Electronic Supplementary Material (ESI) for Chemical Communications

Supporting Information

A Versatile Platform for Highly Sensitive Detection of Kinase Activity Based on Metal Ion-Mediated FRET with an Anionic Conjugated Polymer

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List of Contents:

Supporting Information

1. Materials and reagents

Poly[(9,9-bis(3′-N,N-bis(2′′-carboxyethyl) amino)propyl) fluorenylene phenylene] (PFPaa, 1.0 mM in methanol) was a gift from Prof. Shu Wang's group (Institute of Chemistry, Chinese Academy of Sciences). The PKA-specific peptide substrate (FITC-LRRASLG) and AKT1-specific peptide substrate (TAMRA-CKRPRAASFAE) were synthesized by GL Biochem Ltd. (Shanghai, China). cAMP-dependent protein kinase (PKA), catalytic subunit was obtained from New England Biolabs and AKT1 was obtained form Sigma-Aldrich. H-89 was purchased from EMD Bioscence and zirconium oxychloride ($ZrOCl_2 \cdot 8H_2O$) was acquired from Beijing Chemical Company. ATP was purchased from Sangon Biotech Co., Ltd.

2. Standard experimental procedures for PKA and inhibition assay

At first, PFPaa (2 μM) was mixed with Zr^{4+} (80 μM) in 100 μL of reaction buffer (pH 7.4, 50 mM Tris-HCl, 150 mM NaCl) and incubated at 30 ℃ for 60 minutes with gentle shaking to form the PFPaa-Zr complex.

At the same time, in a typical 100 μL phosphorylation reaction solution, FITC-LRRASLG peptide (1.5 μM) was reacted with certain amount of PKA at 30℃for 60 minutes in a 50 mM Tris-HCl buffer (pH 7.4) containing 10 mM $MgCl_2$ and 3.0 μM ATP. Then, this reaction solution was directly mixed with an equal volume of PFPaa-Zr solution mentioned above and incubated for 30 min at room temperature.

Finally, the fluorescence spectrum of this mixed solution was recorded with an F-4500 fluorescence spectrophotometer (Hitachi, Japan) under the excitation of 370 nm.

For PKA inhibitor assay, the experiments were carried out in the same procedures as those for PKA assay stated above, except for the involvement of a fixed PKA concentration of 0.1 U/μL and varied concentrations of H-89 (0-10 μM) in the reaction mixture.

3. Fluorescence spectra of PFPaa, FITC-peptide and the mixture of PFPaa+FITC-peptide

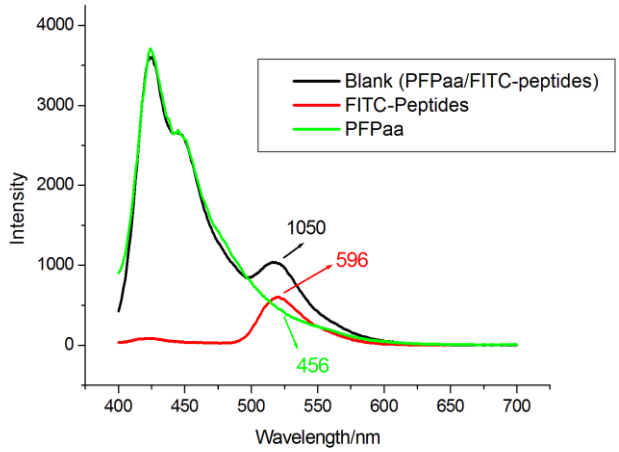

Fig. S1 Fluorescence spectra of 1.0 μM PFPaa, 750 nM FITC-peptide, and the mixture of 1.0 μM PFPaa+750 nM FITC-peptide in the absence of PKA. Excitation: 370 nm.

