μL of the reaction mixture for 5 min and FL spectrum was recorded.

For CK2 inhibitor assay, inhibitors TBCA dissolved in DMSO were diluted to be each concentration with CK2 reaction buffer. The experiments were conducted by preincubating 100 U/mL of CK2 concentration with varied concentrations of inhibitors, then initiating the phosphorylation reaction. After the CPY degradation and binding of Cyt c, the FL spectrum was measured.

Preparation of cell lysate

HeLa cells were grown in RPMI 1640 containing 10% fetal bovine serum and 1% Penn/Strep at 37 °C in a humid atmospherea 5% CO_2. Then the culture medium was replaced by 0.5 % serum RPMI 1640, and the cells were incubated for additional 20 h. The cells were washed three times with ice-cold PBS. Subsequently, a commercial kit (BSP022, Sangon Biotech, Shanghai) was used to extract proteins. Briefly, the cells were removed by scraping, counted, and then collected by centrifugation at 2500 ×g for 10 min at 4 °C. After discarding the supernatant carefully, the cells were resuspended in lysis buffer containing protease inhibitor, dithiothreitol and phenylmethanesulfonyl fluoride. The mixture was rotated for 10 min and then centrifuged at 14000 × g for 15 min at 4 °C. The resulting supernatants were collected

and stored at −80 °C before use. Concentration of cell lines is approximately 3×10^6 cells mL^{-1}. For the reactions containing cell lysates, the lysate solution was diluted 2.5 times with Tris−HCl buffer (20 mM, pH 7.5). Then 1 μL of dilution solution of cell lysates was moved out and added into 20 μL of the CK2 reaction buffer for phosphorylation reaction. The concentration of CPY and Cyt c were 3.87 U/mL and 170 μM, respectively.

Detection of CDK1 activity

A cationic substrate peptide T modified with a fluorophore was rationally designed for CDK1. The decapeptide –PKTPKKAKKL– is a recognized motif of CDK1 and threonine (T) is the phosphorylation site and the four negatively charged aspartic acids at the N-terminal were designed to couple to Cyt c after phosphorylation of threonine and sequential CPY treatment. Because of CDK1 belongs to basophilic kinase, three additional positively charged arginine residues (RRR) were designed to ensure peptide T net charge being positive. The phosphorylation reaction was performed at 30℃ for 60 min in 10 μL of assay solution containing peptide T (8 μM), CDK1 (1.25−1000 U/mL), ATP (100 μM), Tris−HCl buffer (20 mM, pH 7.5), EDTA (0.1 mM) and $MgCl_2$ (10 mM). After phosphorylation, the products were incubated with CPY (2.42 U/mL) for 30 min at 25℃. Then 70 μL of Tris−HCl buffer (100 mM, pH 8.5) and 5 μL of Cyt c (3 mM) was added in 25 μL of the reaction mixture for 5 min and FL spectrum was measured.

Fluorescence anisotropy analysis of CK2 kinase activity under different conditions

Fluorescence anisotropy (FA) analysis can provides the information about rotation of the fluorophore in its microenvironment, thus the adsorption difference of Cyt c toward free dye and peptide-conjugated dye could be monitored by FA because of the remarkable increase of molecular weight after attachment on Cyt c. Hence, FA

has been utilized to study the phosphorylation-mediated peptide-Cyt c interaction. As shown in Fig.S3, the inital FA value of peptide S was 0.0075, and it significantly increased to 0.0812 after adding Cyt c to the solution, indicating that the formation of Cyt c-peptide S complex that restricted the rotation of the dye. When peptide S was digested by CPY and then mixed with Cyt c, the FA value decreased to 0.0046, implying that the effective cleavage of the peptide by CPY released free dye insusceptible to attachment of Cyt c. In comparison, when peptide S was phosphorylated by CK2 and then treated by CPY and Cyt c, a high FA was obtained, though a little lower than that of the Cyt c-peptide S complex, presumably attributed to relatively weak electrostatic interaction between the resulting phosphopeptide fragments and Cyt c, and a 10.8-fold increase was observed compared with free dye. The results suggest that phosphorylation catalyzed by CK2 is what protected the peptide S from degradation by CPY, resulting in the attachment of peptide on Cyt c which restricted the rotational diffusion of dye.

