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Phosphorylation-mediated Assembly of Semisynthetic Fluorescent Protein for Label-free Detection of Protein Kinase Activity

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Abstract

Protein phosphorylation catalyzed by protein kinases plays a critical role in many intracellular processes, and detecting kinase activity is important in biochemical research and drug discovery. Herein, we developed a novel fluorescent biosensor to detect protein kinase activity based on phosphorylation-mediated assembly of semisynthetic green fluorescent protein (GFP). A chimaera peptide, S-peptide, composed of the 10^{th} β-strand of GFP (s10) and a kinase substrate peptide, was synthesized. Kinase-catalyzed phosphorylation of S-peptide can protect its s10 part against cleavage by carboxypeptidase Y (CPY). Then the peptide can bind with the truncated GFP (tGFP, GFP without s10) to assemble intact GFP and recover fluorescence. The unphosphorylated S-peptide would be degraded by CPY, and fluorescent protein assembly cannot happen. Thus, the kinase-catalyzed phosphorylation can switch on the fluorescence signal. This platform has been successfully applied to detect the activity of cAMP-dependent protein kinase (PKA) with a low detection limit of 0.50 mU/μL, and its inhibition of H-89 with IC_{50} value of 23.4 nM. The feasibility of this method has been further demonstrated by assessment of the kinase activity and inhibition in cell lysate. Moreover, based on the reverse principle, this method was expanded to detect the activity of protein phosphatase 1 (PP1). Our method, using semisynthetic GFP as a readout, is facile, sensitive, label-free, and highly versatile, which shows great potential to be a promising platform for protein kinases detection and inhibitor screening.

Introduction

Protein phosphorylation is an important post-translational modification (PTM) mechanism, which plays a critical role in signal transduction, metabolism, gene expression, cell-cycle progression, and immune response.[1,2] Protein kinases and phosphatases, which catalyze protein phosphorylation and dephosphorylation, respecitively, work cooperatively to control the function and activity of proteins, serving as a ubiquitous regulatory mechanism in cellular signal pathways. Aberrant regulation of protein kinase is closely associated with various human diseases such as cancer, immune deficiencies, neurodegenerative diseases, and endocrinological disorder.[3-5] Over the last two decades protein kinases have become the most important class of drug target in anti-cancer drug development.[5] Therefore, developing methods for probing protein kinase activity is of great significance to fundamental biological research, pharmaceutical development, and medical diagnostics.

Protein kinase catalyzes the transfer of the γ-phosphate from adenosine-5'-triphosphate (ATP) to conserved tyrosine, threonine or serine residues in a peptide or protein substrate. Traditional radiometric protein kinase assays[6, 7] using radiolabeled ATP suffer from their unhealthy radioactive waste, and have been largely replaced by non-radiometric assays.[8] In comparison with some heterogeneous kinase assays based on electrochemistry,[9,10] quartz crystal microbalance,[11] surface plasmon resonance,[12] or mass spectrometry,[13] homogenous fluorescence kinase assays[14-16] have some inherent advantages, including high sensitivity, simplicity, high-throughput capability, and the avoidance of tedious surface immobilization and rinsing. For

efficient signal generation, the synthetic substrate peptides labeled with fluorogenic organic molecules or nanomaterials were generally exploited as fluorescent probes in the reported fluorescent kinase assays.[17,18] A number of kinase biosensors have been fabricated by the rationally designed fluorophore-tagged peptide probes solo or their complexes with conjugated polymers, gold nanoparticles, or graphene oxide. Additionally, both Willner's[19] and Stevens's[20] groups prepared quantum dots(QDs)-based kinase sensors, where peptide-modified QDs served as surrogate substrates and fluorescence resonance energy transfer (FRET) donors. Such assays are effective but require sophisticated and costly peptide labeling or the labor-intensive modification of nanomaterials. Hence, it is still a challenge but highly desirable to develop new mechanisms for convenient and label-free detection of protein kinase.

Green fluorescent protein (GFP) coming from Aequorea is a 238-aa fluorescent protein, composed of a β-barrel structure with 11 β-sheets outside and 1 α-helix strand inside. It has been widely recognized as a powerful molecular tool in modern life science because of its intrinsic fluorescence and genetically encoded property.[21] GFP is fluorescently quenched when its barrel structure is cleaved and separated into two parts. If the cleavage site is in a loop between the secondary structural elements, the two parts of the split GFP would noncovalently assemble to a stable, functional, and fluorescent holoprotein via spontaneous self-association or, in some cases, triggered by a protein interaction modular pairs.[22,23] The controllable split GFP reassembly has emerged as a potent approach for analysis of protein-protein interactions, detection of specific nucleic acid sequences or modifications, and even

probing the host-guest supermolecular recognition.[24-26] Currently, Boxer group reported a novel semisynthetic GFP system generated by assembly of a synthetic peptide and a truncated protein (tGFP) that is produced recombinantly.[27] tGFP, derivative from the circularly permuted superfolder GFP but the 10th β-strand (s10) is removed, contains the full chromophore ready for quick fluorescence restoration after addition of the synthetic s10 peptide. This semisynthetic system presents several unique advantages over traditional split GFP systems, such as fast complementary and fluorescence development, high affinity between two parts at picomolar level, intriguing light-activating property, and the great versatility of synthetic peptides with desired sequences containing natural or unnatural amino acids.[27] Furthermore, the low background and fluorescence switch-on of this semisynthetic GFP shows great potential for developing new biosensing mechanisms. However, to our knowledge, the attempts of exploiting this promising split GFP system in bio-sensing are scarce, and, accordingly, much efforts are needed.

