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An Excimer-Based, Turn-On Fluorescent Sensor for the Selective Detection of Diphosphorylated Proteins in Aqueous Solution and Polyacrylamide Gels

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Supporting Information

ABSTRACT: Protein phosphorylation is a ubiquitous post-translational modification, which often acts as a switch to proteins' activation and is frequently perturbed in diseases. Although many general phospho-protein detection tools are available, none of them offers information about the relative spatial arrangement of phosphorylated residues. Specifically, proximally phosphorylated residues are hallmarks of certain activated disease-relevant proteins. We herein report the first turn-on fluorescent sensor for the selective detection of proximally phosphorylated protein sites, suitable for application in both aqueous solutions and polyacrylamide gels.

Protein phosphorylation is a ubiquitous post-translation modification, which serves, among other roles, as a switch to control proteins' activation state.[1] Significantly, perturbed protein phosphorylation levels and/or the overexpression of phosphorylated proteins in signaling pathways are the hallmark of many human disease.[2] Thus, development of molecular methods for the detection and quantification of phosphorylated proteins is of utmost interest and importance.

Pro-Q Diamond, a commercially available fluorescent phospho-protein stain, has been applied for studying the phospho-proteome[3] and for identification of kinase/phosphatase targets.[4] Although highly efficient at determining the total phosphorylation levels (staining all tyrosine (pY), serine (pS) and threonine (pT) residues),[5] it offers no information about the spatial arrangement of these phosphorylated sites. Herein, we report a turn-on fluorescent sensor which selectively detects proximally diphosphorylated protein sites in both aqueous solutions and polyacrylamide gels.

Diphosphorylation on proximal residues is required for the activation of a subset of proteins, including Jak2[6] and ERK2[7] kinases, resulting in pYpY and pTXpY motifs, respectively (X = any amino acid). Importantly, many of these activated kinases are overexpressed in a variety of diseases, notably human cancers.[2] Therefore, a sensor capable of detecting proximal phosphorylated residues will provide valuable information about specific protein activation status and serve as a molecularly targeted diagnostic tool for disease detection. Many of the developed potent phospho-protein sensors were based on transition metals,[8,9] and were coupled to fluorescence or colorimetric detection techniques for solution and gel assays.[10−14] Hamachi and co-workers pioneered research aimed at the detection of proximally phosphorylated protein sites by successfully designing rigid fluorescent sensors.[14−16]

To design a more effective, turn-on sensor, we have employed a mechanistically different approach from those previously reported. Instead of conducting laborious structure−activity relationship studies for identifying a suitable geometrical fit for one sensor targeting a particular diphosphorylated motif, our approach relies upon a fluorescent response resulting from association of two sensor units with a target phosphorylated motif. To achieve this, we utilized pyrene-mediated excimer emission as a turn-on fluorescent reporter component of our sensor. Briefly, when two pyrene molecules associate, there is an observed increase in excimer emission in the 480 nm region. This feature of pyrene has been employed in developing sensors of nucleotides[17] and pyrophosphate,[18] among others.[19,20] As the binding component of our sensor we employed a Lewis Zn(II)-coordination complex that non-specifically binds all phosphorylated sites. Since only proximal pyrene molecules produce an excimer signal, we hypothesized that a pyrene coupled to Zn(II)-cyclen macrocycle would preferentially form a 1:1 complex with a pX-containing peptide/protein site and therefore not produce an excimer signal (Figure 1A). However, for proximally diphosphorylated peptides, we postulated that each pX residue could coordinate a Zn(II)-cyclen unit, facilitating pyrene interaction and resultant excimer emission (Figure 1A). Thus, for proof-of-principle, we synthesized sensor **1** (Figure 1B).

For initial testing, sensor **1** (Figure 1B) was evaluated against peptide sequences containing mono- and diphosphorylated sites (AYpYAA and ApYpYAA) as models of differentially phosphorylated proteins. All experiments were performed in aqueous solutions under physiological conditions (HEPES). As predicted, upon excitation at 350 nm, sensor **1** produced a pronounced shift in emission from the 380 to 480 nm region in response to pYpY-containing peptides at concentrations as low as 10^{-6} M (Figure 2A). This shift, attributed to excimer formation, was at least 5-fold lower in response to mono-phosphorylated pY peptides at all concentrations tested (3−250 μM of peptide), suggesting that strong excimer emission signal is specific to a diphosphorylated motif. As pY peptides induced minor excimer formation, we hypothesize that while

