

Label-Free Fluorescent Kinase and Phosphatase Enzyme Assays with Supramolecular Host-Dye Pairs

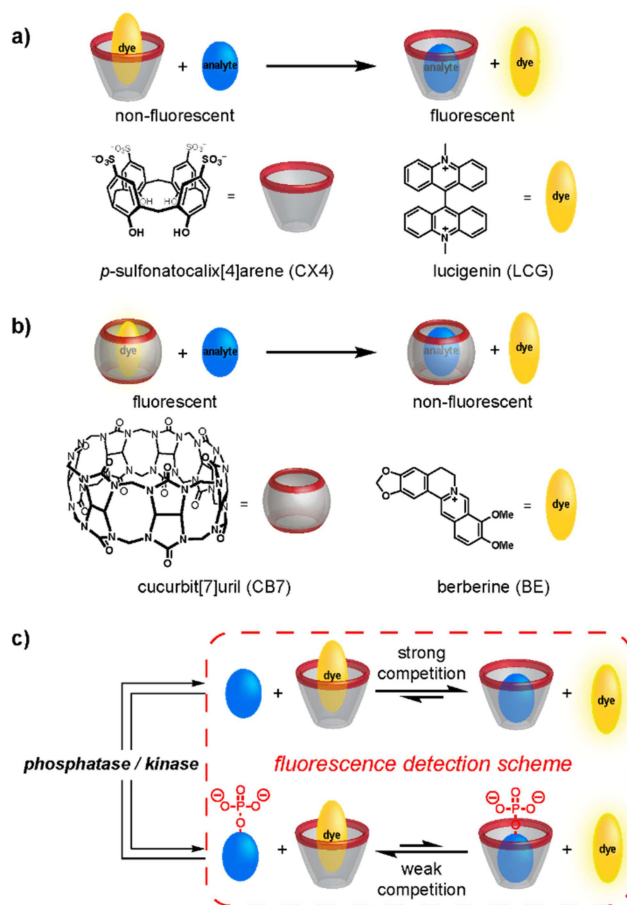
Yan-Cen Liu,^[a] Shu Peng,^[a, b] Lora Angelova,^[a] Werner M. Nau,^[a] and Andreas Hennig^{*[a]}Dedicated to Jean-Marie Lehn on the occasion of his 80th birthday

The combination of the macrocyclic hosts *p*-sulfonatocalix[4]arene and cucurbit[7]uril with the fluorescent dyes lucigenin and berberine affords two label-free enzyme assays for the detection of kinase and phosphatase activity by fluorescence monitoring. In contrast to established assays, no substrate labeling is required. Since phosphorylation is one of the most important regulatory mechanisms in biological signal transduction, the assays should be useful for identification of inhibitors and activators in high-throughput screening (HTS) format for drug discovery.

The phosphorylation and dephosphorylation of serine, threonine, and tyrosine residues in peptides by kinases and phosphatases is one of the most important regulatory mechanisms in biological signal transduction.^[1] Numerous diseases, such as pathogenic infections^[2] or cancer,^[3] involve abnormal activities of these enzymes. As a consequence, convenient and cost-effective enzyme assays to monitor kinase and phosphatase activity are required in pharmaceutical industrial drug discovery as well as in fundamental enzymological research in order to identify suitable inhibitors or activators as potential lead structures for new drugs.^[4] Typical methods include radioimmunoassays^[5] as well as laborious electrophoretic^[6] or chromatographic assays,^[7] which, however, lack the possibility for an easy scale-up to high-throughput screening (HTS) format.^[8] Most desirable are homogeneous fluorescence-based assays, which offer high sensitivity, short detection times, and the possibility for continuous real-time monitoring of enzyme activity.^[9] However, the design of fluorescence-based kinase and phosphatase assays is challenging and often involves expensive antibodies,^[10] phosphate-binding proteins^[11] and

peptides,^[12] biopolymers,^[13] or strategies involving metal particles,^[14] graphene oxide,^[15] as well as metal-ion chelating sites^[16] to generate a detectable fluorescence signal change. Single-labeling strategies based on static or dynamic fluorescence quenching by tyrosine residues have already been explored, but these are not applicable to serine or threonine kinases and the introduction of large aromatic dyes often interferes with substrate recognition by the enzyme.^[17]

