Label-Free and Real-Time Monitoring of Phosphatase Reactions Using a Phosphate-Specific and Fluorescent Probe

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A phosphate-specific and fluorescent probe was prepared for label-free phosphatase assays based on fluorescence polarization. By using the probe, dephosphorylation reactions of DNA and protein substrates by calf intestinal alkaline phosphatase (CIP) could effectively be monitored in real-time. Since this assay method does not require additional materials such as labeled substrates and phosphospecific antibodies to obtain fluorescence polarization signals, it is simple, cost-effective, and expected to be useful not only for measuring activity of phosphatases but also for high-throughput screening of phosphatase inhibitors.

Key Words : Fluorescence polarization, Phosphatase

Introduction

Phosphatases are a family of enzymes that catalyze the specific dephosphorylation of physiological molecules. They are an important class of enzymatic targets for new drug discovery.[1] Nonradioactive, homogeneous, and real-time monitoring methods are of particular interest for high-throughput drug screening applications. Numerous assay methods have been reported to determine the activity of phosphatases, utilizing the change of spectrometric properties such as UV absorption and fluorescence intensity during dephosphorylation reactions. The UV absorption-based assay uses *p*-nitrophenyl phosphate (pNPP) as the substrate of phosphatases.[2,3] The hydrolyzed product of pNPP, *p*-nitrophenol (pNP) has a strong absorption at 400 nm in a basic solution and the absorption can be quantified to estimate the activity of phosphatases. However, the assay based on UV-absorption of pNP is not well suited for detection of a minute (submicromolar) change of substrates in a phosphatase reaction because the extinction coefficient of pNP is about 18,000 $cm^{-1}M^{-1}$.

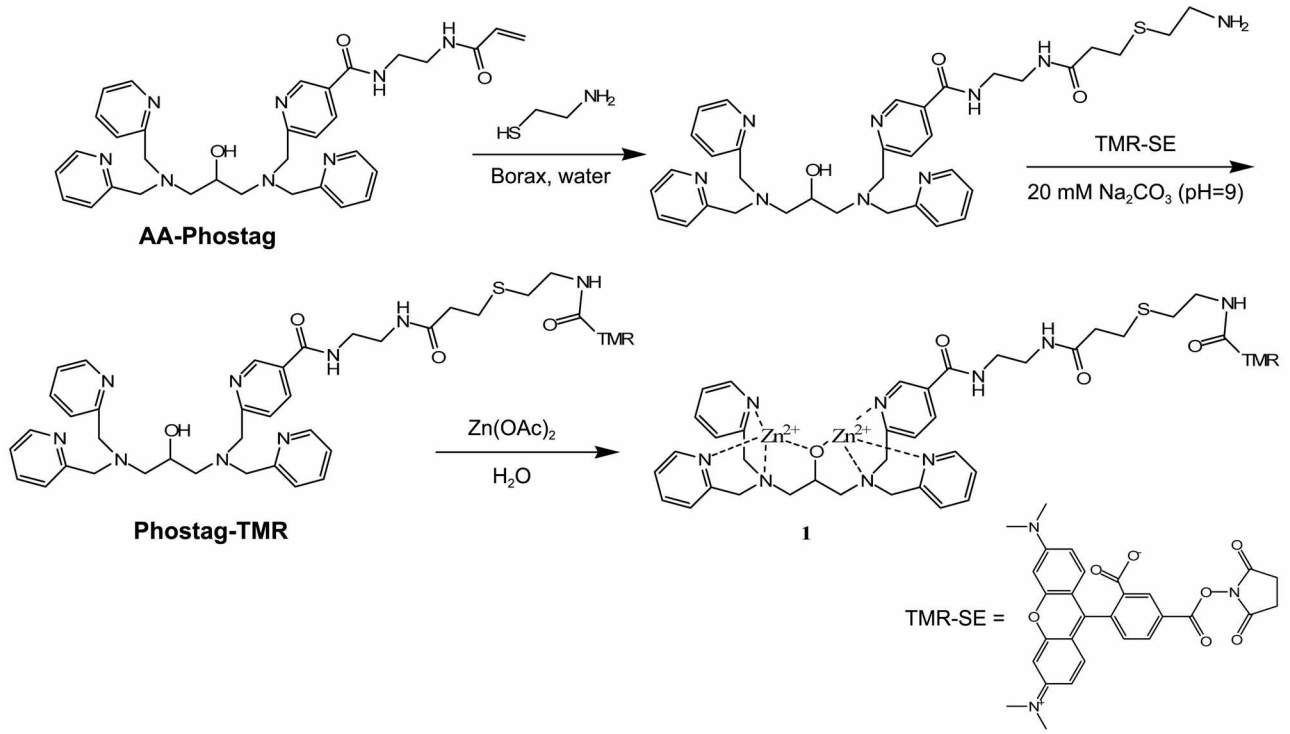
For more sensitive monitoring of phosphatase reactions, assays using substrates such as 4-methyl-7-hydroxycoumarinyl phosphate (MUP),[4-6] 3,6-fluorescein diphosphate (FDP),[7,8] and 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP) have been developed.[9,10] These methods are all based on the change of fluorescence intensity after the hydrolysis reaction on the substrates by phosphatases. Recently, fluorescence polarization-based assays have been gaining popularity since they have a lower detection limit and more reproducible signal compared with UV absorption-based and fluorescence intensity-based methods, respectively.[11] So far all the developed methods based on fluorescence polarization for phosphatase assays need substrates labeled with proper fluorophores and huge molecules that bind specifically to phosphates such as antibodies,[12,13] polyarginine,[14] or IMAP™,[15] and such requirements make the assays less straightforward. Development of methods to detect the phosphatase activity with less number of experimental steps would make assays more cost-effective and less laborious and eventually improve high-throughput screening of phosphatase inhibitors as well as measuring activity of phosphatases. Therefore, we were interested in developing a new assay method for phosphatases without fluorophores labeled on substrates by using the fluorescence polarization signal.