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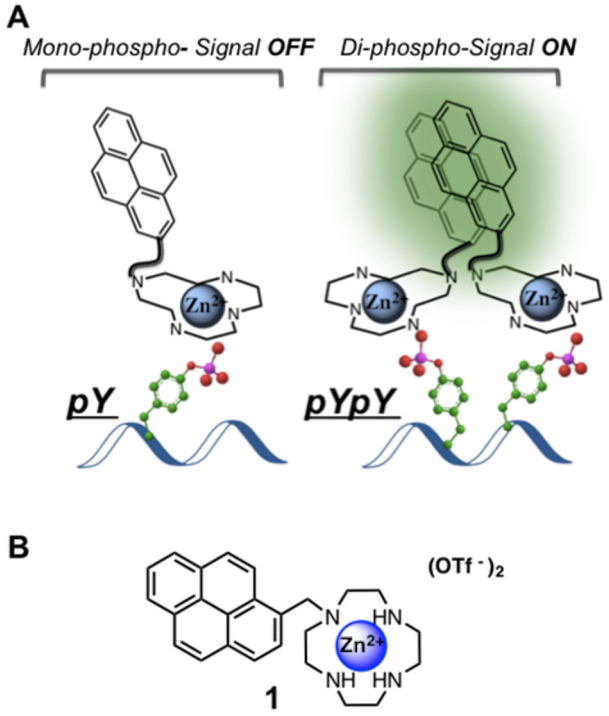

Figure 1. (A) Mechanism for the sensor signaling; (B) chemical structure of proof-of-principle sensor, **1**.

not favorable, pY association with two molecules of **1** can occur.

Next, we performed titration experiments which confirmed that the maximum excimer signal resulted from a 2:1 sensor:peptide complexation stoichiometry (Figure 2B). Consistently, upon addition of excess peptide, reduction in signal was observed, corresponding to a shift in the equilibrium toward a 1:1 complex. Association of the sensor with the pYpY peptide was also found to be cooperative (n_{Hill} = 3.2 ± 0.2; log K_{app}= 4.3 ± 0.1 M^{-1}) with the detection limit of 0.6 μM, which was unaffected by the presence of equimolar amount of the off-target pY peptide (Figures S4−S5). Significantly, it was also possible to observe this selective response by standard fluorescence imaging, further broadening the utility of the sensor (Figure 2C).

While initial experiments demonstrated the efficacy of our sensor in a model system, we next sought to probe whether it could retain selectively for the target diphosphorylated motifs within full-length proteins. Thus, a selection of variably phosphorylated proteins (Table 1) were incubated with **1** at a range of concentrations (1−10 μM of protein) in aqueous solution and assessed for excimer fluorescence enhancement. The results of this experiment are illustrated in Figure 3A.

For nonphosphorylated BSA protein, no significant excimer formation was observed, indicating limited nonspecific binding to nonphosphorylated protein surfaces (Figure 3A). Excimer formation was also not induced upon incubation with Stat5 protein (containing three distal phosphorylated residues). These results correlate with our hypothesis and the initial peptide studies. Importantly, for α- and β-casein, which both contain diphosphorylated motifs, there was observed an almost 30-fold fluorescence enhancement, suggesting that the sensor is selective for proximal diphosphorylated sites. We also tested dephosphorylated α-casein (α-casein-D), which, while partially dephosphorylated, is known to retain two phosphorylated

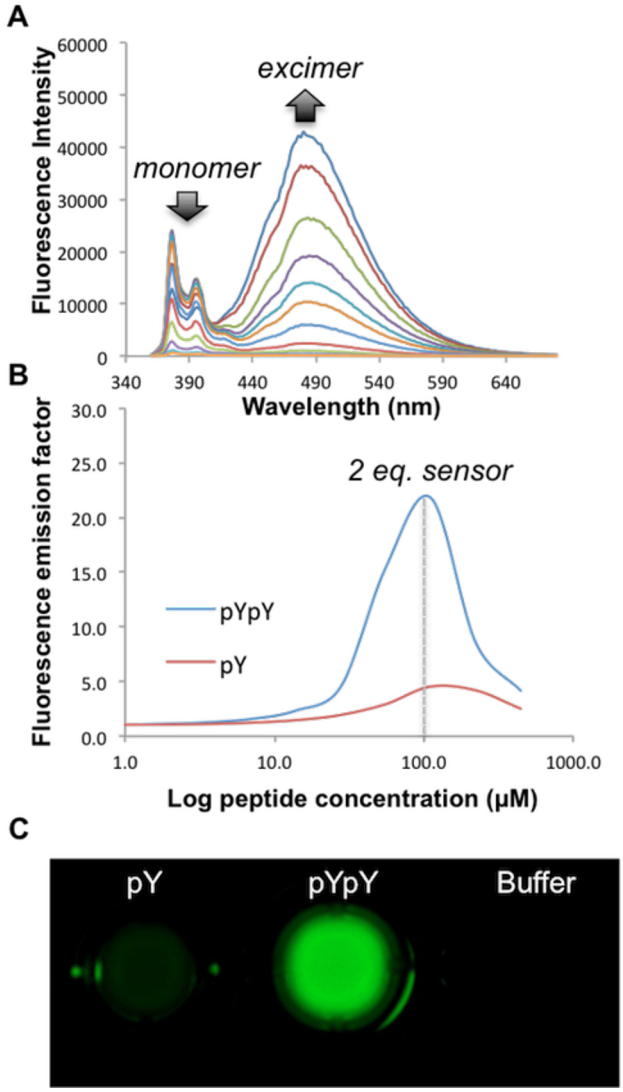

Figure 2. (A) emission spectrum of the 2:1 sensor:pYpY peptide (250−3 μM analyte); (B) titrations of sensor (0.22 mM) with peptides; (C) fluorescence image of 50 μM sensor and 25 μM peptides acquired with Bio-Rad ChemiDoc MP imaging system.