Herein, we proposed a novel semisynthetic fluorescent protein assembly platform for label-free detection of the activity of protein kinase and phosphatase. The rationally designed synthetic peptide (S-peptide) integrating the s10 strand and the kinase substrate sequence was utilized as the peptide probe, and the truncated GFP with pre-matured chromophore served as the signal reporter ready to light up. The recognition of kinase-catalyzed phosphorylation relied on the phosphorylation protection of S-peptide against the digestion of carboxypeptidase Y (CPY), a specific protease that hydrolyzes a peptide bond from the carboxy-terminal (C-terminal) end

of a protein or peptide.[28,29] Upon phosphorylation, the phosphorylated S-peptide can prohibit CPY digestion and keep the s10 peptide segment intact, and then the peptide can assemble with tGFP to form intact GFP and recover fluorescence. Because of phosphorylation-mediated assembly of semisynthetic GFP and fluorescence development, we could realize a facile turn-on quantitative analysis for protein kinase without requirement of peptide/protein labeling or modification. It has been demonstrated by using cAMP-dependent protein kinase (PKA) as a model. Such assay was also feasible for the detection of PKA inhibition and the PKA activity in cell lysate. Moreover, based on the reverse principle, this method was expanded to probe the activity of protein phosphatase 1 (PP1), an enzyme participating in protein dephosphorylation process.[30,31] Since the synthetic peptide module can be easily replaced by the substrate of other protein kinases, this method has the potential to be a versatile platform for protein kinases assay and inhibitor screening.

Experimental Section

Materials and Measurements

Cyclic adenosine 3', 5'-monophosphate-dependent protein kinase (catalytic subunit of PKA) and protein phosphatase 1 (PP1) were purchased from New England Biolabs (Beverly, MA, USA). Generally, PKA was diluted using the storing solutions (50 mM NaCl, 1 mM EDTA, 2 mM DTT, 50 % glycerol) in 20 mM Tris-HCl buffer (pH 7.5, 25 °C) and stored in the refrigerator at -70 °C. s10 peptide (LPDN**HYLSYQTVLS**KDPNE, the 10th β-sheet sequence of GFP (in bold) with the flank loop sequences around it), S-peptide (LPDN**HYLSYQTVLS**KDPNE*LRRASLG*,

and the sequence in italics is the PKA substrate peptide), S'-peptide (**HYLSYQTVLS***LRRASLG*) and the phosphorylated peptide (P-peptide, LPDN**HYLSYQTVLS**KDPNE*LRRAS{p}LG*) were synthesized by GL Biochem (Shanghai, China). ATP was bought from Generay (Shanghai, China). H-89 was obtained from EMD Bioscience (Calbiochem-Novabiochem. La Jolla, CA, USA). Carboxypeptidase Y (CPY) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Human breast cancer cells (MCF-7) were obtained from the Cell Bank of Xiangya Central Experiment Laboratory of Central South University (Changsha, China). PP1 was diluted using the storing solutions (200 mM NaCl, 50 mM HEPES, 0.1 mM $MnCl_2$, 0.1 mM EGTA, 2 mM DTT, 0.025 % Tween 20, 50 % glycerol pH 7.5, 25 °C) and stored in the refrigerator at -70 °C. All solutions were prepared using ultrapure water (18.25 MΩ.cm) from the Millipore Mill-Q system.

The UV-vis absorption spectra were recorded on a UV-vis spectrophotometer (Beckman DU-800) in a wavelength range from 350 nm to 550 nm. Fluorescence measurements were performed on Photon Technology International (PTI) QM4 fluorescence spectrophotometer. The CD spectra were determined using a Bio-Logic MOS-500 Spectrometer and recorded at 25 °C from 200 nm to 280 nm. Cell-breaking was performed using a JY92-IIN ultrasonic cell disruption system (Scientz, Ningbo, China). All samples were excited at wavelength of 488 nm, and the fluorescence emission was scanned from 500 to 600 nm at 25 °C. The fluorescence measurements were performed three times for each sample (n=3). The results showed the average of measurements with error bars indicating the relative standard deviation.

Protein Expression and tGFP Purification

A circularly permuted GFP construct pET-15b (s10:loop:GFP) was kindly given by professor Steven Boxer in Stanford University. It was transformed and expressed in *Escherichia coli* strain BL21. Cells grew in LB medium containing 30 μg/mL ampicillin at 37 °C to an OD_{600} of 0.6. After addition of 0.25 g/L IPTG, cells were further grown for 20 hours at 17 °C, collected by centrifugation, and then washed for 3 times by the lysis buffer (50 mM HEPES, 300 mM NaCl, and 10% glycerol at pH 8.0). The resultant cell suspension was lysed by JY92-IIN ultrasonic cell disruption system. After centrifugation at 8000 rpm for 30 min, the supernatant was loaded onto a Ni-NTA column (ÄKTA, GE) for purification. The manipulation for protein purification followed that described in the manual. The target protein was finally obtained after rinsing with elution buffer (10 mM Tris-HCl, 2 M NaCl, and 500 mM imidazole, at pH 7.4). The buffer of the final sample was exchanged back into lysis buffer using a desalting column (ÄKTA, GE). The purified GFP was reloaded onto a Ni-NTA column equilibrated with cleavage buffer (50 mM Tris and 20 mM $CaCl_2$ at pH 8.0). Trypsin was dissolved in 1M HCl to make 10 U/μL trypsin solution, and used for on-line digestion for 60 min at 37 °C with 100 U trypsin per 1 mg protein. The column was washed with diluted binding buffer (50 mM HEPES, 300 mM NaCl, 20 mM imidazole and 10 % glycerol at pH 8.0) to remove trypsin, and then washed with 8 M urea to get the denatured protein. The eluted products were applied to a size exclusion column (Superdex TM 75 10/300, ÄKTA, GE) equilibrated with lysis buffer to separate tGFP from s10 peptide. At the same time the separated

tGFP was refolded in lysis buffer.