An alternative strategy to monitor enzyme activity utilizes supramolecular reporter pairs composed of a suitable combination of host and dye molecules (Scheme 1). Fluorescent dyes typically change their fluorescence spectroscopic properties



Scheme 1. (a,b) Principle of indicator displacement assays and structures of (a) the CX4/LCG and (b) the CB7/BE reporter pair used for (c) a supramolecular tandem enzyme assay for kinases and phosphatases.

[a] Y.-C. Liu, Dr. S. Peng, L. Angelova, Prof. Dr. W. M. Nau, Dr. A. Hennig
Department of Life Sciences and Chemistry
Jacobs University Bremen gGmbH
Campus Ring 1, 28759 Bremen (Germany)
E-mail: a.hennig@jacobs-university.de

[b] Dr. S. Peng
College of Chemistry, State Key Laboratory of Elemento-Organic Chemistry,
Key Laboratory of Functional Polymer Materials, Ministry of Education,
Nankai University, Tianjin 300071 Tianjin (China)

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when they bind to macrocyclic hosts. A more strongly binding analyte, which displaces the fluorescent dyes from the host, can, thus, be sensed through a change in fluorescence (Scheme 1a and 1b).^[18] As a refinement of this indicator displacement assay strategy, we^[19] and others^[20] have developed and applied the supramolecular tandem enzyme assay principle (Scheme 1c). Therein, the differential binding affinity of the substrate and product of an enzymatic reaction is exploited to afford an overall change in fluorescence intensity when a weakly binding substrate is converted into a more strongly binding product or *vice versa*.

Herein, we report that supramolecular tandem enzyme assays afford a simple and label-free method to continuously monitor phosphatase and kinase activity by fluorescence spectroscopy. We use, first, *p*-sulfonatocalix[4]arene (CX4) in conjunction with the fluorescent dye lucigenin (LCG) to monitor phosphorylation of the two peptides P1 (H-LRRWSLG-OH) and P2 (H-WKRTLRL-OH) by the serine kinase protein kinase A (PKA) and the threonine kinase protein kinase C (PKC), respectively. Second, we establish that the reporter pair cucurbit[7]uril (CB7) and berberine (BE) can also be used to follow the dephosphorylation of *O*-phospho-L-tyrosine (pTyr) by alkaline and acid phosphatase.

We have previously reported that P1 and P2 are bound to CX4 with higher affinity than their phosphorylated counterparts,^[21] and we now determined the binding constants of the two peptides in 10 mM Hepes, pH 7.0. This indicated that the unphosphorylated peptides bind to CX4 10–100 times more strongly than their phosphorylated counterparts (Figure 1), which is sufficient for a tandem enzyme assay.^[19c] Obviously, the phosphate group lowers the overall positive charge of the peptides, such that the affinity to the negatively charged CX4 decreases significantly.

The supramolecular tandem enzyme assay was set up by using substrate concentrations, which are sufficiently high to afford a significant displacement of LCG, but sufficiently low to afford minimal displacement after phosphorylation by the kinases. Considering the excellent discrimination of substrate and product by the reporter pair, a wide range of concentrations can be set up,^[19c] and we have selected a range of 20 μM to 100 μM peptide concentration in the following experiments, which is a typical range for kinase assays.^[17]

Addition of PKA to a solution containing 20 μM P1 and 1.0 μM CX4 and 0.5 μM LCG of the CX4/LCG reporter pair led to a time-dependent decrease in fluorescence intensity (Figure 2), which is in accordance with the tandem assay principle. In detail, phosphorylation of P1 decreases its affinity to CX4 such that more LCG can be bound leading to an overall decrease in fluorescence due to quenching of LCG by CX4. Evidently, the initial rate of fluorescence decrease depended linearly on the enzyme concentration (inset of Figure 2).

