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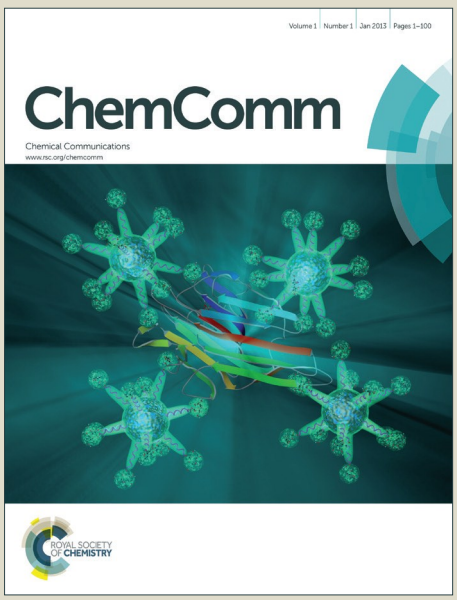

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Phosphorylation-induced formation of cytochrome c-peptide complex: a novel fluorescent sensing platform for protein kinase assay

Cite this: DOI: 10.1039/xoxxoooooox

Received ooth January 2012,
Accepted ooth January 2012

DOI: 10.1039/xoxxoooooox

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A novel fluorescent sensing platform has been developed for protein kinase assay based on phosphorylation-induced formation of cytochrome c-peptide complex.

The phosphorylation of cellular protein by kinases play a pivotal regulatory role in many cellular processes, including gene expression, cell differentiation, metabolism and apoptosis.[1,2] Casein kinase II (CK2) is a multifunctional and pleiotropic acidophilic Ser/Thr protein kinase, which was found to phosphorylate more than 300 different proteins already recognized. [3] Significantly, recent research demonstrates that dysregulation of CK2 is closely associated with a variety of human diseases, especially cancers.[4] As a result, identification of CK2 activity, their substrates and their potential inhibitors is of utmost importance in the study of many fundamental cellular processes and the development of kinase-targeted drugs discovery.

Conventional techniques for protein kinase assays typically rely on the use of radioactive ATP (γ -^{32}P-ATP).[5,6] Radioactive methods are well established but require demanding laboratory environments, and suffer from a high risk of harmful radioactive contamination. To eliminate the hazardous effects of radiometric assays, various approaches have been established for nonradioactive assays of protein kinase activity, such as electrogenerated chemiluminescent (ECL),[7] electrochemical,[8-11] fluorescent,[12-17] colorimetric,[18,19] mass spectrometry,[20,21] quartz-crystal microbalance–based,[22] and surface plasmon resonance–based methods.[23] Among these method, fluorometric approach is highly preferred owing to easy readout, low sample volume and high sensivity. More recently, the strategies for detecting kinase activities based on the use of fluorescent peptide probes have attracted more interest.[24,25] Andreas et al described a new fluorescence (FL) method for CK2 activity assay by utilizing a ratiometric peptide probe.[26] However, it requires expensive double-labeled peptide probe which is difficult to synthesize and the FL quenching degree of dye is limited. Zhou et al developed a novel FL biosensor for investigation of the activity of CK2 using graphene oxide (GO) and a fluorophore-tagged peptide probe.[27] Although this method is effective, however, it involves the sophisticated oxidation of GO to require water-solubility. Unfortunately, such oxidation treatment inevitably will result in the decrease of FL quenching ability because of the reduced percentage of sp2 carbon atoms. Hence, it is still challenging to develop facile, simple and cost-effective protein kinase assays.

Cytochrome c (Cyt c) is a well-known electron transfer (ET) protein due to its heme confactor that can quench the FL of many molecule probe efficiently.[28-33] More attractively, Cyt c is a highly water-soluble protein and its isoelectric point (pI) is 10.0–10.5.[34] Inspired by this phenomenon, we for the first time develop a novel FL peptide/Cyt c sensing platform for the detection of CK2 activity and inhibition based on phosphorylation protection against CPY degradation.[35]