Assembly of tGFP and S-peptide

For assembly, tGFP and S-peptide (s-10) were mixed with different ratio in lysis buffer, and then incubated for different time at room temperature, under natural light or 405 nm light. The fluorescence intensity, absorbance and CD spectra of the assembled GFP were recorded.

Treatment of S-peptide and P-peptide by CPY

S-peptide (1 μM) and P-peptide (1 μM) was treated with CPY (2 U/mL) for 90 min. The resulting solution (50 μL) was mixed with an equal volume of tGFP solution (0.5 μM) and then measured by fluorescence spectrophotometry. A range of concentration (0-4 U/mL) of CPY was exploited to optimize the quantity of CPY. For optimization experiment of incubating time, the same experiments were conducted with 2 U/mL CPY for different incubating time (0-120 min).

Detection of Activity and Inhibition of PKA

For PKA-catalyzed phosphorylation, 25 μL of the PKA reaction solution composed of PKA (0-1 U/μL), S-peptide (2 μM)，$MgCl_2$ (2 mM), ATP (0.2 mM) in 50 mM Tris-HCl buffer (pH 7.5, 25 ℃) were incubated at 30 ℃ for 60 min, and the resulting solution was incubated with 2 U/mL CPY at 25 ℃ for 90 min. Then 50 μL of the mixture was mixed with tGFP (50 μL, 0.5 μM), incubated under 405 nm light for 30 min, and measured by fluorescence spectrometry.

For PKA inhibition assay, 250 mU/μL PKA and H-89 at different concentrations (0-2.5 μM) were added into the phosphorylation reaction solution. The

following procedures were carried out under the above-mentioned conditions.

Detection of PKA Activity in MCF-7 Cell Lysate

The MCF-7 breast cancer cells ($1x10^6$ cells) were supplemented with ten percents fetal bovine serum, 0.1 mM Mimimum Eagle's essential medium (MEM) nonessential amino acid solution, 1 % insulin-transferrin-selenium A supplement, 100 U/mL of penicillin, 100 mg/mL of streptomycin, and 0.25 mg/mL amphotericin B. The MCF-7 cells were incubated under a humidified atmosphere with 5% CO_2 at 37 °C. After 4 h incubation in the serum-free medium, the forskolin/IBMX mixture diluted with DMSO was added into the medium at various concentrations to stimulate intracellular PKA activity. Equal volume of DMSO was added into the medium as an unstimulated control. After thirty minutes of stimulation, the cultured cells were scraped and lysed in Dulbecco's phosphate-buffered saline, followed by sonication (200 W) for 2 s 60 times at a 3 s interval. The samples were clarified by centrifugation for 60 min at 22000 rpm, 4 °C, and the extracted supernatants were stored in the freezing tubes at -20 °C and used in the following experiments. The total protein concentration of the cell lysate was determined by the Bradford method with BSA as the standard, and in the following experiments the cell lysate samples were diluted to 12.5 μg/mL.

Detection of PP1 Activity

For PP1-catalyzed dephosphorylation, 25 μL of the PP1 reaction solution composed of PP1 (0-5 U/μL), P-peptide (2 μM) in PP1 reaction buffer (50 mM HEPES, 100 mM NaCl, 0.1 mM EGTA, 2 mM DTT, 0.025 % Tween, 1 mM $MnCl_2$,

pH 7.5, 25 °C) was incubated at 30 °C for 60 min，and then incubated with 2 U/mL CPY at 25 °C for 90 min. Then 50 μL of the mixture was mixed with tGFP (50 μL, 0.5 μM), incubated under 405 nm light for 30 min, and measured by fluorescence spectrometry.

Results and Discussion

Assembly of Semisynthetic GFP by the Association of tGFP with S-peptide

Our kinase detection platform relied on the association of the truncated GFP (tGFP) with the synthetic peptide probe (S-peptide) to form whole semisynthetic GFP. tGFP was derived from a recombinantly expressed full-length GFP with modified primary sequence containing a loop insertion with a trypsin cleavage site. tGFP was prepared by the trypsin cleavage, urea denaturation of the full-length GFP, and size exclusion chromatography separation to isolate the larger segment (tGFP) and remove the terminal peptide (s10).[32] S-peptide, which consists of the s10 peptide and the substrate peptide of PKA at its carboxyl terminal, was designed and synthesized as a surrogate PKA substrate. In order to discriminate the semisynthetic GFP from the original GFP on spectroscopic characteristics, a T203Y mutation (Thr to Tyr at the 203th residue of the original GFP) that would lead to a red-shift of fluorescent emission was introduced in S-peptide, as that in yellow fluorescent proteins (YFPs).[33] To confirm whether this S-peptide can assemble with tGFP, several experiments were carried out. Figure 1A presents the fluorescence emission spectra of tGFP in the absence or presence of S-peptide. The solo tGFP shows weak fluorescence with a maximum emission at 500 nm, similar to that of the original GFP, because the

conformational flexibility after removing s10 leads to non-radiative decay of the chromophore. However, the addition of S-peptide induced a significantly enhanced fluorescence of tGFP with an 8-fold increase as well as a red-shift in emission from 500 nm to 515 nm, which came from the mutation of T203Y in S-peptide. It is worth to note that the red-shift caused by T203Y mutation decreases the fluorescence background (at 515 nm) and remarkably increases the signal-to-background ratio (S/B). Correspondingly, as shown in the inset of Figure 1, the fluorescence recovery of the tGFP/S-peptide complex is observed by naked eye under an ultraviolet lamp (with the excitation wavelength of 365 nm). Moreover, the assembled semisynthetic GFP has green color, which can also be distinguished from the original GFP by naked eye. The assembly of tGFP and S-peptide was further proved by comparison of the UV-vis absorption spectrum of tGFP/S-peptide complex with those of tGFP and the original GFP, respectively (Figure 1B). The deprotonated absorbance band of the original GFP at 460 nm is significantly decreased in tGFP. Upon incubation with S-peptide, the disappeared deprotonated absorbance peak of tGFP was recovered and red shifted to 510 nm as the result of T203Y substitution. The circular dichroism (CD) spectrum was employed to characterize the structure change during the tGFP/S-peptide complex assembly. The CD spectrum of tGFP was indistinguishable from those of the intact GFP and the tGFP/S-peptide complex with the peak at 220 nm (Figure S-1), which is the typical peak of β-sheet structure. These coincident spectra indicated that removing of s10 did not perturb the secondary structure of tGFP, which is one of the foundations of fast reassembly of tGFP/s10 pair.[34] Furthermore, all the

