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Array-based fluorescence assay for serine/threonine kinases using specific chemical reaction

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ABSTRACT

We report herein the development of an efficient fluorescence assay for serine/threonine kinases using a peptide array. Our approach is based on chemical reactions specific to phosphoserine and phosphothreonine residues, that is, base-mediated β-elimination of the phosphate group and subsequent Michael addition of a thiol-containing fluorescent reagent. This procedure enables the covalent introduction of a fluorescent moiety into the phosphorylated peptide. Novel fluorescent reagents were designed for this purpose and synthesized. With these reagents, protein kinase A (PKA) and Akt-1 activities were readily detected. Our method can also be used to measure the activity of kinase inhibitors. This assay is expected to be widely applicable in kinase research.

1. Introduction

Protein kinases catalyze phosphoryl group transfer from adenosine 5′-triphosphate (ATP) to serine, threonine and/or tyrosine residues of target proteins, and play an important role in intracellular signal transduction. Protein kinases represent an emerging class of drug targets, since malfunctions of cellular signaling have been associated with many common diseases, such as cancers, cardiovascular disease, and inflammation.[1,2] Five hundred and eighteen kinase genes have been identified, which correspond to 1.7% of all human genes.[3] To study and differentiate these enzymes, a robust and efficient kinase assay for drug screening and mechanism studies is needed. Considerable efforts have been made to develop analytical techniques for high-throughput screening of kinase activity and kinase-related drugs.[4,5] Although various methods have been reported, the major detection principles can be classified into three categories: radiolabel, antibody, or phosphate chelator.

The first methodology is based on radiolabeling of target peptides or proteins using γ-^{32}P/^{33}P-ATP.[6–15] Kinase activity is measured in terms of the incorporation of ^{32}P or ^{33}P into a kinase substrate. This method is popular, but radioisotopes present a potential risk to human health and to the environment. Cost is also a concern. Alternative nonradioactive methods are considered preferable.

Antibodies directed against phosphorylated residues are also commonly used.[7,16–18] The readout may be fluorescence, luminescence, or polarization measurements. Antibody-based fluorescence kinase assays are especially useful for tyrosine kinases, for which high-affinity antibodies are available. However, antibodies that bind phosphoserine and phosphothreonine are generally less readily available, which is a problem in the development of antibody-based methods for the majority of known kinases.

Recently, several methods using phosphate chelators[19–21] have been reported, such as Zn^{2+}-based artificial phosphate receptors,[22–28] peptide substrate bearing a phosphate sensor,[29–32] metal ion-coated particles,[33] or fluorescent polymers.[34] These probes are elegantly designed, but the major disadvantage is that anionic compounds, such as ATP, phospholipids, and other carboxylate-containing compounds, compete with the phosphopeptide for binding to the metal ions. Also the affinity and sensitivity could be dependent upon the amino acid sequence of the target phosphopeptide.[23] These drawbacks limit the assay to low ATP concentrations and reduce the signal-to-background ratio.

Several groups, including ours, have also reported chemical methods to detect phosphate using mass spectrometry[35–37] and fluorescence.[38,39] The labeling methods can be summarized as follows: (1) β-elimination of phosphoserine and phosphothreonine residues followed by Michael addition,[35,37,38] and (2) modification of all electrophilic side chains followed by selective deprotection and relabeling of the phosphate.[36,39] We have reported a fluorescence-based on-bead assay using the former strategy.[38] This methodology overcomes some of the limitations of previous techniques. Our method is nonradioactive; selective fluorescent derivatization of phosphorylated residues is possible; and no side reaction with anionic compounds occurs. However, several problems emerged. The major drawback is poor compatibility of kinases with the

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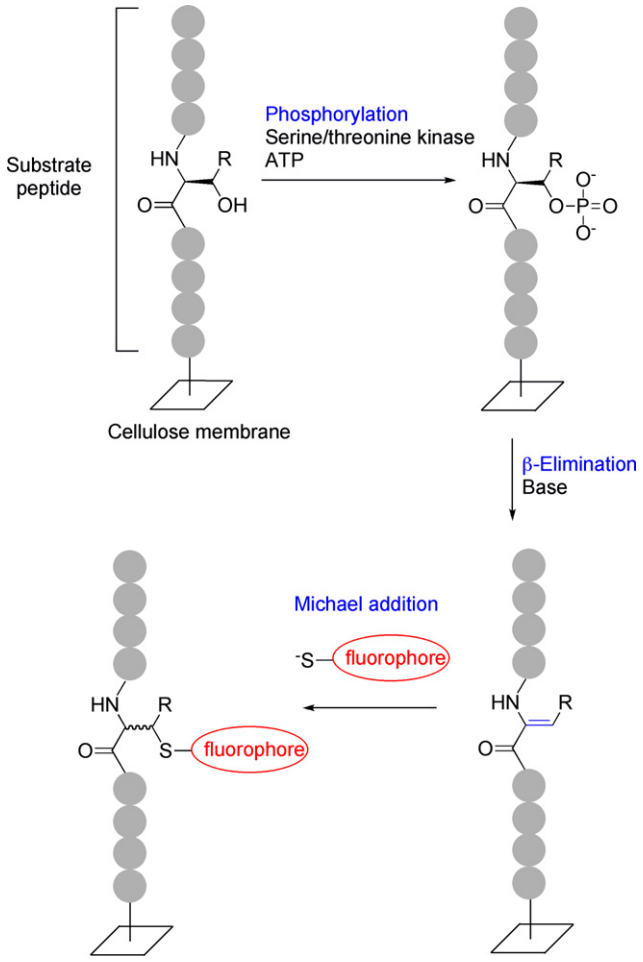
peptide-bound resin beads. The efficiency of enzymes in transforming substrates that are bound on solid supports is strongly influenced by the permeability of enzymes into gel network. The $PEGA_{1900}$ resin we used was compatible with relatively small kinases, but problems arose with larger enzymes.[40,41] Another drawback is the efficiency; this on-bead assay is difficult to use in a high-throughput format because of the fluorescence microscope-based detection. Therefore, we considered application of this methodology to peptide arrays to circumvent these limitations.