In this proposed sensing system, relatively high concentrations of FITC-peptides are used (750 nM in the final detection solutions) to obtain wide dynamic ranges for detection of PKA activity. Therefore, although 370 nm are not the optimal excitation wavelength of FITC, the FITC-peptides can also be excited partly under the irradiation of 370 nm and show a relative weak emission at 520 nm (red line in Fig. S1). As can be seen from Fig. S1, the fluorescence intensities at 520 nm of FITC-peptides, PFPaa and FITC-peptides+PFPaa are 596, 456 and 1050, respectively. Therefore, the weak signal at 520 nm for the blank sample in the absence of PKA (black line in Fig. S1) is not originated from FRET but from the direct excitation of FITC under the 370 nm irradiation (596+456=1052).

4. Optimization of Zr^{4+} concentration for the FRET-based PKA assay

The experiments were carried out according to the standard procedures stated in the above section. Firstly, by fixing the concentration of PFPaa at 2 μM, the PFPaa-Zr^{4+} complexes were prepared by controlling different Zr^{4+}/PFPaa ratios at 0, 2, 5, 10, 20, 25, 35, 40, 80 and 100, respectively. Then, the series of PFPaa-Zr^{4+} complexes were respectively mixed with the PKA (0.02 U/μL)-treated FITC-peptide (1.5 μM) solutions. The fluorescence signals of the mixtures containing the series of PFPaa-Zr^{4+} complexes and phosphorylated FITC-peptide were measured with F-4500 fluorescence spectrophotometer. The FITC-peptide without the treatment with PKA was used as the blank control.

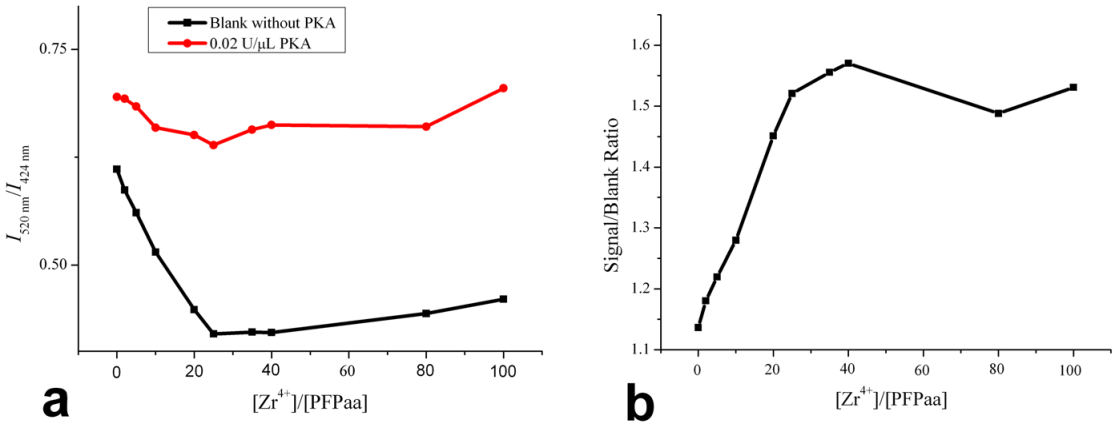

Fig. S2 (a) Effect of Zr^{4+} concentration on the FRET ratios ($I_{520\ nm}/I_{424\ nm}$) in the PFPaa-based FRET system. Black line: blank without PKA; red line: 0.02 U/μL PKA. (b) Plot for the dependence of the ratios of the signals ($I_{520\ nm}/I_{424\ nm}$) produced by 0.02 U/μL PKA to the blank at different Zr^{4+} concentrations. Other experimental conditions in the final detection solutions: PFPaa, 1.0 μM; FITC-peptide, 750 nM; ATP, 1.5 μM.

The effect of Zr^{4+} concentration on the FRET ratios ($I_{520\ nm}/I_{424\ nm}$) in the PFPaa-based FRET system was shown in Fig. S2a. As can be seen from Fig. S2a that at lower concentrations of Zr^{4+} ions, the $I_{520\ nm}/I_{424\ nm}$ ratios of the blank were higher, which can be sharply decreased when the ratios of [Zr^{4+}]/[PFPaa] increased from 0 to 25 and kept a relatively low value with further increasing Zr^{4+} concentration. Meanwhile, the $I_{520\ nm}/I_{424\ nm}$ ratios of the reaction systems treated with PKA showed a much slower decrease when [Zr^{4+}]/[PFPaa] ratios changed from 0 to 25. When further increasing the [Zr^{4+}]/[PFPaa] ratios from 25 to 40, the $I_{520\ nm}/I_{424\ nm}$ ratios displayed a slow increasing tendency and would keep a relatively stable values when [Zr^{4+}]/[PFPaa] ratios was exceeded 40. As shown in Fig. S2b, the highest ratio of the signals ($I_{520\ nm}/I_{424\ nm}$) produced by 0.02 U/μL PKA to the blank (S/B) was obtained at the [Zr^{4+}]/[PFPaa] ratio of 40, which was selected as the optimal condition for further analytical applications in this study.