fluorescence, absorbance and CD spectra of the tGFP/S-peptide complex cannot be distinguished from that of the tGFP/s10 complex (Figure S-1, 2). Therefore, all these results demonstrated that the addition of the substrate peptide at the carboxyl terminal of s10 did not affect its function of assembly with tGFP, and the resulting functional peptide, S-peptide, with tGFP can be used to design a semisynthetic GFP assembly platform for PKA activity assay.

The Principle of Protein Kinase Activity Detection

The applications of semisynthetic fluorescent protein assembly in enzyme activity bioanalysis, especially protein kinase detection, are seldom reported. By the assistance of CPY and based on the above-mentioned successful assembly of tGFP and S-peptide, we proposed a novel semisynthetic fluorescent protein assembly platform for label-free detection of protein kinase activity. Scheme 1 depicts its detailed detection mechanism. It has been demonstrated that the phosphorylation of peptides greatly suppresses their proteolytic degradation by endoprotease or exopeptidases. Herein, carboxypeptidase Y (CPY), as a representative exopeptidase, was used to discriminate the phosphorylated peptide from the un-phosphorylated ones.[35, 36] Without PKA treatment or with coexisting of PKA inhibitor, the unphosphorlated S-peptide would be digested by CPY, leading to its degradation. The resulting fragments of s10 would be unable to assemble with tGFP, and the fluorescence signal would keep quenching. After PKA treatment, the phosphorylated S-peptide (P-peptide) can resist to CPY digestion and retain the intact s10. Thus P-peptide would bind to tGFP to assembly semisynthetic GFP and induce the fluorescence recovery. Therefore,

a turn on detection for PKA activity was developed based on phosphorylation-mediated assembly of semisynthetic GFP.

The preliminary experiments were performed to demonstrate the feasibility of the assay. First of all, the different digesting effects of CPY on S-peptide and P-peptide were investigated. As illustrated in Figure 2 line 3, after S-peptide was treated by CPY and mixed with tGFP, there is only weak fluorescence detected (18 % of that without CPY digestion, Figure 2 line 2), indicating CPY can effectively digest the unphosphorylated S-peptide, and the digestive product cannot assemble with tGFP. However, when the synthetic P-peptide control is treated by CPY and then incubated with tGFP, the fluorescence intensity is similar to that of tGFP/S-peptide complex (Figure 2 line 4), indicating the successful reconstitution of GFP. Hence, CPY digestion can effectively distinguish S-peptide from P-peptide. Then, PKA catalysis was involved. It was found that after S-peptide being treated with PKA for 1 h and digested by CPY, the resulting peptide can bind to tGFP and dramatically recover the fluorescence, which is comparable to that of P-peptide (Figure 2 line 5). However, the fluorescence failed to recover when phosphorylation was carried out by PKA without ATP (the co-substrate of PKA, Figure 2 line 6) or coexistence with 2.5 μM H-89 (Figure 2 line 7), a PKA inhibitor, which further confirmed that it was PKA-catalyzed phosphorylation that protected S-peptide from digestion of CPY, resulting in the assembly of GFP. Moreover, in the presence of a control protein kinase, casein kinase II (CKII), there was no obvious fluorescence signal detected (Figure 2 line 8). These results suggest that the integration of CPY digestion with semisynthetic fluorescent

protein assembly was an effective way to probe PKA-catalyzed phosphorylation.

Detection of the Activity and Inhibition of PKA

To improve the assay efficiency, the assembly of tGFP and S-peptide, as a signal readout, was optimized. The relative fluorescence intensity, defined as $(F-F_0)/F_0$, was used to evaluate the fluorescence recovery efficiency, where F referred to the fluorescence intensity of the assembled semisynthetic GFP, and F_0 referred to that of tGFP only. Firstly, two different sequences of substrate peptides, S-peptide and S'-peptide, were tested and compared. It was found that addition of the flanking loop sequences of the 10^{th} β-sheet (LPDN……KDPNE) made S-peptide (LPDN**HYLSYQTVLS**KDPNE*LRRASLG*) had a higher assembly efficiency than that of S'-peptide (**HYLSYQTVLS***LRRASLG*), which only contains the sequence of the 10^{th} β-sheet of GFP and the substrate peptide sequence of PKA (Figure S-3A). Moreover, the kinase assay based on S-peptide/tGFP showed lower detection limit than that based on S'-peptide/tGFP (Figure S-3B, for detailed description, please see Supplementary Information). Hence, S-peptide rather than S'-peptide was chosen as the substrate peptide for this semi-synthesized GFP sensor. Secondly, the binding ratio of S-peptide to tGFP was measured. As shown in Figure S-4A, the fluorescence intensity increases with the increase of the ratio of S-peptide to tGFP. The relative fluorescence intensity is nearly linear to the ratio, and reaches a relative platform when S-peptide:tGFP was 1:1 (Figure S-4B). The binding stoichiometry of tGFP/S-peptide was further established by Jobs method of continuous variations[38] (Figure S-5), which also indicated that the preferred binding stoichiometry of