The translation of conventional solution-phase assays into immobilized formats has markedly increased the rate and scope of discoveries in basic science and biotechnology. Peptide arrays have recently generated widespread interest, because many enzymatic processes, including kinase activities,[7,12–14,28,42–45] can be studied using peptides as model substrates. However, the detection of kinase activity was carried out using RI, antibody or phosphate-selective Zn^{2+}-complex, which have the drawbacks mentioned above. The peptide array seems more compatible with enzymatic reaction than bead-bound peptide, and should be suitable for a high-throughput format. We described herein a novel and efficient fluorescence assay of kinases using peptide arrays.

2. Results and discussion

2.1. Strategy for detection of phosphorylated peptides

Our approach is summarized in Scheme 1. After phosphorylation of solid-supported substrate peptides by the corresponding kinases, each phosphorylated peptide is selectively transformed into a fluorescent derivative in two steps. The reactions are the base-mediated β-elimination of the phosphate group, followed by the Michael addition of a thiol-containing fluorescent molecule. In order to make this strategy more practical, the use of substrate peptide array was examined. As a solid support, a glass slide[6–10] or a cellulose membrane[11–15,44,46,47] has been widely used for the preparation of peptide arrays.



Scheme 1. Strategy for detection of serine/threonine kinase activity. R = H: serine, R = CH_3: threonine.

We chose a cellulose membrane because the hydrophilic nature of cellulose is expected to suppress non-specific interaction of the fluorescent reagent with the solid support. Also, the preparation of peptide arrays on cellulose membrane has been established as 'SPOT'[46,47] synthesis and the compatibility of cellulose with kinase reactions was suggested by several researchers using radiolabel detection[11–15,44] or antibody-based detection.[18]

2.2. Design and synthesis of fluorescent reagents

For on-bead assay, we previously developed thiol-containing coumarin derivatives.[38] These molecules worked well for on-bead assay, but were not appropriate for the array-based assay. Coumarin derivatives typically have an excitation maximum around 360 nm, which is not compatible with a standard microarray scanner. Thus, we undertook the development of new fluorescent reagents.

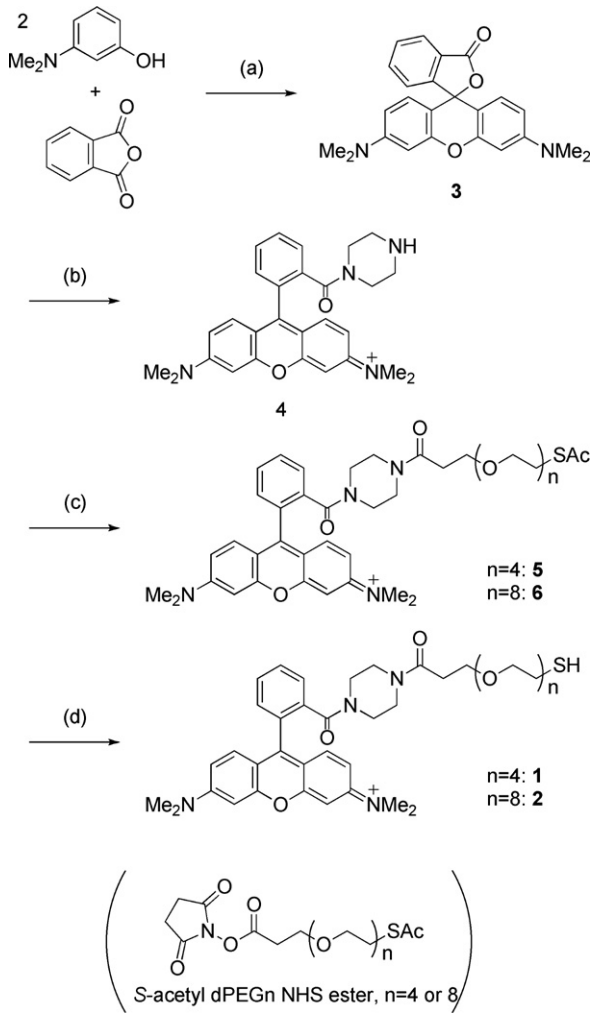
Tetramethylrhodamine was chosen as a fluorophore, because the fluorescence of rhodamine derivatives can be readily detected with standard microarray scanner. Compounds **1** and **2** were designed as new fluorescent reagents for peptide-array based assay (Scheme 2). A thiol group, which is necessary for the Michael addition, was introduced via hydrophilic oligo(ethylene glycol) linker. The hydrophilicity of the linker would be expected to suppress non-specific interaction of the fluorescence compounds with the solid support and to increase the reactivity. The designed molecules were synthesized according to Scheme 2, and purified with reverse-phase HPLC (RP-HPLC).