Optimization of Experimental Conditions

In order to obtain a high effective hydrolysis performance of CPY, the concentration and digestion time of CPY were optimized for full cleavage of peptide S. As shown in Fig. S4-A, the FL signal of peptide S increased gradually with the increase of CPY concentration until it reached 2.42 U/mL, followed by a much smaller decrease at higher concentration of CPY. Hence, the optimal concentration of CPY was chosen to be 2.42 U/mL. For reaction time of CPY, with the incubation time increasing, the FL signal gradually increases and then turns to be a plateau at reaction time 20 min. The results are consistent with that of previous research. Thus, 20 min of reaction time were employed for further analytical applications in this study (Fig. S4-B).

It is known that Mg^{2+} acts as the cofactor of CK2 and ATP is indispensible for the CK2-induced phosphorylation reaction, so their effect on kinase activity was investigated. As shown in Fig. S5A, the FL intensity gradually decreased with

increasing Mg^{2+} concentration, and the minimum intensity was obtained when 10 mM Mg^{2+} was used. Therefore, 10 mM Mg^{2+} was chosen in the subsequent research. The ATP concentration was further optimized, too. It was observed that the FL signal decreased with the increasing concentrations of ATP and remain nearly unchanged after 50 μM (Fig. S5B). Therefore, 50 μM ATP was selected for further experiments.

Supplementary Figures

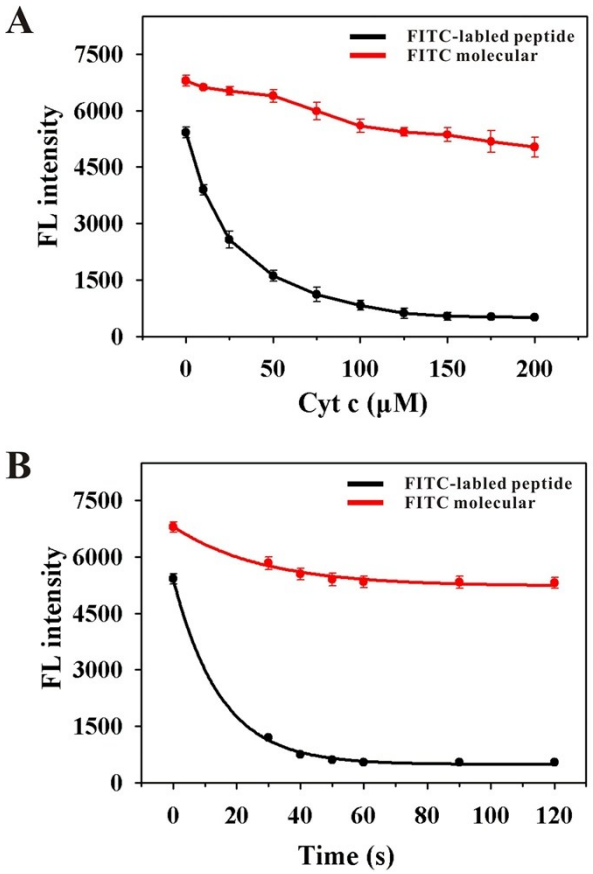

Fig. S1 (a) FL intensity changes of the 0.8 μM of FITC (red) and 0.8 μM of peptide S (black) at 517 nm as a function of Cyt c concentrations. Incubation time：10 min. (b) FL intensities changes of 0.8 μM FITC (red) and 0.8 μM of peptide S (black) at 517 nm via time in the presence of 150 μM Cyt c. Standard deviations were obtained from three experiments.

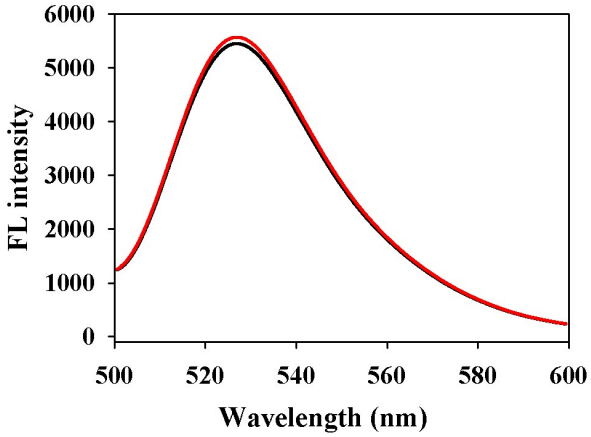

Fig. S2 FL spectra of the peptide S (black), and the peptide S mixed with haemoglobin (red). Conditions: peptide S, 0.8 μM; haemoglobin, 150 μM.