tGFP/S-peptide assembly is 1:1. Thus, a dissociation constant (K_d) of 142.9±52.4 pM was calculated (described in SI), which is close to that in the literature.[27] Such low K_d value indicated the high affinity between tGFP and S-peptide and, consequently, their highly efficient assembly. Thirdly, it was reported that the assembly of split GFP (tGFP/s11) can be light-activated, which is wavelength-dependent and light intensity-independent.[39] In our assay, 2 kinds of wavelength lights, natural light and 405 nm light were chosen. Figure S-6 depicts that the relative fluorescence intensity increases with the increase of irradiation time, and it reaches a platform for 90 min under natural light and for 30 min under 405 nm light. Traditionally, it will take 12 h to 2 days for the assembled split GFP to recover its fluorescence, because time is required for chromophore maturation.[40,41] Comparatively, with the matured chromophore in tGFP, our semisynthetic GFP assembly is time-saving, irradiated by not only 405 nm light but also natural light. Thus, 1:1 of S-peptide and tGFP and 405 nm light illumination for 30 min were chosen for the semisynthetic GFP assembly in the following experiments.

The detection strategy of our work was also depended on the phosphorylation protection against CPY digestion. The CPY concentration and the digestion time were further optimized, with 1 μM S-peptide and P-peptide as two different substrates. The relative fluorescence intensity of S-peptide and P-peptide were compared and the signal to background ratio, defined as the relative fluorescence intensity ratio of S-peptide to P-peptide, was used to evaluate the distinguishing ability of CPY. As shown in Figure S-7, 2 U/mL of CPY digestion for 90 min can distinguish S-peptide

and P-peptide better, and was used in the following experiments.

Since ATP acts as a co-substrate and Mg^{2+} is the co-factor of PKA, their possible interferences on the GFP assembly were investigated, respectively. As shown in Figure S-8, the effect of ATP (0-0.5 mM) and Mg^{2+} (0–10 mM) on the synthetic GFP assembly can be negligible. Therefore, under the optimized conditions above, the activity of PKA was analyzed based on this semisynthetic GFP assembly platform. The detection was quantitatively carried out by incubation of 2 μM S-peptide with different amounts of PKA (0–1.00 U/μL) in PKA reaction buffer at 30°C for 1 h. After being digested by CPY and assembling with tGFP, the fluorescence spectra of samples were recorded and depicted in Figure 3A. The fluorescence intensity was found to increase along with the increase of PKA concentration. It is reasonable that since the more PKA, the more S-peptide peptide is phosphorylated, protected from digestion, and bound with tGFP, and the more fluorescence is recovered. The logarithmic plot of the relative fluorescence intensity versus the PKA concentration indicated an EC_{50} value (enzyme concentration at which 50 % substrate is converted) of 24.50 mU/μL (Figure 3B). The relative fluorescence intensity also exhibited a linear correlation to the logarithmic PKA concentration in the range of 2.50 mU/μL to 125.00 mU/μL, with a minimum detectable concentration of 0.50 mU/μL (Figure 3B inset). The EC_{50} and the detection limit of this assay was highly comparable with the recently reported PKA methods.[42,43] Although CPY already had some applications in phosphorylation analysis, most of them were fluorescence quenching (signal turn-off) methods. This kind of methods often suffers from the disadvantages that it is difficult to know

whether the signal reduction comes from the target or the nonspecific quench. In our work, because only phosphorylation can effectively hinder the S-peptide digestion by CPY, the turn-on fluorescence signal reflects a real PKA activity. Combined with the S-peptide mutation-based red-shift of fluorescence, a signal to background ratio (S/B) of above 5 was gotten, which was higher than that of other CPY-based turn-off methods for PKA detection (with S/B of 2 or 3).[44,45]

In order to investigate whether this assay could be further used to study the PKA inhibition, the experiment was performed in the presence of a protein kinase inhibitor, H-89 (a known cell permeable inhibitor of PKA),[46] with different concentrations and 250.00 mU/μL PKA. Since the effect of 0-2.5 μM H-89 on tGFP and S-peptide assembly can be ignored (Figure S-9), the decrease of fluorescence intensity in Figure 4 was mainly because that with more H-89, more PKA was inhibited, and less fluorescence was recovered. Accordingly, a sigmoidal profile of the relative fluorescence intensity versus the H-89 concentration was obtained, and the IC_{50} value (the half maximal inhibitory concentration) of H-89 was calculated to be 24.3 nM, which was comparable to that reported inliteratures.[47,48] These results indicated that our detection was feasible for the study of protein kinase inhibition and had the potential for PKA inhibitor screening.

Detection of PKA Activity in Cell Lysates

Since protein kinases have significant impact on cell signaling, the measurement of PKA in cell lysates is crucial for related research. Here, our method was applied to investigate PKA activity in cell lysate. MCF-7 breast carcinoma cell

was chosen as a cell model, because PKA activity in MCF-7 cell lysate, without drug stimulation, was too low to be detected.[49] The cells were stimulated by various concentrations of Forskolin and IBMX, which had already been reported as an efficient activator combination.[50,51] The combination can largely increase the intracellular level of cyclic adenosine monophosphate (cAMP), and further activate PKA. Figure 5 illustrates that the fluorescence intensity gradually increased along with the increase of the stimulators concentration, and the induced increase of fluorescence intensity was almost inhibited by inhibitor H-89. Previous works have proven that more stimulators can induce more PKA in MCF-7 cell,[52] and the preliminary experiment showed that cell lysates had no obvious interference on assembly of tGFP and S-peptide (Figure S-10), which indicated that these responses in Figure 5 were directly derived from the PKA activity. Therefore, with excellent specificity and sensitivity, our semisynthetic GFP assembly platform revealed great potential *in vitro* cell kinase assays and inhibitor screening.