2.3. Preparation of peptide array

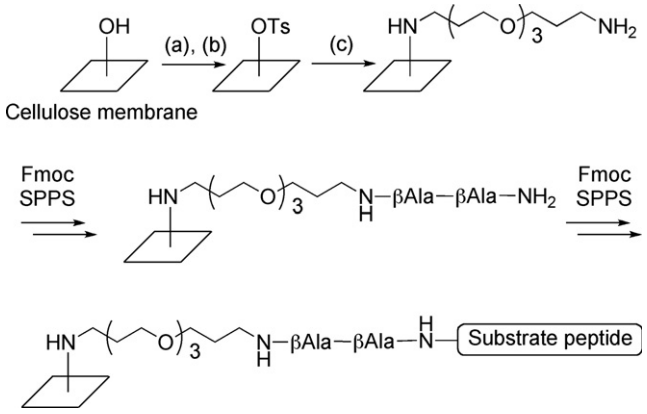
Peptide arrays prepared by SPOT synthesis are generally base-cleavable.[46,47] Since our strategy requires base treatment, a modified cellulose membrane was needed. The base-stable peptide array was generated according to Scheme 3. The amino-modified membrane was reported as an intermediate of an acid-cleavable linker-derivatized support.[48] A flexible diamino 'spacer' unit, 4,7,10-trioxa-1,13-tridecanediamine, was introduced onto the tosylated support through standard nucleophilic substitution. The additional βAla-βAla spacer, which is frequently used in SPOT synthesis, was incorporated before the synthesis of the substrate peptides. The substrate peptides were synthesized by standard Fmoc solid-phase peptide synthesis. The peptide sequences used in this study are summarized in Table 1.

2.4. Detection of phosphorylated peptides

Using the synthesized fluorescent reagents, we examined the detection of phosphorylated peptides. As a model peptide, a well-known PKA consensus sequence 'Kemptide'[49] was chosen. Phosphorylated peptide (Ac-LRRApSLG; PKA-pS) and non-phosphorylated peptide (Ac-LRRASLG; PKA-S) were synthesized on a cellulose membrane. Alanine substituted peptide (Ac-LRRAALG; PKA-A) was also synthesized as a negative control. These peptides were treated with base and then fluorescent reagent **1** or **2**. The fluorescence from the membrane was observed with a fluorescence image scanner (Typhoon 9400, GE Healthcare UK Ltd).



Scheme 2. Synthesis of fluorescent reagents. Reagents and conditions: (a) 180 °C, 29%; (b) piperazine, $AlMe_3$, CH_2Cl_2, 0 °C → reflux, 54%; (c) *S*-acetyl-dPEG$_n$ NHS ester (n = 4, 8), triethylamine, CH_3CN, 71% (for **5**), 60% (for **6**); (d) NaOH, H_2O/CH_3OH, 86% (for **1**), 80% (for **2**).



Scheme 3. Preparation of base-stable peptide array on cellulose membrane. Reagents and conditions: (a) 20% TFA in CH_2Cl_2, rt; (b) 2 M TsCl in pyridine, rt; (c) 4,7,10-trioxa-1,13-tridecanediamine, 80 °C.

As shown in Figure 1, the fluorescence from a spot of phosphorylated peptide was stronger than that of non-phosphorylated peptides. This result indicated that our method could successfully derivatize phosphorylated peptides to fluorophore-containing peptides. The fluorescence of PKA-S differed slightly from that of non-phosphorylated PKA-A, suggesting that the non-specific interaction of the fluorescent reagent may be peptide sequence-dependent, although the difference was small. In the case of cysteine-containing substrate peptides, the thiol group of the reagents might form a disulfide linkage with the substrate peptides. However, the oxidation of thiols could be suppressed by the addition of a reducing agent during Michael addition, as we did in this work. The yields of β-elimination and Michael addition were difficult to estimate on cellulose membrane. In solution, the yield of β-elimination was approximately 80% determined by HPLC.[38] The condition of Michael addition was optimized for this assay and the yield was supposed to be low, since greater introduction of rhodamine chromophore resulted in fluorescence self-quenching. Although such reaction conditions might lead to experimental error, the results presented in this manuscript were well reproducible. Compound **2** gave a slightly better signal-to-noise ratio than **1**, probably because the longer hydrophilic linker of **2** would facilitate the Michael addition and/or suppress non-specific interaction with the solid support. Therefore, we chose compound **2** for further investigation.

Table 1
Cellulose-immobilized peptides and their sequences

Code	Target kinase	Sequence	Comment
PKA-A	PKA	Ac-LRRAALG	(Negative control)
PKA-S	PKA	Ac-LRRASLG	(Substrate)
PKA-pS	PKA	Ac-LRRApSLG	(Positive control)
Akt-A	Akt-1	Ac-RPRAAAF	(Negative control)
Akt-S	Akt-1	Ac-RPRAASF	(Substrate)
Akt-pS	Akt-1	Ac-RPRAApSF	(Positive control)

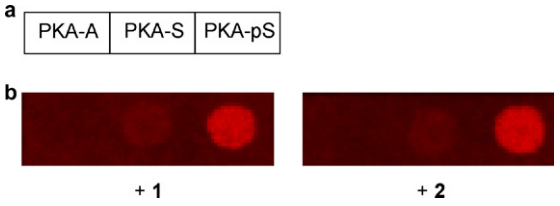


Figure 1. Detection of phosphorylated peptide. (a) Spotting pattern of peptides (Ac-LRRAXLGβAβA, pS: X = pS; S: X = S; A: X = A). (b) Fluorescence images (λ_{ex}: 532 nm, λ_{em}: 565–595 nm) of cellulose membranes treated with base and **1** or **2**.