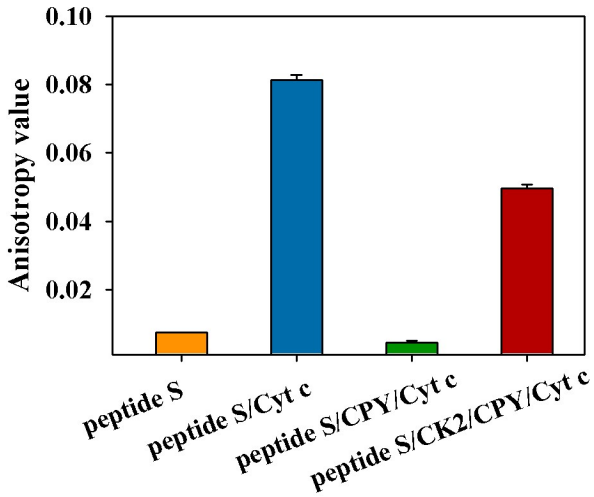

Fig. S3 FL anisotropy values at 517 nm obtained in assays of CK2 kinase activity by the Cyt c/peptide system under different conditions: peptide S (orange), peptide S mixed with Cyt c (blue), peptide S reacted with the CPY and mixed with Cyt c (green), and peptide S phosphorylated by CK2 and then treated by CPY and Cyt c (red).

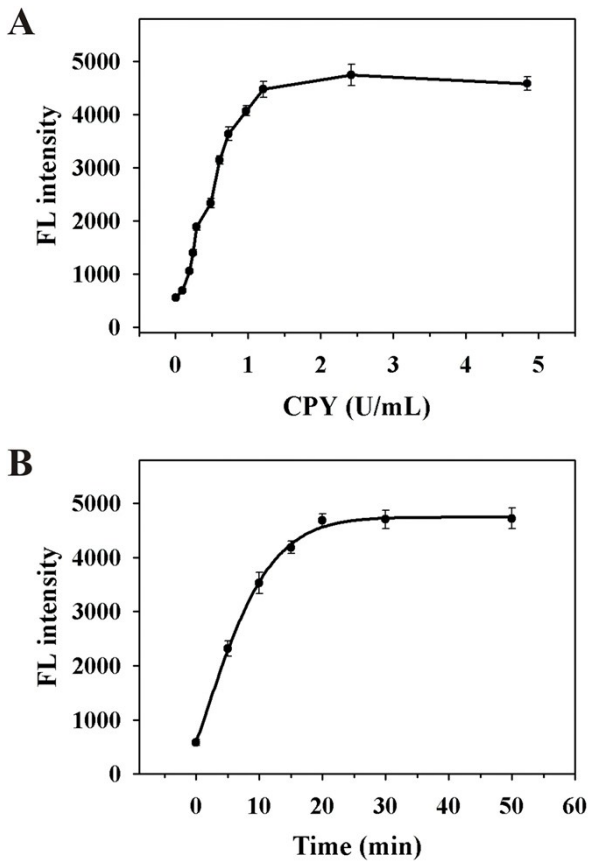

Fig. S4 (A) FL responses at 517 nm emission of peptide S digested by different concentrations of CPY for 30 min and then treated with Cyt c. (B) FL responses at 517 nm emission of peptide S digested by 2.42 U/mL CPY for different time and then mixed with Cyt c. Other conditions in the final detection solutions: peptide S, 0.8 μM; Cyt c, 150 μM. Standard deviations were obtained from three experiments.