Table 1. Phosphorylation Motifs of Proteins Selected for Treatment with Sensor 1

Protein	Phosphorylation motif
BSA	No phosphorylation
α-casein-D	2 pS residues; motif unknown
β-casein	pSLpSpSpS
α-casein	pSEpS, pSIpSpSpS
Stat5	3 distal pY residues

residues,[5] but their relative spatial arrangement is unknown. As can be seen, our data suggests that these two phosphate esters might be relatively close in proximity, as indicated by a 5-fold increase in excimer formation. Titration experiments indicated that proximally diphosphorylated proteins were detectable at concentrations of 0.6 μM and the signal was not significantly affected by the presence of other nontarget proteins (Figures S7−S9).

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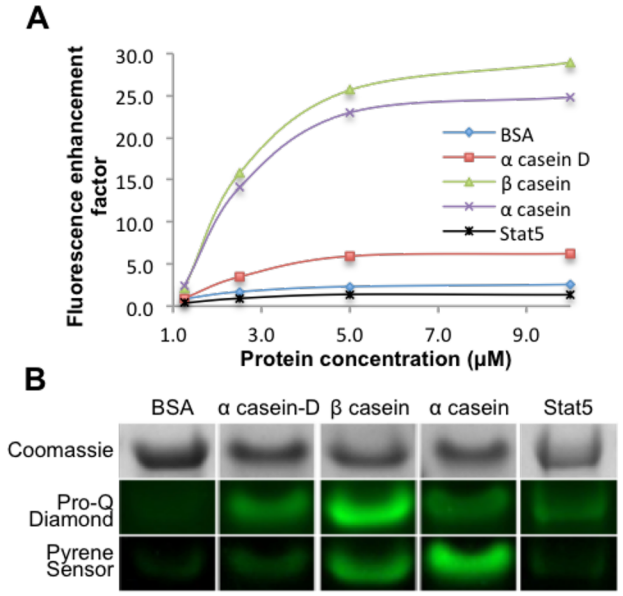

Figure 3. (A) fluorescent response of the sensor to proteins in HEPES buffer; (B) stained polyacrylamide gels (full gel images are presented in Figure S4).

Since proteins are, in the prevailing majority of studies, detected by staining following separation on polyacrylamide gels, we next examined the utility of our sensor in this medium. Briefly, on the same polyacrylamide gel, approximately equal amounts of proteins (1.2 μg $\approx$ 50 pmols) were run and stained with the universal Coomassie Blue protein stain (Figure 3B, top row). To determine the relative levels of total protein phosphorylation, we stained the gel with the Pro-Q Diamond stain. As expected, staining of nonphosphorylated BSA protein with Pro-Q Diamond was negligible. While phosphorylated Stat5, α-casein and α-casein-D exhibited comparable phosphorylation levels, β-casein was stained to a greater extent. We then treated gels with sensor **1** as follows. The gel was fixed for 40 min in a solution of 50% methanol/10% acetic acid in water and then incubated in a solution of 300 μM sensor for 40 min and rinsed with acidic sodium acetate-acetonitrile buffer. Gels were then imaged with a BIORAD ChemiDoc MP fluorescent imaging system.

As expected, the BSA band was negligibly stained by both Pro-Q Diamond and **1**. Excitingly, differential staining of STAT5 (bearing distal pY motifs) by Pro-Q Diamond and **1** was observed: sensor **1** did not stain the Stat5 band, which was detected by Pro-Q Diamond. This data strongly suggested that **1** does not form excimers with monophosphorylated protein motifs. In addition, sensor **1** more intensely stained α-casein over β-casein, despite the higher total phosphorylation of the latter (as determined by Pro-Q Diamond). This observation further supported our hypothesis, since α-casein has an additional diphosphorylated site (Table 1), and would therefore facilitate increased excimer formation per protein molecule. As can be seen, the distinct pattern of staining by sensor **1** relative to that of Pro-Q Diamond, strongly suggest that **1** is selective for diphosphorylated protein motifs. Owing to its unique excimer turn-on mechanism, stained gels have essentially no background fluorescence and therefore do not require destaining, making it possible to complete an entire protocol in under 1.5 h.

In conclusion, to the best of our knowledge, this is the first reported turn-on fluorescent sensor specific to proximally diphosphorylated protein sites with demonstrated utility for both solution and gel-based fluorescent detection techniques and detection limits of 0.6 μM and 0.6 μg of protein, respectively. We believe that the developed excimer-based sensor offers another dimension of phospho-proteome characterization and significant potential as a disease diagnostic tool. Current efforts in our lab are directed at optimizing excimer-based sensors for increased potency and selectivity.

■ ASSOCIATED CONTENT

Supporting Information

Synthetic schemes, compound characterization, biophysical and biological protocols. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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