2.5. Detection of kinase activity

The activity of PKA and Akt-1 was evaluated using suitable substrate peptides (Kemptide[49] for PKA, Ac-RPRAASF[50] for Akt-1) synthesized on a cellulose membrane. PKA and Akt-1 are well studied, representative serine/threonine kinases. For the detection of their activity, the substrate peptide array was incubated with PKA or Akt-1. Incubation in the absence of kinase was also performed as a negative control. These membranes were treated with base and then with compound **2**. As shown in Figure 2b, the spot of PKA substrate peptide (PKA-S) showed obviously stronger fluorescence than the spot of PKA-S on the control membrane. Detection of Akt-1 activity was also successful. The molecular weight of Akt-1 is approximately 85 kDa, which is too large for on-bead assay to detect the activity.[40,41] As shown in Figure 2d, the fluorescence from Akt-1 substrate (Akt-S) was clearly stronger than that on the control membrane. This result demonstrated that the on-cellulose assay is useful for the detection of large enzymes. The quantitative data are shown in Figure 2e. These results indicated that our simple methodology is useful for the fluorescence detection of kinase activity.

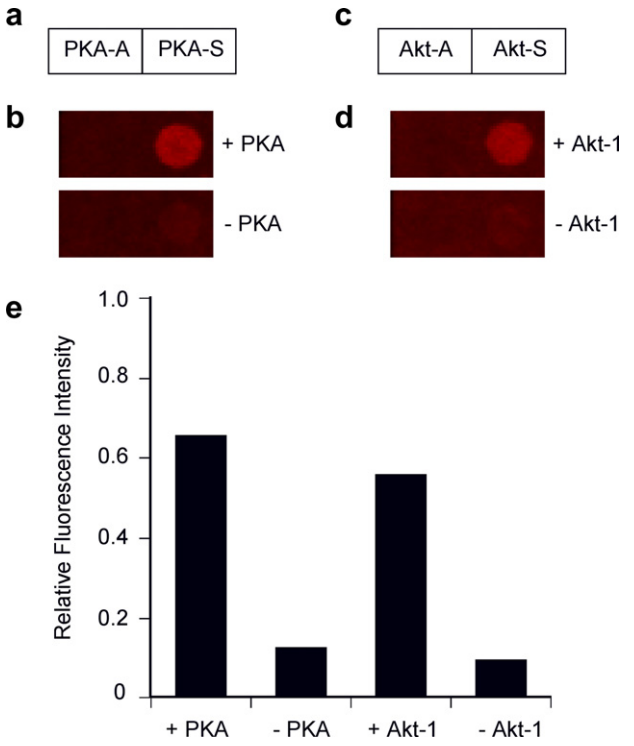


Figure 2. Detection of kinase activity. (a) Spotting pattern of PKA substrate peptides. (b) Fluorescence images of cellulose membranes incubated with or without PKA, treated with base and **2**, and detected with a fluorescence scanner (λ_{ex}: 532 nm, λ_{em}: 565–595 nm). (c) Spotting pattern of Akt-1 substrate peptides. (d) Fluorescence images of cellulose membranes incubated with or without Akt-1, treated with base and **2**, and detected with a fluorescence scanner (λ_{ex}: 532 nm, λ_{em}: 565–595 nm). (e) Relative fluorescence intensity of substrate peptides [(fluorescence intensity of substrate peptide (S) – negative control peptide (A))/(fluorescence intensity of positive control peptide (pS) – that of negative control peptide (A))].

As regards detection sensitivity, it is quite difficult to compare the present method directly with other reported methods, since there are many differences in the reaction conditions employed. Among the reported assays, that of Shigaki et al. seems to be among the most sensitive, and the reaction conditions are similar to ours.[28] They developed a peptide microarray to detect protein kinase activity even in cell lysates by using biotinylated Phos-tag (a Zn^{2+}-based artificial phosphate receptor) and fluorescently-labeled streptavidin. In the phosphorylation reaction for PKA, they used 200 U/mL of PKA and obtained a five- to sixfold increase in fluorescence. In our case, 100 U/mL PKA gave a similar fluorescence enhancement, suggesting that our assay has comparable sensitivity with this reported methodology.

The peptide array is also useful for the determination of the substrate specificity of kinases. Several groups have reported the discovery of specific substrate peptides of various kinases by using SPOTs membrane and radiolabel-based detection of phosphorylation.[12–14,44] Although an array-based technique is not suitable for measurement of kinetic constants, the results obtained with SPOTs were in good agreement with the values obtained using the conventional methods.[12–14,44] Thus, our method could be useful in initial screening to identify substrate peptides.

2.6. Effect of kinase inhibitor

We also examined the effect of kinase inhibitor. A well-studied kinase inhibitor, staurosporine, was added at various concentrations (0–50 μM) and incubated with PKA. After the usual procedure, the fluorescence intensities of the spots were observed. The fluorescence intensity was weaker in the presence of the inhibitor, and was inversely correlated with the inhibitor concentration, suggesting that the kinase activity could be monitored in terms of fluorescence intensity (Fig. 3b). The IC_{50} value was calculated as 2 μM (Fig. 3c), which is different from the reported values (15 nM,[51,52] and 0.12 μM[53]). The inhibition of protein kinases by staurosporine is known to be competitive with respect to ATP.[51,54] Thus, the IC_{50} depends on the intrinsic affinity of the inhibitor (the dissociation constant, K_i), as well as the competition from ATP under the specific assay conditions, namely ATP concentration ([ATP]) and K_m of the kinase for ATP ($K_{m,ATP}$). The IC_{50} and K_i are related to each other by the Cheng–Prusoff equation[55,56]:

$$IC_{50} = K_i(1 + [ATP]/K_{m,ATP})$$

The reported K_i value of staurosporine for PKA is 5 nM,[51] and the reported IC_{50} value, 0.12 μM,[53] can be converted to a K_i value of 67 nM, since the $K_{m,ATP}$ value for PKA was reported to be 14 μM.[51] Our IC_{50} value can be similarly converted to a K_i of 250 nM, which is still different from the reported values. The differences in reaction conditions (phosphorylatable substrate, reaction time, buffer conditions, etc.) could explain the difference between our result and the values reported previously. In addition, substrate peptide immobilization could affect the K_i value. These results suggest that our method can be used for initial inhibitor screening, but additional experiments would be necessary for detailed characterization of active compounds.

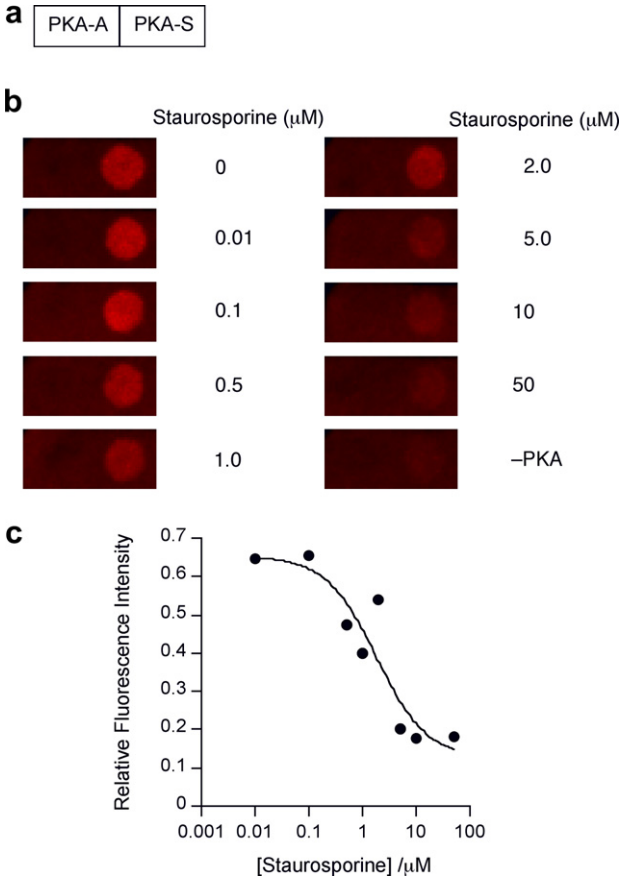


Figure 3. Effect of PKA inhibitor, staurosporine. (a) Spotting pattern of PKA substrate peptides. (b) Fluorescence images of cellulose membranes, incubated with PKA in the presence of staurosporine. The membranes were treated with base and **2**, and detected with a fluorescence scanner (λ_{ex}: 532 nm, λ_{em}: 565–595 nm). (c) Inhibition of PKA activity by staurosporine. The images shown in (b) were quantified.

3. Conclusion

We have developed an efficient fluorescent assay for serine/threonine kinases using a peptide array, which enables simultaneous measurement of kinase activities. Our strategy combines unique chemical reactions of phosphoserine and phosphothreonine with the array technology, and is more general and efficient than the previously reported on-bead assay. The activity of a large kinase, Akt-1 (85 kDa), could be readily detected, which was impossible with the on-bead assay. Although development of our assay is at an early stage, potential applications include enzyme functional analysis and drug discovery. Further study is currently under way in our laboratory.

4. Experimental

4.1. General

All reagents and solvents were of the highest commercial quality and were used without further purification. PKA (EC 2.7.11.11; catalytic subunit from bovine heart) and Akt-1 (EC 2.7.11.1; active human) were purchased from Sigma–Aldrich, and used without activation. Melting point (Mp) determinations were performed with a Yanaco Micro Melting Point Apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a JEOL GSX-400 at 400 MHz. ^{13}C NMR spectra were recorded on either a JEOL JNM-LA 500 or a Bruker AVANCE 600 at 125 MHz or 150 MHz, respectively. Chemical shifts are expressed as parts per million (ppm) using solvent as the internal standard. Infrared (IR) spectra were recorded on a JASCO FT/IR-680 Fourier-transform infrared spectrophotometer. Electrospray ionization mass spectra (ESI-MS) were measured with a Bruker APEX III mass spectrometer. High-resolution mass (HRMS) data were obtained with a Bruker APEX III mass spectrometer (ESI) and JEOL JMS-SX102A mass spectrometer (FAB). Fluorescence images were obtained with a Typhoon 9400 image scanner (GE Healthcare UK Ltd, Amersham Place, England). Preparative RP-HPLC was performed using a Shimadzu LC-6AD pump and a Shimadzu SPD-M10AVP variable-wavelength UV detector. For chromatographic separations, an Inertsil ODS-3 column (10 × 250 mm, GL Sciences) was employed.