To demonstrate the utility of our supramolecular tandem enzyme assay in identifying and characterizing enzyme inhibitors, we selected *N*-ethylmaleimide (NEM) as a reported suicide inhibitor of PKA.^[22] The normalized time-dependent fluorescence changes clearly showed decreasing PKA activity as the incubation time with a constant NEM concentration was

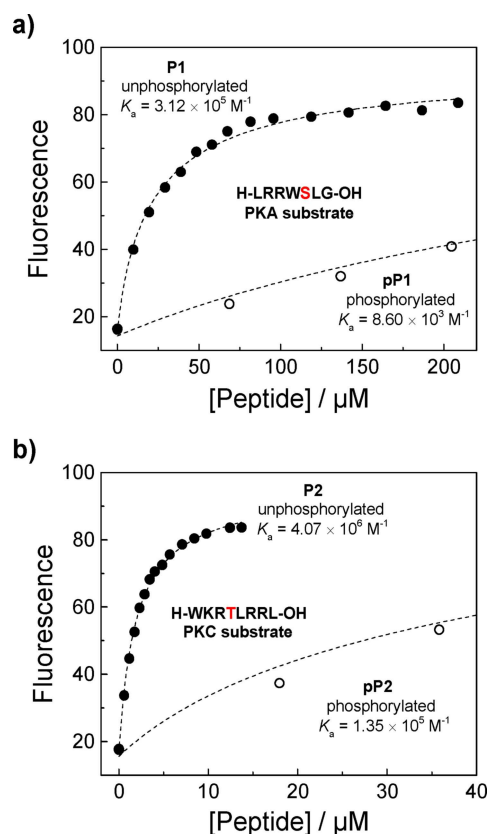


Figure 1. Competitive fluorescence titrations ($\lambda_{\text{exc}} = 367 \text{ nm}$, $\lambda_{\text{em}} = 506 \text{ nm}$) with CX4 (1.0 μM) and LCG (0.5 μM) to determine the binding constants of a) P1 and b) P2 (closed circles) and their respective phosphorylated kinase products (open circles) in 10 mM Hepes, pH 7.0.

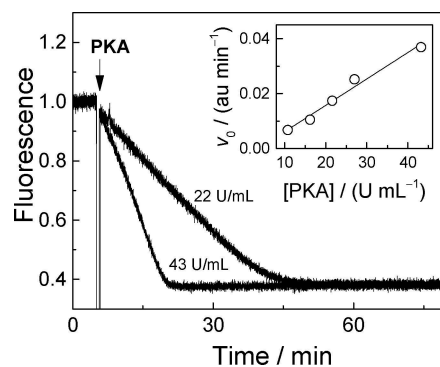


Figure 2. Continuous, label-free fluorescence monitoring of kinase activity with the CX4/LCG reporter pair. PKA was added to 20 μM P1, 0.5 μM LCG, 1.0 μM CX4, and 0.2 mM ATP in 10 mM Hepes, 10 mM $\text{Mg}(\text{NO}_3)_2$, pH 7.0 ($\lambda_{\text{exc}} = 367 \text{ nm}$, $\lambda_{\text{em}} = 506 \text{ nm}$). The inset shows the linear dependence of the initial rate on enzyme concentration.

increased (Figure 3), which is in accordance with a suicide inhibition mode.^[19g] Noteworthy, we observed that the initial decrease of the fluorescence intensity up to ca. 7 min was independent of the incubation time. This originates presumably from a comparably slow reorganization of the different host-guest complexes during addition of the enzyme substrate P1. This hypothesis was confirmed by addition of P1 to a solution containing all assay components except the enzyme PKA, which