Scheme 1 illustrated the principle of the approach. A specific substrate peptide of CK2 modified with a fluorescein (peptide S: FITC-DEDADISDEEDYDLGL) is used,[36] in which the serine (S) serves as the phosphorylation site and two amino acids (D and E) with negative charged side chain allow the peptides to be bound tightly on cationic Cyt c. CPY is capable of digesting any amino acids of the nonphosphorylated peptide from C-terminal.[37] However, phosphorylated serine significantly reduces the ability of CPY cleavage. As depicted in Scheme 1, without CK2 treatment or coincubated with CK2 inhibitor, the FITC labeled substrate peptide (peptide S) is promptly hydrolyzed by CPY, leading to the release of the FITC. Then the free FITC possesses a negative charge (-1) and would unable to absorb on Cyt c because of their weaker electrostatic interaction between the FITC and Cyt c, and thus induce

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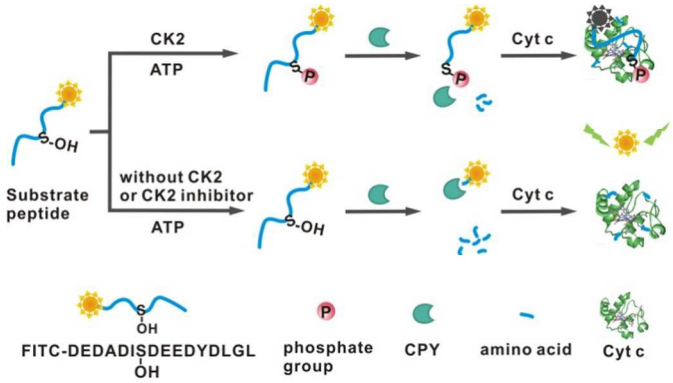
† Electronic Supplementary Information (ESI) available: Experimental details and supplementary figures. See DOI: 10.1039/b000000x



Scheme 1 Schematic illustration of the Cyt c-peptide sensing platform for the detection of CK2 activity and inhibition based on phosphorylation against CPY degradation.

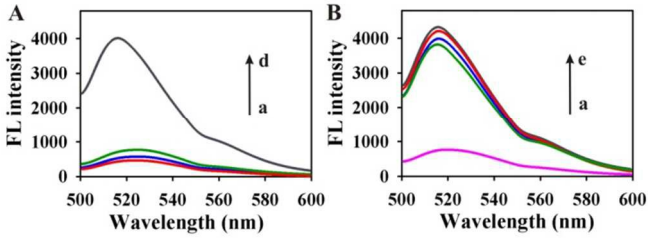


Fig. 1 (A) FL spectra of (d) 4 μM peptide S and (c) 4 μM peptide pS digested by 2.42 U/mL CPY and then mixed with 150 μM Cyt c, (b) peptide S, or (a) peptide pS mixed with the Cyt c without digestion by CPY. (B) FL spectra obtained in assays of CK2 kinase activity by the Cyt c/peptide system under different conditions: (a) 4 μM peptide S incubated with 200 U/mL CK2 and 50 μM ATP, followed by 2.42 U/mL CPY degradation and then mixed with 150 μM Cyt c, (b) a with the inhibitor TBCA, 0.8 μM, (c) a with 200 U/mL control protein kinase PKA, (d) a without ATP, (e) a with 4 μM control peptide kemptide.

the negligible FL quenching by Cyt c. In contrast, after CK2 treatment, the phosphorylated peptide S (peptide pS) can resist CPY digestion, yielding anionic phosphopeptides (FITC-DEDADIpS) with a net charge of -7 in the reaction buffer. Upon the addition of Cyt c, the FL was greatly quenched due to the strong absorption of peptide fragments on Cyt c by electrostatic interaction and the effective ET from the FITC to the heme cofactor of Cyt c. Therefore, the activity of CK2 can be easily and facilely monitored by FL signal change.