These phenomena shown in Fig. S2 can be explained as follows. In the FRET system, PFPaa is an anionic conjugated polymer with negative charges and the PKA-specific substrate peptide (LRRASLG) (pI ~12.00) has positive charges under the experimental conditions (pH 7.4). Therefore, at low Zr^{4+} concentrations for the blank solutions without PKA, the electrostatic interactions are strong between the PFPaa and the non-phosphopeptides, which will result in non-specific FRET signals. With increasing ratios of [Zr^{4+}]/[PFPaa], the PFPaa-Zr^{4+} complexes becoming positively charged and the nonspecific electrostatic interaction between the peptides and the PFPaa will be greatly weakened, resulting in the sharply decreased $I_{520\ nm}/I_{424\ nm}$ ratios. At higher [Zr^{4+}]/[PFPaa] ratios, the electrostatic interaction would be mostly suppressed and thus, the $I_{520\ nm}/I_{424\ nm}$ ratios would keep relatively stable.

On the other hand, for the reaction systems treated with PKA, the Zr^{4+}-mediated specific phosphopeptides recognition coexisted with the nonspecific electrostatic interaction under low Zr^{4+}concentration. Therefore, a much slower decrease tendency of the $I_{520\ nm}/I_{424\ nm}$ ratios was observed in the presence of PKA when initially increasing the [Zr^{4+}]/[PFPaa] ratios to 25. When further elevating the [Zr^{4+}]/[PFPaa] ratios, the electrostatic interaction effect will be completely eliminated and the Zr^{4+}-mediated specific phosphopeptides recognition became the dominant and therefore, the $I_{520\ nm}/I_{424\ nm}$ ratios tended to be stable. Therefore, in the PFPaa-based system, Zr^{4+} ions not only acts as a "bridge" to bring the PFPaa (donor) and fluorescein-labeled phosphopeptides (acceptor) in close proximity to produce efficient FRET, but also efficiently eliminate the nonspecific electrostatic interaction between PFPaa and unphosphorylated peptides, which can greatly improve the selectivity of the PFPaa-based assay for detection of protein kinase activity.

5. Effect of PFPaa concentration on the PKA assay

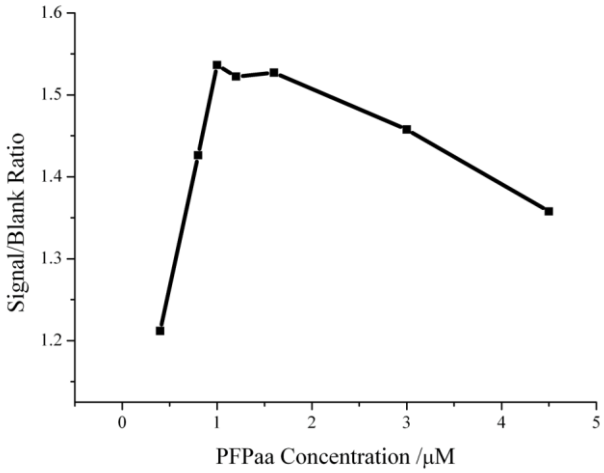

Fig. S3 Effects of PFPaa concentration on the performance of the PKA assay. The signal/blank (S/B) ratios refer to the ratios of the $I_{520\ nm}/I_{424\ nm}$ values of 0.02 U/μL PKA-treated reaction systems to those of the blank solutions without addition of PKA. Other experimental conditions in the final detection solutions: FITC-peptide, 750 nM; ATP, 1.5 μM; Zr^{4+}/PFPaa ratios were maintained at 40.