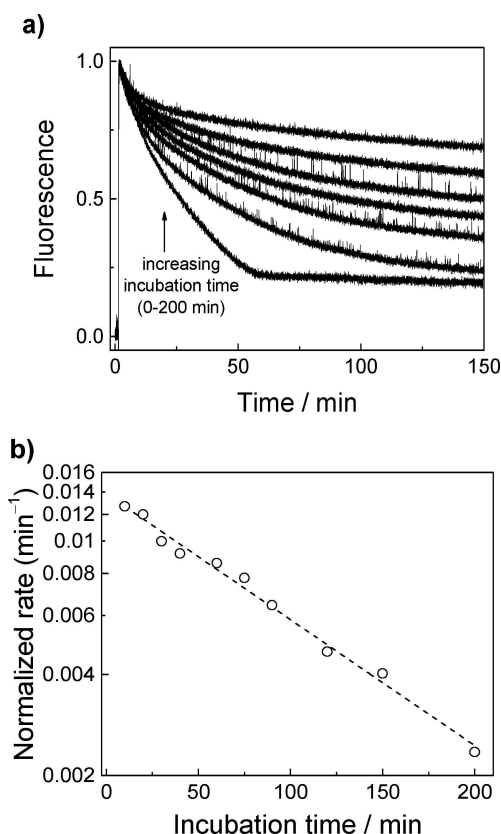


Figure 3. Inhibition of PKA activity by $12 \mu\text{M}$ *N*-ethylmaleimide (NEM). a) Fluorescence traces ($\lambda_{\text{exc}} = 367 \text{ nm}$, $\lambda_{\text{em}} = 505 \text{ nm}$) upon addition of $100 \mu\text{M}$ P1 at 1.4 min to a solution containing 10 mM $\text{Mg}(\text{NO}_3)_2$, $2.0 \mu\text{M}$ cAMP, 0.2 mM ATP, $1.0 \mu\text{M}$ CX4, $0.5 \mu\text{M}$ LCG, $12 \mu\text{M}$ NEM, and 24.6 U/mL PKA in 10 mM Hepes buffer, pH 7.5, at 25°C at varying incubation times of NEM with PKA. b) Dependence of the enzymatic reaction rate on incubation time of PKA with NEM.

also showed a similar initial time-dependent fluorescence decrease. Consequently, we fitted the fluorescence data between 10 and 20 min instead of the initial decrease to afford a measure of PKA activity. The logarithm of the resulting normalized enzymatic reaction rates was plotted against the incubation time, which afforded the expected linear relationship for a suicide inhibitor (Figure 3b).

It is noteworthy that our tandem kinase assay based on CX4/LCG uses a *cation receptor* to follow the introduction of a *negative charge* into the substrate. This contrasts established strategies, which have commonly utilized the selective recognition of phosphate groups by anion receptors in kinase and phosphatase assays.^[16b,23] For example, metal-ion chelating sites were previously engineered into peptide kinase substrates to enable an interaction of the metal cation with the phosphate group after phosphorylation,^[16b] and we have previously used positively charged anion receptor macrocycles to follow the hydrolysis of the negatively charged cofactor adenosine triphosphate.^[23] Only recently, Hooley and coworkers have utilized a negatively charged cavitant to distinguish phosphorylated and unphosphorylated versions of cationic peptides and, thereby, to monitor the activity of kinases and phosphatases.^[20e] This prompted us to explore this rather counterintuitive

strategy further by investigating whether we can also monitor the enzymatic conversion of uncharged or even negatively charged molecules with a cation receptor.