Since the FL quenching of FITC by peptide bound to the Cyt c is the precondition of the developed method, we first studied the FL quenching ability of Cyt c toward FITC-labeled peptide (peptide S) and FITC molecule. As shown in Fig. S1A (ESI†), with the increasing of Cyt c concentration, the FL intensity of FITC-labeled peptide S (with a net charge of -10) decreased dramatically. When concentration of Cyt c was up to 150 μM, the FL intensity of FITC-labeled peptide S was quenched down to 9.7 % of the initial FL signal upon the addition of Cyt c within 5 min (Fig. S1B, ESI†). In contrast, 80 % FL intensity still remained when free FITC was mixed with Cyt c under identical condition. This result indicates that remarkable FL quenching of FITC-labeled peptide S by Cyt c may be ascribed to polyanionic peptide-caused proximity. Here, 150 μM was chosen as optimized Cyt c concentration because it yields the maximal signal in FL between peptide S and free FITC. To further demonstrate that the FL quenching was caused by electrostatic interactions, peptide S was mixed with haemoglobin, another protein containing heme cofactor, and no noticeable FL quenching could be observed (Fig. S2, ESI†). These results verified that both the binding of peptide and Cyt c and highly efficient ET caused the FL quenching.

It has been reported that phosphorylated serine residue drastically decreased the proteolytic activity of exopeptidases and endoproteases.[31] Thus, phosphorylation of serine residues could be monitored by the increasing resistance of phosphopeptides to protease cleavage. To verify the feasibility of the proposed principle, we investigated the digestion influence of CPY on peptide S and peptide pS. As shown in Fig. 1A, after the peptide S reacted with CPY and Cyt c, a strong FL signal was obtained. This suggests the effective cleavage of peptide S by CPY released free FITC insusceptible to Cyt c quenching. However, a much lower FL signal was observed when peptide pS (with net charge of -12) bound to Cyt c or peptide pS treated with CPY and Cyt c. These result clearly indicate that the binding of peptide on Cyt c results in FL quenching of FITC. Hence, CPY can discriminate the peptide S from the peptide pS based on effective phosphorylation against CPY degradation. Next, we investigated CK2 catalysis. The resulting FL emission spectra were shown in Fig. 1B. After peptide S was treated with CK2 and digested by CPY, the resulting peptide could adsorbed onto Cyt c, leading to a dramatic FL quenching through efficient ET from FITC to Cyt c (curve a). Fluorescence anisotropy analysis also demonstrated that decreased FL was ascribed to the resulting peptide absorbed on Cyt c (Fig. S3, ESI†). In contrast, a high FL response was observed in the control experiment, in which peptide S was incubated with CK2 but without ATP (curve d) or with inhibitor, TBCA (curve b). Moreover, when the CK2 was replaced by control protein kinase PKA, no obvious FL quenching was obtained (curve c). A similar response was observed using the control FITC-labeled kemptide. These results suggest that CK2-catalyzed phosphorylation can be specifically and selectively reflected by the FL signals change of this peptide/Cyt c system. Some important experimental parameters, such as CPY concentration and reaction time, and the concentrations of Mg^{2+} and ATP, were all optimized (Fig. S4, S5, ESI†). Then, quantitative detection of CK2 activity was carried out under the optimized experimental conditions. As shown in Fig. 2A, the FL signals gradually decreased as the concentrations of CK2. The dependence of FL signal on CK2 concentration was displayed in Fig. 2B. The FL signal was linearly decreased with the logarithm of CK2 concentration in the range from 0.8 to 50 U/mL. The calibration equation was I_F = -1725 log c + 4334 (where R = 0.990, I_F is the FL intensity observed at 517 nm, c is the concentration of CK2). The EC50 value (enzyme concentration producing 50 percent substrate conversion) of CK2 was estimated to be 12.8 U/mL. The detection limit of CK2 was calculated to be 0.15 U/mL (S/N = 3), which was better than that of the FL assay based on quantum dots (0.5 U/mL).[14]

To demonstrate the potential applications of our method in protein kinase inhibitor screening, we examined the potent CK2 inhibitors (TBCA), which is a highly selective inhibitor of CK2 by