The effect of PFFaa concentrations on the proposed PKA assay was also investigated and the results are shown in Fig. S3. One can see that the maximum S/B value was obtained when 1 μM PFPaa (in the final 200 μL detection solutions) was used. Therefore, 1 μM of PFPaa was chosen as the optimal concentration for subsequent work.

6. Effect of ATP concentration on the PKA assay

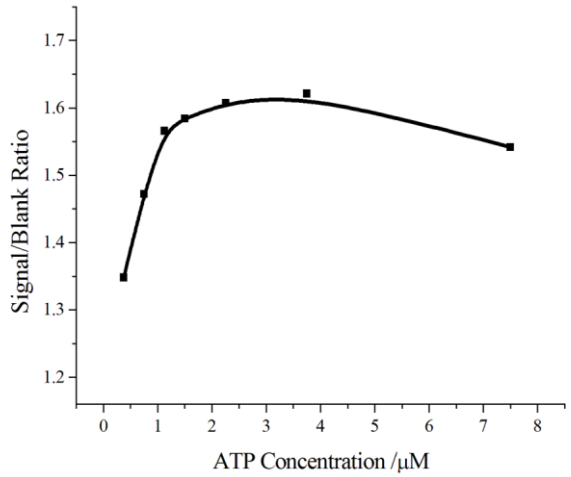

Fig. S4 Effects of ATP concentration on signal/blank (S/B) ratios which is the same as mentioned above in Fig. S3. Other experimental conditions in the final detection solutions: PFPaa, 1 μM; Zr^{4+}, 40μM; FITC-peptide, 750 nM.

ATP was indispensible for the PKA-induced phosphorylation reaction, so ATP concentration was optimized by changing the [peptide]/[ATP] ratios with the peptide concentration fixed at 750 nM. As can be seen from Fig. S4, the S/B ratio reached its maximum value and almost stable when the [peptide]/[ATP] ratio was in the range of 1:2 to 1:5. Finally, [peptide]/[ATP] ratio of 1:2 was selected as the optimum, which was also the recommended ATP dosage in the product manual of PKA.

7. Optimization of FITC-peptide concentration

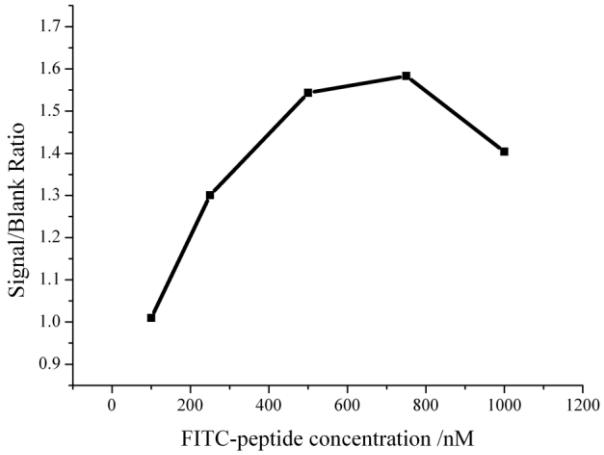

Fig. S5 Effects of FITC-peptide concentration on the signal/blank (S/B) ratios which is the same as mentioned above in Fig. S3. Experimental conditions in the final detection solutions: PFPaa, 1 μM; Zr^{4+}, 40μM; the ATP/peptide ratios were kept at 2.

Fig. S5 shows the effect of FITC-peptide concentrations on the S/B ratios. It can be seen that the S/B value reached to the maximum at FITC-peptide concentration of 750 nM. Therefore, 750 nM of FITC-peptide was selected for subsequent work.