To demonstrate, we have chosen the reporter pair CB7/BE to follow the dephosphorylation of the negatively charged pTyr by acid and alkaline phosphatase, which affords the uncharged zwitterionic L-tyrosine (Tyr) as product. Competitive titrations at the optimal pH values for acid and alkaline phosphatase yielded binding constants of $2.4 \times 10^5 \text{ M}^{-1}$ and $1.9 \times 10^5 \text{ M}^{-1}$ for Tyr and $6.9 \times 10^4 \text{ M}^{-1}$ and $2.7 \times 10^4 \text{ M}^{-1}$ for pTyr at pH 6.0 and 8.8, respectively (Figure 4). The pH dependence probably originates from a partly deprotonated α -ammonium group at pH 8.8 ($\text{p}K_a(\text{Tyr}) = 9.11$) and a partly protonated phosphate group at pH 6.0 ($\text{p}K_a = 5.8$), which both lead to less favorable interactions with the carbonyl portals of CB7 at alkaline pH.

Although the affinity of Tyr and pTyr differed only by a factor of 3.5 and 7.0 at pH 6.0 and 8.8, enzyme-activity monitoring was clearly possible at both pH values (Figure 5). Addition of either acid or alkaline phosphatase to a mixture containing the CB7/BE reporter pair at micromolar concentrations and the weak competitor pTyr resulted in a continuous fluorescence decrease until a plateau value was reached. This is in agreement with dephosphorylation of pTyr affording the stronger binder Tyr, which can displace the fluorescent dye BE from CB7, thereby, lowering its fluorescence intensity. Addi-

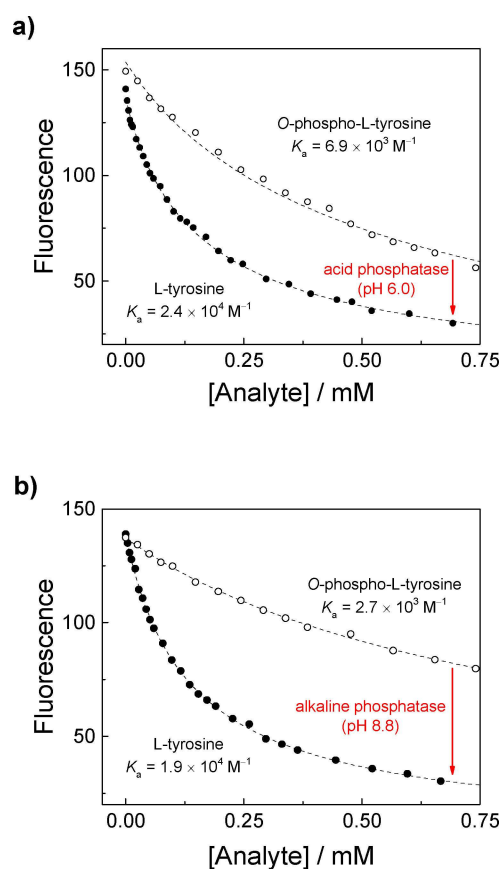


Figure 4. Fluorescence displacement titrations of L-tyrosine and O-phospho-L-tyrosine using $1.0 \mu\text{M}$ CB7 and $1.0 \mu\text{M}$ berberine in a) 10 mM NaH_2PO_4 , pH 6.0 and b) 10 mM boric acid, pH 8.8.