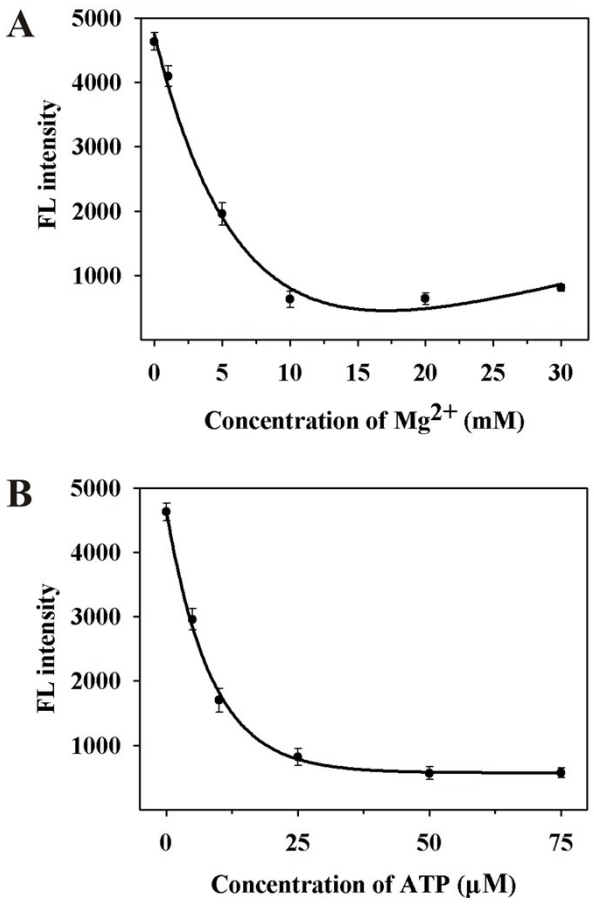

Fig. S5 (A) Optimization of Mg^{2+} concentration. The experiments was all carried out in the Tris−HCl (pH 7.5) solution containing different concentrations of Mg^{2+}, 4 μM peptide S and 50 μM ATP. (B) Optimization of ATP concentration. The experiments was all carried out in the Tris−HCl (pH 7.5) solution containing different concentrations of ATP, 4 μM peptide S and 10 mM Mg^{2+}. Standard deviations were obtained from three experiments.

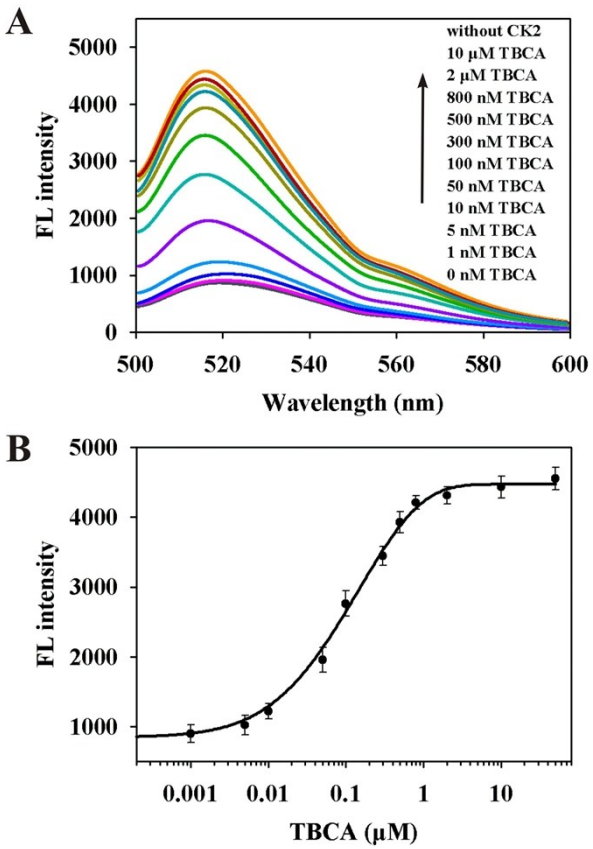

Fig. S6 (A) FL spectra of peptide/Cyt c system in the presence of 100 U/mL CK2, 50 μM ATP and TBCA with various concentrations. (B) Plot of the FL intensity at 517 nm vs TBCA concentrations.

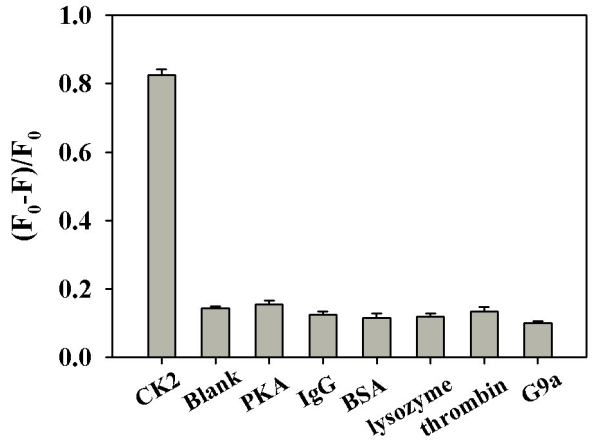

Fig. S7 Relative FL intensity profiles of the peptide/Cyt c system toward CK2 or some other proteins. The concentration of CK2 was 200 U/mL (about 1.76 nM) and the concentration of PKA was 2000 U/mL (about 10.4 nM). The concentrations of Bovine serum albumin (BSA), lysozyme, thrombin, G9a histone acetyltransferase were all 17.6 nM. Standard deviations were obtained from three experiments.