Detection of the Activity of Protein Phosphatase

In vivo protein phosphorylation is a reversible process. The hydrolytic cleavage of phosphate esters by protein phosphatase (PP) represents the reverse reaction of protein phosphorylation. Protein phosphatase 1 (PP1) belongs to a certain class of PP, which takes phosphorylated serine/threonine as substrate and acts pivotal roles in mediating diverse signal transduction pathways.[53] To the best of our knowledge, the previously reported PP1 activity biosensors were limited. Based on the reverse principle of PKA activity detection (Scheme 1), our semisynthetic GFP

assembly platform also showed the ability for the detection of PP1, where a synthesized phosphorylated substrate (P-peptide) was used. As mentioned above, P-peptide resisted CPY degradation and can bind to tGFP and recover its fluorescence (Figure 2). After P-peptide incubated with PP1 in the PP1 reaction solution at 30 ℃ for 1 h and sequentially mixed with CPY (under the same condition as that for the PKA activity detection), the product lost most all of its ability to bind with tGFP and the fluorescence kept quenching, which was comparable to that of S-peptide (Figure S-11 and Figure 2). Such result suggested that the phosphate ester on P-peptide was hydrolyzed by PP1, and the peptide losing its phosphate group, like S-peptide, was digested by CPY and cannot bind to tGFP. As shown in Figure 6, more PP1 leads to less fluorescence intensity. A linear correlation between the relative fluorescence intensity ratio and PP1 logarithmic concentrations was observed in the range of 0.10 mU/μL to 800.00 mU/μL, with a minimum detection concentration of 0.06 mU/μL， which was lower than the colorimetric assay for PP1 detection based on the gold nanoparticles developed by Kim *et al*.[54]

Conclusion

In summary, a semisynthetic GFP assembly platform was firstly developed to detect the enzyme activities involved in the reversible protein phosphorylation process. The assay has been successfully applied for detection of the activity and inhibition of PKA in the stimulated cell lysate, as well as PP1 activity. The effective protection of peptide by phosphorylation against CPY degradation and assembly of tGFP and S-peptide, along with the mutation-based red shift of fluorescence, give our assay

excellent specificity and sensitivity. Compared with the traditional methods based on radioactive techniques or phosphorylation-specific antibodies, our label-free method is much more simple, cost-effective, environment-friendly, and biocompatible. Due to its simplicity and high sensitivity, the proposed approach has the potential to be developed as a high-throughput method for protein kinase-targeted drug screening and *in vitro* kinase activity analysis in cell lysate. Since the design of peptide sequence is flexible, this semisynthetic GFP assembly-based method may expand to a versatile platform to detect different protein kinases activities and their inhibitions.

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Supporting Information Available

Additional information including the description of extensive methods and figures as noted in text is available free of charge via the Internet at http://pubs.acs.org.

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Figure captions

Figure 1. Fluorescence (A) and UV absorption spectra (B) of tGFP/S-peptide (S-p) complex, tGFP, and GFP with the same concentrations of 5 μM. The inset is the fluorescence photograph of GFP (1), tGFP (2) and tGFP/S-p (3) under an ultraviolet lamp with the excitation wavelength of 365 nm.

Scheme 1. Concept of phosphorylation-mediated assembly of semisynthetic fluorescent protein for label-free detection of protein kinase activity.

Figure 2. Fluorescence spectra of (1) tGFP only, (2) tGFP/S-peptide complex, (3) S-peptide digested by CPY, and then incubated with tGFP, (4) P-peptide digested by CPY, and then incubated with tGFP, (5) S-peptide incubated with PKA followed by CPY digestion, and then incubated with tGFP, (6) 5 without ATP, (7) 5 with inhibitor H-89, (8) S-peptide incubated with CKII followed by CPY digestion, and then incubated with tGFP.

Figure 3. A, fluorescence spectra of the fluorescent protein assembly system in response to different concentrations of PKA (b-l). b, 0; c, 0.63 mU/μL; d, 1. 25 mU/μL; e, 2.50 mU/μL; f, 5.00 mU/μL; g, 12.50 mU/μL; h, 25.00 mU/μL; i, 62.50 mU/μL; j, 125.00 mU/μL; k, 500.00 mU/μL; l, 1.00 U/μL; a, 0.50 μM tGFP only. B, the relative fluorescence intensity ($(F-F_0)/F_0$) as a function of PKA concentration (0 – 1.00 U/μL). Inset shows a linear correlation of the relative fluorescence intensity to the logarithmic concentration of PKA in the range of 2.50 mU/μL to 125.00 mU/μL.

Figure 4. A, fluorescence spectra of the fluorescent protein assembly system in response to different concentrations of PKA inhibitor H-89 (0-2.50 μM). B, the

relative fluorescence intensity as a function of the logarithmic concentration of H-89.

Figure 5. The relative fluorescence intensity of the fluorescent protein assembly system in response to different cell lysates, where PKA activity was activated by different concentrations of activator combination or inhibited by H-89. Inset indicates the different concentrations of the activators and H-89 for cell samples.

Figure 6. Fluorescence spectra of the fluorescent protein assembly system in response to different concentrations of PP1 (a-o): a, 0; b, 0.10 mU/μL; c, 0.25 mU/μL; d, 0.50 mU/μL; e, 1.00 mU/μL; f, 2.50 mU/μL; g, 5.00 mU/μL; h, 12.50 mU/μL; i, 50.00mU/μL; j, 0.20 x 10^3mU/μL; k, 0.80 x 10^3 mU/μL; l, 1.00 x 10^3mU/μL; m, 2.00 x 10^3 mU/μL; n, 5.00 x 10^3mU/μL; o, tGFP only. Inset is the linear correlation of the relative fluorescence intensity to the logarithmic concentration of PP1.