4.2. Synthesis of fluorescent reagents

4.2.1. Synthesis of 3

N,*N*-dimethyl-3-aminophenol (5.49 g, 40.0 mmol) and phthalic anhydride (2.96 g, 20.0 mmol) were mixed thoroughly, and the mixture was stirred for 2.5 h at 180 °C. It was then cooled to rt, and the crude product was purified by silica gel column chromatography (10% MeOH/CH_2Cl_2). The product was dissolved in 600 mL of 1 N NaOH, and the solution was saturated with NaCl and extracted with EtOAc (3× 400 mL). The combined organic layers were washed with brine (300 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure to give a purple solid (2.22 g, y. 29%). Mp: 219 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.00 (d, J = 7.3 Hz, 1H), 7.63 (t, J = 7.3 Hz, 1H), 7.57 (t, J = 7.3 Hz, 1H), 7.18 (d, J = 7.3 Hz, 1H), 6.60 (d, J = 8.9 Hz, 2H), 6.48 (d, J = 2.5 Hz, 2H), 6.39 (dd, J = 8.9, 2.5 Hz, 2H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 169.9, 153.3, 152.9, 152.0, 134.6, 129.2, 128.7, 127.5, 124.7, 124.0, 108.6, 106.8, 98.5, 40.2. IR (neat, cm^{-1}): 2896, 1753, 1614, 1520, 1429, 1111. HRMS (ESI$^+$): calcd for $C_{24}H_{23}N_2O_3$ $(M+H)^+$ 387.1703, found 387.1698.

4.2.2. Synthesis of 4

Compound **4** was synthesized according to the reported procedure[57] with slight modifications. A 1.8 M solution of trimethylaluminum in toluene (4.5 mL, 8.1 mmol) was added dropwise to a solution of piperazine (1.35 g, 15.6 mmol) in 6 mL of CH_2Cl_2 at 0 °C under an Ar atmosphere. The solution was warmed to rt and stirred for 60 min. A solution of **3** (1.00 g, 2.59 mmol) in 5 mL of CH_2Cl_2 was added dropwise, and the whole was refluxed for 24 h. It was then cooled to 0 °C, and 0.1 N HCl was added dropwise to quench the reaction. The resulting solution was evaporated. The residue was diluted in 300 mL of 0.5 N HCl, and washed with CH_2Cl_2 (4× 150 mL), then the aqueous layer was saturated with NaCl and extracted with isopropanol/CH_2Cl_2 (2:1) (6× 150 mL). The combined organic layers were dried over anhydrous Na_2SO_4 and evaporated. The residue was dissolved in 300 mL of 2.5% aqueous solution of Na_2CO_3, saturated with NaCl, extracted with CH_2Cl_2 (3× 150 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure to give a dark purple powder (0.64 g, y. 54%). Mp: >300 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.69–7.64 (m, 2H), 7.56–7.54 (m, 1H), 7.37–7.35 (m, 1H), 7.28–7.25 (m, 2H), 7.10–7.07 (m, 2H), 6.82 (s, 2H), 3.42–3.33 (m, 16H), 2.67 (br s, 4H). ^{13}C NMR (125 MHz, CD_3OD): δ 169.4, 159.0, 157.8, 157.6, 136.9, 132.8, 132.4, 131.8, 131.5, 131.4, 128.9, 115.5, 115.0, 97.5, 46.7, 45.0, 41.0. IR (KBr, cm^{-1}): 3399, 1597, 1494, 1408, 1348, 1190, 1135. HRMS (ESI$^+$): calcd for $C_{28}H_{31}N_4O_2$ $(M)^+$ 455.2442, found 455.2438.

4.2.3. Synthesis of 5

A 100 mg/mL solution of *S*-acetyl-dPEG$_4$ NHS ester (Quanta BioDesign, OH, USA) in MeOH (200 μL, 48 μmol) was added to a solution of **4** (26 mg, 57 μmol) in 3 mL of CH_3CN, and the mixture was stirred for 24 h at rt. It was then evaporated, the residue was dissolved in 1.7 mL of H_2O, and the product was purified by RP-HPLC with a linear gradient from 38% to 40% CH_3CN containing 0.1% TFA in H_2O containing 0.1% TFA for 30 min at a flow rate of 1 mL/min to give a dark purple, sticky oil (30 mg, y. 71% as a TFA salt). ^{1}H NMR (400 MHz, CD_3OD): δ 7.79–7.76 (m, 2H), 7.71–7.67 (m, 1H), 7.52–7.47 (m, 1H), 7.30 (d, J = 9.5H, 2H), 7.10 (dd, J = 9.5, 2.4 Hz, 2H), 6.95 (d, J = 2.4 Hz, 2H), 3.70 (t, J = 6.1 Hz, 2H), 3.61–3.52 (m, 14H), 3.43 (br s, 4H), 3.40 (br s, 4H), 3.31 (s, 12H), 3.00 (t, J = 6.6 Hz, 2H), 2.60 (t, J = 6.1 Hz, 2H), 2.29 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 195.7, 170.2, 167.7, 157.5, 157.4, 135.1, 131.9, 131.6, 130.4, 130.3, 130.1, 127.6, 114.4, 114.2, 113.9, 113.8, 96.9, 96.6, 70.3, 70.2, 70.1, 69.7, 66.9, 47.5, 47.1, 45.5, 45.0, 40.9, 33.3, 30.6, 28.7. IR (neat, cm^{-1}): 2919, 1692, 1633, 1597, 1495, 1410, 1349, 1189, 1133. HRMS (ESI$^+$): calcd for $C_{41}H_{53}N_4O_8S$ $(M)^+$ 761.3579, found 761.3576.