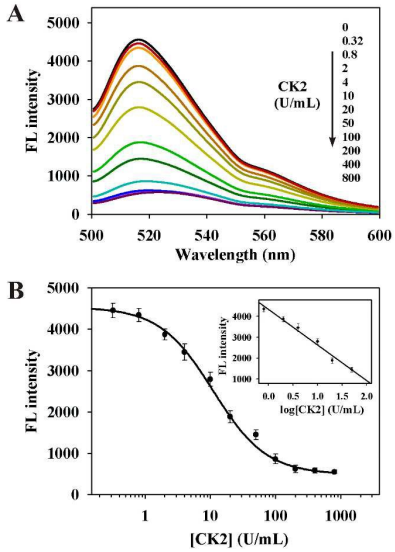


Fig. 2 (A) FL spectra of peptide/Cyt c system in the presence of CK2 with different concentrations. (B) Calibration curve for CK2 detection. Inset: dependence of FL intensity with respect to logarithmic CK2 concentrations. Error bars were estimated from three independent measurements. Experimental conditions: peptide S, 4 μM; ATP, 50 μM; Mg^{2+}, 10 mM; CPY, 2.42 U/mL; Cyt c, 150 μM.

competitively binding with ATP. The inhibition test was conducted by coincubating CK2 solution with a fixed concentration with a series of different concentrations of inhibitor. After the CPY degradation and binding of Cyt c, the FL signals were measured. Fig. S6 shows that the FL intensity obviously increased with the increase in the concentration of inhibitors, the IC50 value of TBCA (inhibitor concentration producing 50% inhibition) was estimated to be 100.92 nM, which is consistent with the value reported in the literature.[38] The result suggests that the proposed assay has the potential ability to qualitatively screen the kinase inhibitors. To assess the specificity of Cyt c-based biosensor for CK2 assay, the responses of the biosensor towards some other proteins were explored. As shown in Fig. S7 (ESI†), there was smaller fluorescence intensity ratio (F_0/F) in the presence of the other proteins compared with target CK2, clearly suggesting that they did not interfere with our assay.

To demonstrate the feasibility of our method in complex samples, the activity analysis of CK2 in Hela cell lysates was performed. The cell lysates of Hela cells in the absence and presence of inhibitor TCBA[39,40] were first tested. As shown in Fig. S8, the FL response induced by cell lysate could be completely inhibited by TCBA, demonstrating that this response is directly derived from CK2 activity. Furthermore, the Hela cell lysates with different concentrations of CK2 were prepared by adding different concentrations of standard CK2 solution and then detected using our method. The recoveries ranged from 96.3 % to 105.0 % (Table S1, ESI†). These results show the complex cell extracts do not influence the detection of CK2, suggesting that this Cyt c-based method is feasible for kinase analysis in complex samples.

To demonstrate the generality of the peptide/Cyt c system, another kinase, cyclin-dependent kinase (CDK1), was also tested. A peptide sequence, FITC-DDDDPKTPKKAKKLRRRLL, was designed as the substrate. The results are shown in Fig. S9 (ESI†). It was observed that the fluorescence intensity of peptide/Cyt c system dynamically decreased with the increasing CDK1 concentration varying from 0 to 1000 U/mL, and the fluorescence intensity at 517 nm was linearly related to the CDK1 concentration in the range from 2.5 U/mL to 200 U/mL, with a detection limit of 0.54 U/mL, suggesting that the peptide/Cyt c system can be applied to the detection of other kinase activity.

In summary, a novel fluorescent biosensor for assay of CK2 activity and inhibition has been developed based on peptide/Cyt c complex and phosphorylation against CPY degradation. The effective suppression of phosphorylation to CPY digestion and high quenching capacity of Cyt c both contribute to sensitive and selective screening of CK2 activity. In comparison with phospho-recognizing antibodies-based method or graphene oxide–based kinase assay with the involvement of sophisticated oxidation, our approach is much more cost-effective and simple. Moreover, in contrast to the radioactive techniques with involvement of hazardous material, our method is much more environmentally friendly by use of biodegradable Cyt c. This work provides a new application of Cyt c as a FL quencher for probing activity of post-translational modification enzyme. Moreover, this proposed approach can readily expand to probe different kinase activity by adding some negatively-charged amino acids in substrate peptide. Given the key roles of kinases in many biological processes, this fluorescent peptide/Cyt c sensing platform shows great potential in kinase-related drug discovery and clinical diagnostics.

This work was supported by the National Natural Science Foundation of China (No. 21275045), NCET-11-0121, and Hunan Provincial Natural Science Foundation of China (Grant 12JJ1004).

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