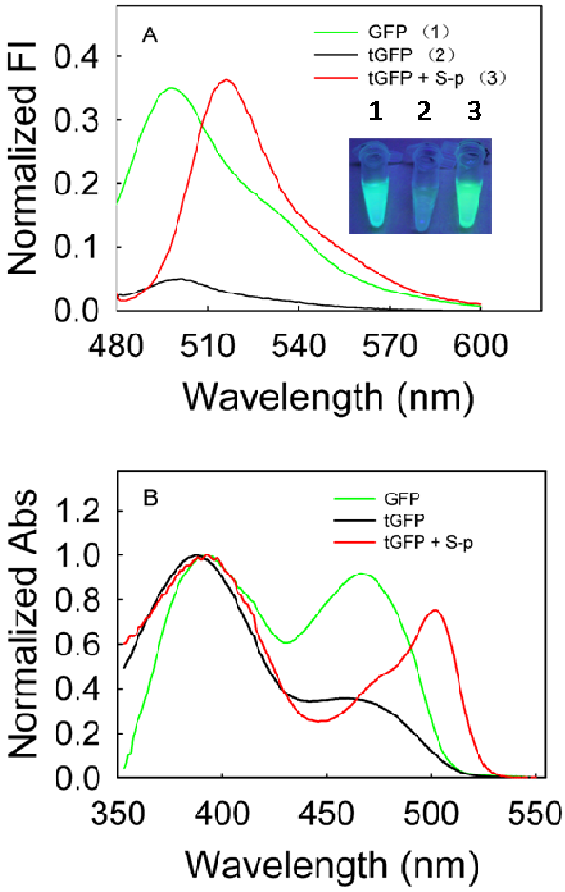
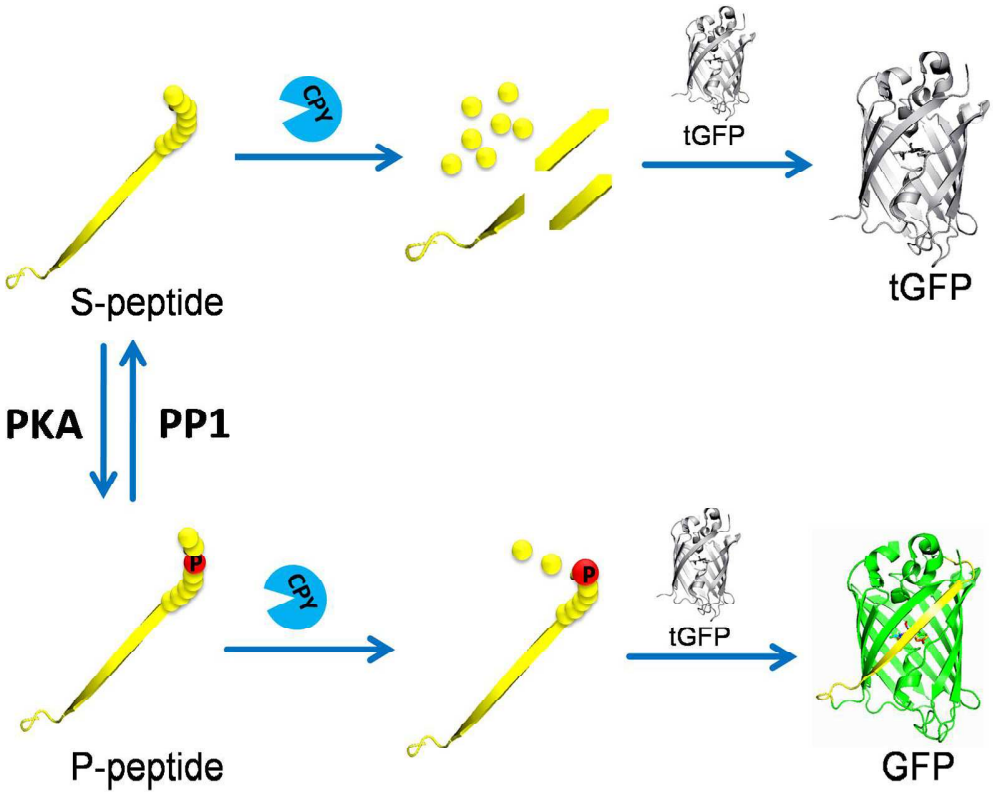

A
GFP (1)
tGFP (2)
tGFP + S-p (3)
1 2 3
Normalized FI
0.0
0.1
0.2
0.3
0.4
480 510 540 570 600
Wavelength (nm)
B
GFP
tGFP
tGFP + S-p
Normalized Abs
0.0
0.2
0.4
0.6
0.8
1.0
1.2
350 400 450 500 550
Wavelength (nm)

Figure 1



Scheme 1.

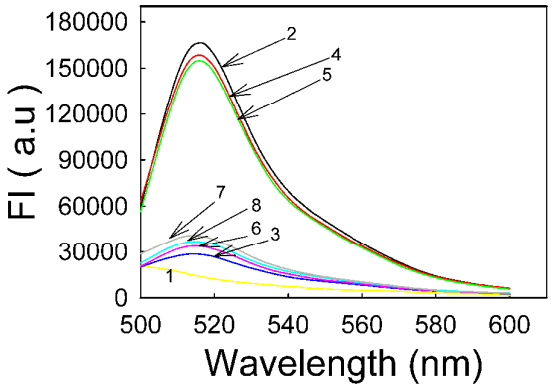

180000
150000
120000
90000
60000
30000
0
FI (a.u)
500
520
540
560
580
600
Wavelength (nm)
1
2
3
4
5
6
7
8