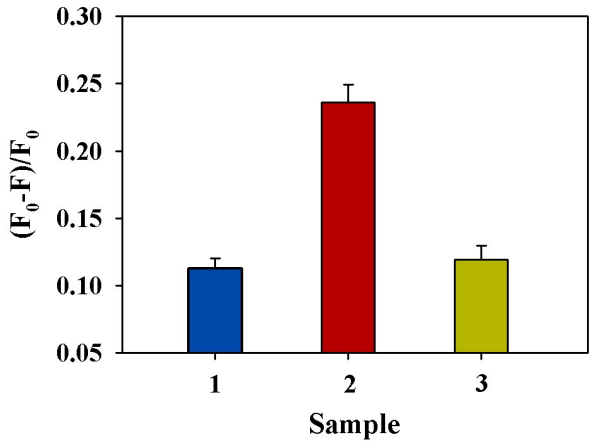

Fig. S8 FL quenching signal of peptide/Cyt c system in response to three different samples in Hela cell lysate assay; (1) blank control without cell lysates; (2) the cell lysates; (3) the sample (2) in the prescence of 10 μM inhibitor TCBA. The activity of CK2 was evaluated by the fluorescent quenching calculated by $(F_0 - F)/F_0$, F_0 and F represent the FL intensity at 517 nm before and after the addition of Cyt c, respectively. The concentration of cell lines is 6×10^4 cells mL^{-1}. Standard deviations were obtained from three experiments.

Table S1. Determination of CK2 levels in Hela cell lysates using the proposed method

Sample[α]	Measured ($U\ mL^{-1}$)	Added ($U\ mL^{-1}$)	Found ($U\ mL^{-1}$)	Recovery (%)	RSD (%, n = 3)
1	4.05				
2	4.05	2	6.35 ± 0.15	105.0	2.4
3	4.05	10	13.53 ± 0.37	96.3	2.7
4	4.05	20	24.70 ± 1.02	102.7	4.1

[α]samples 1−4 were same Hela cell lysates, the concentration of cell lines is 6×10^4 cells mL^{-1}.

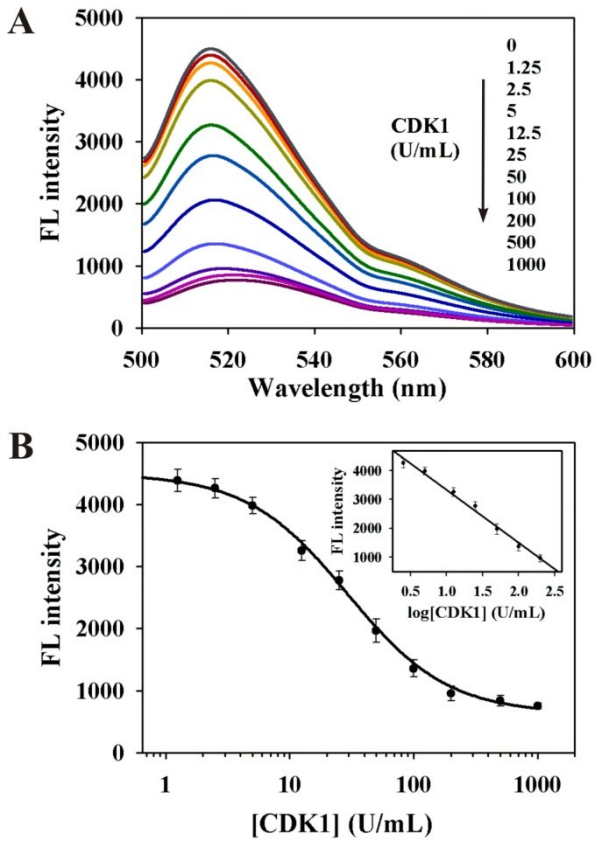

Fig. S9 (A) FL spectra of peptide/Cyt c system in the presence of CDK1 with different concentrations. (B) Calibration curve for CDK1 detection. Inset: dependence of FL intensity with respect to logarithmic CDK1 concentrations. Error bars were estimated from three independent measurements. Experimental conditions: peptide T, 8 μM; ATP, 100 μM; Mg^{2+}, 10 mM; EDTA, 0.1 mM; CPY, 2.42 U/mL; Cyt c, 150 μM.