8. Application for H-89-based inhibition assay

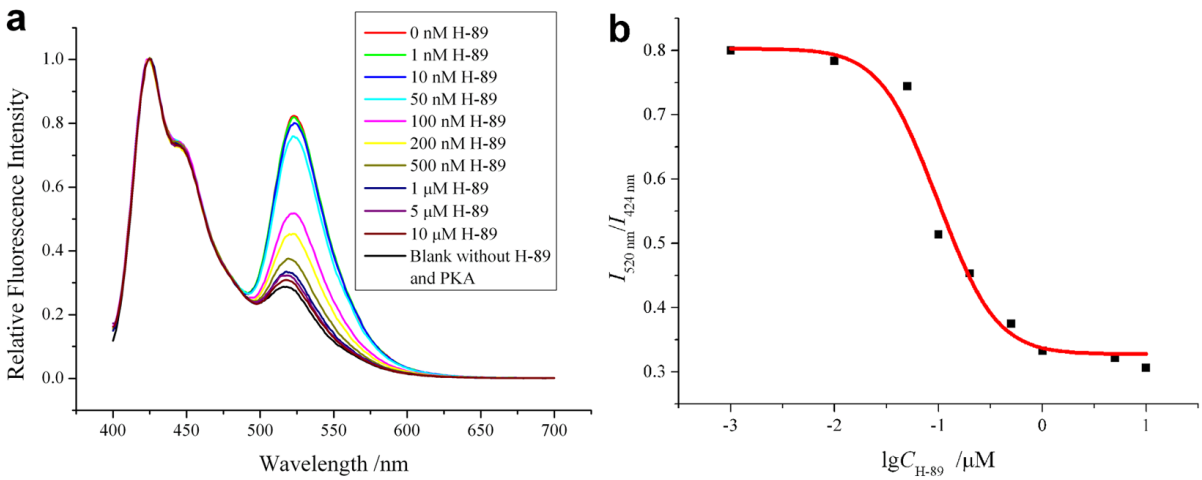

Fig. S6 (a) Fluorescence spectra of the PFPaa-based FRET assay in the presence of different concentrations of inhibitor H-89. PKA: 0.1 U/μL. (b) Plot of fluorescence intensity ratios of $I_{520\ nm}/I_{424\ nm}$ with H-89 concentrations. Other experimental conditions are the same as those in Fig. 3.

9. Evaluation of the universal applicability of the proposed assay

To test whether the PFPaa-based assay is universally applicable, the activity of another randomly chosen kinase AKT1 is also evaluated by the PFPaa-based assay by using a TAMRA-labeled substrate peptide (TAMRA-CKRPRAASFAE with a maximum emission at 580 nm). As can be seen from Fig. S7, the FRET ratio (I_{580nm}/I_{424nm}) increase gradually with increasing concentration of AKT1, indicating that the PFPaa-based method can be easily applicable to detection of other protein kinase activities and is suitable to different fluorescence labels on the peptide substrates.

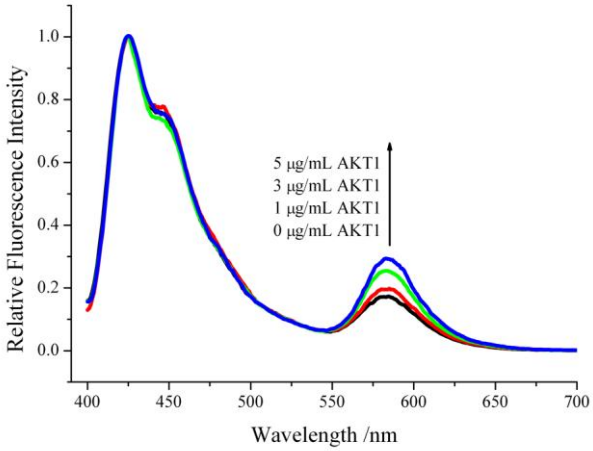

Fig. S7 Fluorescence spectra of the PFPaa-based FRET assay for the detection of AKT1 activity by using TAMRA-labeled substrate peptide. The AKT1 concentrations from the bottom to the top according to the peak at 580 nm: 0 (control), 1, 3, 5 μg/mL. The excitation wavelength is 370 nm and the intensities at 424 nm are all normalized to 1. Other experimental conditions in the final detection solutions: PFPaa, 1.0 μM; TAMRA-peptide, 750 nM; ATP, 1.5 μM, Zr^{4+}, 40 μM.