Figure 2

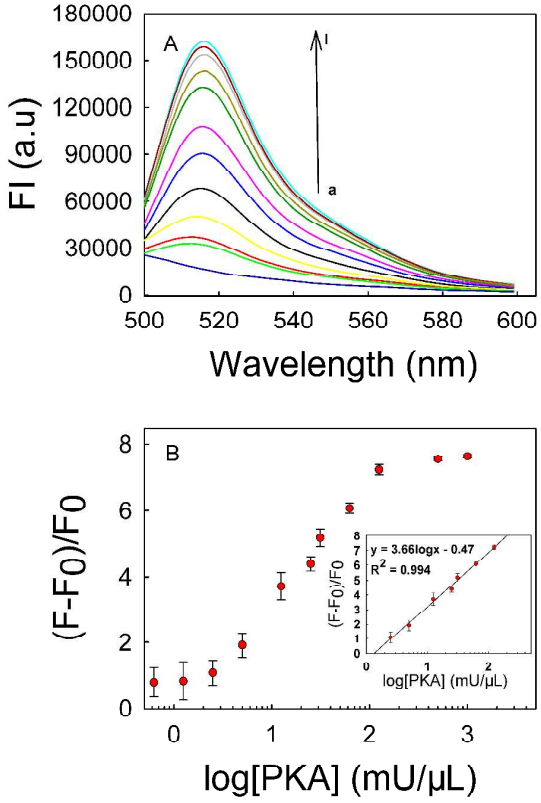

A
I
a
FI (a.u)
Wavelength (nm)
B
(F-F0)/F0
log[PKA] (mU/μL)
y = 3.66logx - 0.47
R2 = 0.994

Figure 3

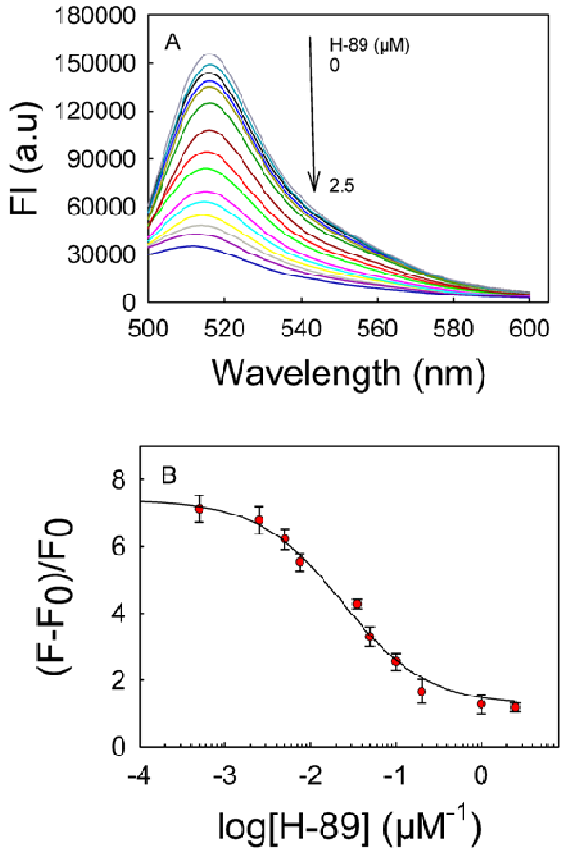

A
H-89 (μM)
0
2.5
FI (a.u)
180000
150000
120000
90000
60000
30000
0
500
520
540
560
580
600
Wavelength (nm)
B
(F-F0)/F0
8
6
4
2
0
-4
-3
-2
-1
0
log[H-89] (μM-1)

Figure 4

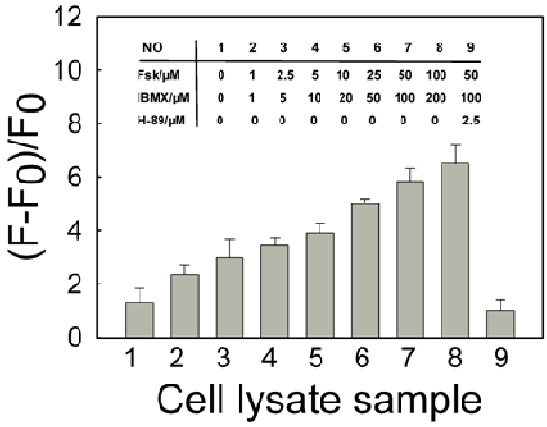

NO 1 2 3 4 5 6 7 8 9
Fsk/μM 0 1 2.5 5 10 25 50 100 50
IBMX/μM 0 1 5 10 20 50 100 200 100
H-89/μM 0 0 0 0 0 0 0 0 2.5
(F-F0)/F0
0 2 4 6 8 10 12
1 2 3 4 5 6 7 8 9
Cell lysate sample

Figure 5

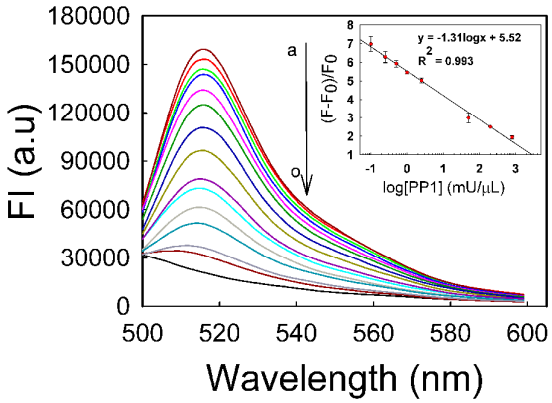

180000
150000
120000
90000
60000
30000
0
FI (a.u)
500
520
540
560
580
600
Wavelength (nm)
a
o
(F-F0)/F0
y = -1.31logx + 5.52
R2 = 0.993
log[PP1] (mU/μL)

Figure 6

For TOC only