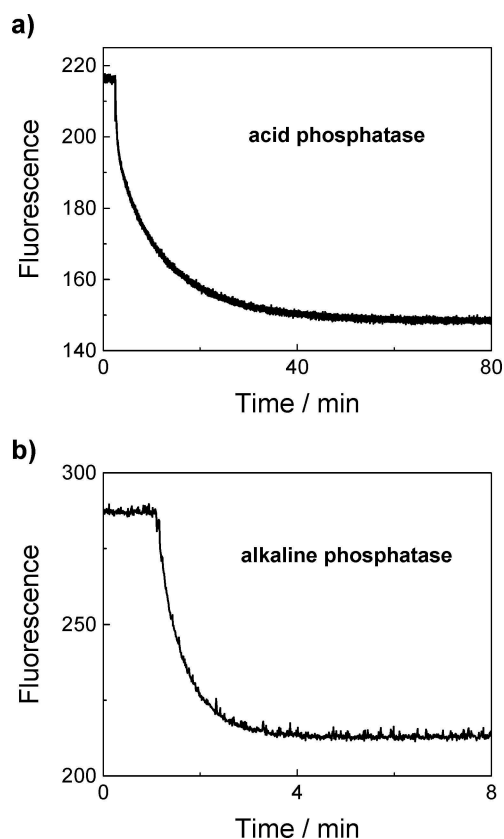


Figure 5. Fluorescence phosphatase assays with the CB7/BE reporter pair (3.0 μM CB7, 3.0 μM BE, $\lambda_{\text{exc}} = 400$ nm, $\lambda_{\text{em}} = 500$ nm) to follow dephosphorylation of 125 μM pTyr with a) 0.27 U/L acid phosphatase in 10 mM Na_2HPO_4 , pH 6.0, and b) 13.05 U/L alkaline phosphatase in 10 mM boric acid, pH 8.8.

tional experiments demonstrated that CB7-based acid and alkaline phosphatase assays can also be set up with other fluorescent dyes displaying different fluorescence output, such as Dapoxyl^[19a] and acridine orange,^[24] and that the transfer to phosphatase peptide substrates, e.g., to monitor dephosphorylation of EEEpYGE-NH₂, is possible.

In conclusion, we have established that supramolecular tandem assays afford a label-free, fluorescent method for the continuous monitoring of kinase and phosphatase activity. Unlike sensors that were designed to recognize the negatively charged phosphate group with an increased affinity, we have utilized two different cation receptors with reduced affinity after phosphorylation, which should render the assay more tolerant to phosphate cofactors required in kinase assays, such as ATP and cAMP (<5% influence on fluorescence at 1 mM ATP or cAMP). Due to their comparably low binding affinity,^[25] Mg²⁺ and Ca²⁺ also have only minor influences on the performance of the tandem assay when their concentrations are kept low in comparison to the substrate concentrations. Overall, this renders the tandem assay very attractive for drug discovery due to its potential to be scaled up to high-throughput screening (HTS) format.

Experimental Section

Reagents and compounds for buffer preparation and analytical measurements including CX4, acid phosphatase (from sweet potato, ammonium sulfate suspension), alkaline phosphatase (from bovine intestinal mucosa), and protein kinase A (from bovine heart) were from Sigma-Aldrich (Steinheim, Germany). CB7 was prepared according to the literature procedure.^[24] Peptides were from BIOSYNTAN GmbH (Berlin, Germany) and obtained in >95% purity as confirmed by HPLC and MS by the supplier. For all experiments, Millipore water (<18.2 M Ω cm) from an ELGA Labwater Classic water purification system was used. Buffers were prepared from solid HEPES, boric acid, sodium dihydrogen phosphate and the pH was adjusted by addition of NaOH. Peptide and amino acid stock solutions were prepared in water and their concentration was determined using the extinction coefficient of tryptophan at 280 nm ($\epsilon = 5540 \text{ M}^{-1} \text{ cm}^{-1}$) and that of tyrosine at 280 nm ($\epsilon = 1280 \text{ M}^{-1} \text{ cm}^{-1}$). Absorption spectra were recorded with a Varian Cary 4000 spectrophotometer and fluorescence was measured with a Varian Cary Eclipse equipped spectrofluorimeter with temperature controller. All spectroscopic measurements were performed in 0.5 ml or 3.5 ml quartz glass cuvettes from Hellma Analytics (Mülheim, Germany). The fluorescence displacement titrations were analyzed with a previously reported competitive binding model assuming a 1:1 host-competitor complex.^[21]

Acknowledgements

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: fluorescence · enzyme assay · host-guest chemistry · kinase · phosphatase

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