4.2.4. Synthesis of 6

A 100 mg/mL solution of *S*-acetyl dPEG$_8$ NHS ester (Quanta BioDesign, OH, USA) in MeOH (240 μL, 40 μmol) and triethylamine (4 mg, 40 μmol) was added to a solution of **4** (20 mg, 44 μmol) in 3 mL of CH_3CN, and the mixture was stirred for 16 h at rt. It was then evaporated, the residue was dissolved in 3.0 mL of H_2O, and the product was purified by RP-HPLC with a linear gradient from 38% to 40% CH_3CN containing 0.1% TFA in H_2O containing 0.1% TFA for 30 min at a flow rate of 1 mL/min to give a dark purple, sticky oil (25 mg, y. 60% as a TFA salt). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.72–7.70 (m, 2H), 7.71–7.67 (m, 1H), 7.56–7.54 (m, 1H), 7.39–7.38 (m, 1H), 7.27–7.25 (m, 2H), 6.97–6.80 (m, 4H), 3.73 (t, J = 6.3 Hz, 2H), 3.69–3.59 (m, 30H), 3.40–3.27 (m, 20H), 3.09 (t, J = 6.3 Hz, 2H), 2.57 (t, J = 6.3 Hz, 2H), 2.33 (s, 3H). ^{13}C NMR (150 MHz, $CDCl_3$): δ 195.6, 170.2, 167.7, 157.5, 157.4, 135.1, 132.0, 131.6, 130.4, 130.3, 130.1, 127.6, 114.5, 114.2, 113.9, 113.8, 97.0, 96.6, 70.5, 70.4, 70.3, 69.7, 67.0, 47.5, 47.1, 45.5, 45.1, 40.9, 33.1, 30.6, 28.8. IR (neat, cm^{-1}): 2873, 1691, 1634, 1597, 1496, 1410, 1349, 1190, 1123. HRMS (ESI$^+$): calcd for $C_{49}H_{69}N_4O_{12}S$ $(M)^+$ 937.4627, found 937.4617.

4.2.5. Synthesis of 1

A solution of **5** (28 mg, 32 μmol) in 4 mL of MeOH was degassed by means of freeze–pump–thaw cycles (three times). Then 1.2 mL of 1 N NaOH (1.2 mmol) was added dropwise, and the solution was stirred for 30 min at rt under an Ar atmosphere. It was then cooled to 0 °C, and 0.65 mL of 2 N HCl was added to quench the reaction. MeOH in the reaction mixture was removed by evaporation, and tris(2-carboxyethyl)phosphine hydrochloride (TCEP) was added to the remaining solution. This solution was neutralized with 1 N NaOH, then stored in a refrigerator overnight, and the product was purified by RP-HPLC with a linear gradient from 37% to 39% CH_3CN containing 0.1% TFA in H_2O containing 0.1% TFA for 20 min at a flow rate of 1 mL/min to give a dark purple, sticky oil (23 mg, y. 86% as a TFA salt). The purity of this compound was determined as >90% by HPLC analysis. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.71–7.69 (m, 2H), 7.56–7.54 (m, 1H), 7.30–7.29 (m, 1H), 7.27–7.23 (m, 2H), 7.06–7.04 (m, 1H), 6.93–6.90 (m, 2H), 6.78 (s, 1H), 3.73 (t, J = 6.1 Hz, 2H), 3.63–3.59 (m, 14H), 3.40–3.29 (m, 20H), 2.71–2.59 (m, 4H), 1.60 (t, J = 8.3 Hz, 1H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 170.0, 167.7, 157.5, 157.4, 135.1, 132.0, 131.6, 130.4, 130.3, 130.1, 127.6, 114.5, 114.1, 113.8, 97.1, 96.6, 72.8, 70.5, 70.4, 70.3, 70.2, 67.1, 47.5, 47.1, 45.6, 45.1, 40.9, 33.4, 24.3. IR (neat, cm^{-1}): 2859, 1631, 1597, 1494, 1410, 1349, 1189, 1125. HRMS (FAB^+): calcd for $C_{39}H_{51}N_4O_7S$ $(M)^+$ 719.34784, found 719.34708.

4.2.6. Synthesis of 2

A solution of **6** (43 mg, 41 μmol) in 5 mL of MeOH was degassed by means of freeze–pump–thaw cycles (three times). Then 1.5 mL of 1 N NaOH (1.5 mmol) was added dropwise, and the solution was stirred for 30 min at rt under an Ar atmosphere. It was then cooled to 0 °C, and 1.55 mL of 1 N HCl was added to quench the reaction. MeOH in the reaction mixture was removed by evaporation, and TCEP (30 mg, 105 μmol) was added to the remaining solution. The mixture was neutralized with 1 N NaOH, then stored in a refrigerator overnight, and the product was purified by RP-HPLC with a linear gradient from 37% to 39% CH_3CN containing 0.1% TFA in H_2O containing 0.1% TFA for 20 min at a flow rate of 1 mL/min to give a dark purple, sticky oil (33 mg, y. 80% as a TFA salt). The purity of this compound was >92% as determined by HPLC. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.71–7.69 (m, 2H), 7.56–7.54 (m, 1H), 7.39–7.37 (m, 1H), 7.27–7.23 (m, 2H), 7.00–6.76 (m, 4H), 3.73 (t, J = 6.3 Hz, 2H), 3.71–3.58 (m, 30H), 3.40–3.30 (m, 20H), 2.72–2.67 (m, 2H), 2.57 (t, J = 6.3 Hz, 2H), 1.60 (t, J = 8.3 Hz, 1H). ^{13}C NMR (150 MHz, $CDCl_3$): δ 170.1, 167.7, 157.5, 157.4, 135.1, 132.0, 131.6, 130.4, 130.1, 127.6, 114.5, 114.0, 113.8, 97.1, 96.6, 72.9, 70.6, 70.5, 70.4, 70.3, 70.2, 47.5, 47.1, 45.6, 45.1, 41.0, 33.3, 24.3. IR (neat, cm^{-1}): 2872, 1633, 1597, 1495, 1410, 1349, 1189, 1133. HRMS (FAB^+): calcd for $C_{47}H_{67}N_4O_{11}S$ $(M)^+$ 895.45274, found 895.45334.

4.3. Preparation of peptide array on cellulose membrane

A sheet of cellulose membrane was amino-functionalized by a reported procedure.[48] All peptides were synthesized on cellulose according to the slight modification of the literature procedure, with C-terminal βAla-βAla as a linker.[46,47] Commonly, DIC and HOBt are used as coupling reagents for peptide synthesis on cellulose. Instead of them, we adopted HATU and HOAt because the common coupling reagents did not work well to prepare phosphorylated peptides. After peptide synthesis, the cellulose membrane was treated with a solution of 90% TFA, 3% TIPS, 2% H_2O, 1% phenol, and 4% DCM (2 × 60 min) at rt to deprotect side-chain protective groups. The size of a peptide spot was approximately 3 mm in diameter, and the distance between spots was approximately 6 mm.

4.4. Detection of phosphorylated peptide

The membrane was treated with 100 mM NaOH in H_2O/DMSO/EtOH (4:3:1) solution (2 mL) at rt for 60 min, then washed with 50 mM Tris–HCl buffer (pH 7.4) (2 mL; 2 × 1 min) and H_2O (2 mL; 4 × 2 min). The membrane was incubated with 20 μM **1** or **2** in 50 mM borate/NaOH buffer (pH 10.5) containing 0.2% *N*,*N*-dimethyldodecylamine *N*-oxide (DMDANO) (1.8 mL) at rt for 60 min on a rotating shaker. The solution was decanted off, and the sheet was washed with 50 mM Tris–HCl buffer (pH 7.5) containing 0.2% DMDANO (2 mL; 2 × 2 min), 50 mM Tris–HCl buffer (pH 7.5) containing 1 mM TCEP and 0.2% DMDANO (2 mL; 2 × 2 min, 1 × 30 min), 6 M guanidium hydrochloride and 1 mM TCEP solution (2 mL; 1 × 2 min, 1 × 30 min), H_2O (2 mL; 4 × 2 min), MeOH (2 mL; 2 × 2 min, 1 × 10 min) and CH_2Cl_2 (2 mL; 1 × 0.5 min), and dried under a stream of air. The membrane was observed with an image scanner (λ_{ex}: 532 nm, λ_{em}: 565–595 nm).

4.5. Kinase assay

The reaction vessels were rinsed with H_2O (2 mL; 3 × 0.5 min) and incubated with buffer for kinase reaction (2 mL; for PKA: 30 mM Tris–HCl (pH 7.4) containing 15 mM $MgCl_2$; for Akt-1: 50 mM Tris–HCl buffer (pH 7.5) containing 10 mM $MgCl_2$) containing 1.0 mg/mL BSA for 60 min at rt. A sheet of peptide-immobilized membrane was washed with H_2O (1.5 mL; 3 × 0.5 min) and buffer for kinase reaction containing 0.1 mg/mL BSA (1.5 mL; 3 × 0.5 min), and incubated with the same buffer (1.5 mL) at rt for 30 min on a rotating shaker. The solution was decanted off, and the sheet was incubated with the kinase reaction solution (1.2 mL; for PKA reaction: 30 mM Tris–HCl (pH 7.4) containing 100 U/mL PKA, 200 μM ATP, 15 mM $MgCl_2$, and 0.1 mg/mL BSA; for Akt-1 reaction: 50 mM Tris–HCl (pH 7.5) containing 0.93 U/mL Akt-1, 200 μM ATP, 10 mM $MgCl_2$, 0.05% 2-mercaptoethanol and 0.1 mg/mL BSA) for 2 or 6.5 h (2 h for PKA reaction, 6.5 h for Akt-1 reaction) at 37 or 30 °C (37 °C for PKA reaction, 30 °C for Akt-1 reaction) on a rotating shaker. The reaction conditions were similar to those described in the literature.[26,28,38,50] The incubation time might be rather long for general kinase reactions, but we took into account the lower reactivity of immobilized substrate peptide with kinase.[26,38] The reaction solution was then decanted off, and the paper was washed with washing solution (6 M guanidine hydrochloride, 1 mM TCEP aqueous solution) (2 mL; 2 × 2 min, 2 × 60 min at 37 °C), H_2O (2 mL; 4 × 2 min at rt), MeOH (2 mL; 4 × 0.5 min at rt) and CH_2Cl_2 (2 mL; 1 × 0.5 min at rt) on a rotating shaker. The membrane was dried under a stream of air. Procedures for detection of phosphorylation were the same as described above.

4.6. Effect of kinase inhibitor

Except for the conditions of the kinase reaction, the procedures were the same as for PKA assay. The cellulose membrane was incubated with a staurosporine-containing solution (30 mM Tris–HCl (pH 7.4) containing 100 U/mL PKA, 0–50 μM staurosporine, 100 μM ATP, 15 mM $MgCl_2$, and 0.1 mg/mL BSA) for 2 h at 37 °C. The membrane was washed, treated with base and **2**, and detected with an image scanner.

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