A strategy to make this method feasible is to use substrates with high molecular weight (> 5 kd) and a small fluorescent probe binding specifically to phosphate in phosphatase reactions. Then, the fluorescence polarization signal of the substrate-probe complex before the phosphatase reaction should be relatively higher than that of the free phosphate-probe complex after the reaction. In the present article, we report our results from studies based on the strategy.

Experimental Section

Materials. Tetramethylrhodamine succinimidyl ester was purchased from Invtrogen (Carlsbad, USA). 2-aminoethanethiol hydrochloride was purchased from TCI (Tokyo, Japan). All other chemicals were purchased from Aldrich (St. Louis, USA). AA-Phostag was synthesized according to the previous report.[16] Alkaline phosphatase calf intestinal (CIP), and the buffer (NEBuffer 2™, 50 mM NaCl, 10 mM Tric-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH = 7.9) for phoshpatase reaction were purchased from New England Biolabs (Ipswich, USA). α-casein was purchased from Sigma (St. Louis, USA). All reactions and the measurement of fluorescence polarization signals were performed in NEBuffer 2™.

Instruments. The nuclear magnetic resonance (^{1}H-NMR) spectrum of **1** was measured on Varian Unity Plus 300 (300 MHz) spectrometer using DMSO-d_6 as the solvent and reported in *ppm* relative to DMSO (δ 2.50). MALDI-TOF mass spectrum of Phostag-TMR was measured on Voyager DE-Pro (Applied Biosystem, Foster City, USA) using α-



Synthesis of 1.

cyano-4-hydroxycinnamic acid (CHCA) as the matrix. Fluorescence polarization was measured at room temperature (25 °C) by LB50B luminescence spectrometer (Perkin-Elmer, Waltham, USA). The excitation wavelength and emission wavelength were 555 and 580 nm, respectively. HPLC (1100 series, Agilent, Santa Clara, USA) and C18 (Agilent, Santa Clara, USA) column were used to separate derivatives of Phostag. Water and acetonitrile containing 0.1% TFA were used as the eluents.

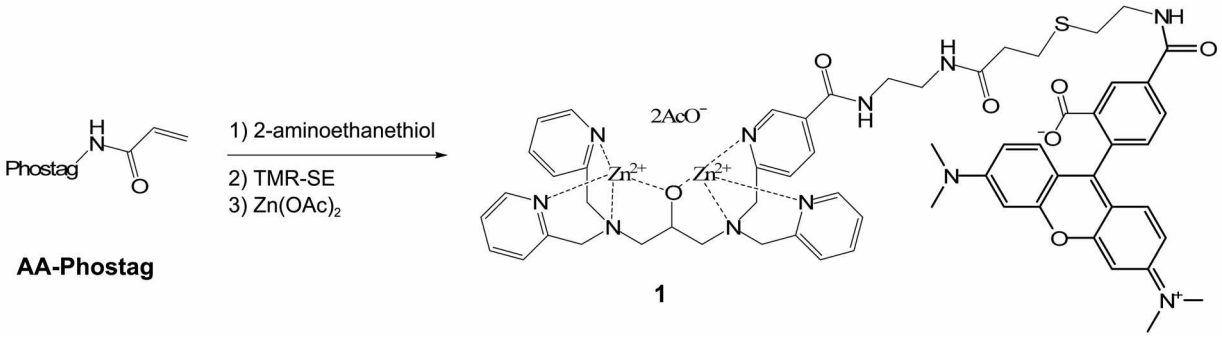
Synthesis. To the solution of **AA-Phostag** in DMSO (50 mM, 40 μL) were added borax (16 mg, 42 mmol), water (1 mL), and 2-aminoethanethiol hydrochloride (5 mg, 44 mmol) at room temperature. The mixture was stirred for 1 hr at room temperature. The phostag derivative containing amino group was obtained by reverse-phase HPLC purification. The purified compound was redissolved in DMSO (50 μL). To the solution were added tetramethylrhodamine succinimidyl ester (TMR-SE, 0.25 mg, 500 mmol) and sodium carbonate solution (20 mM, 200 μL) at room temperature. The reaction mixture was incubated at room temperature overnight. The mixture was purified by reverse-phase HPLC to obtain **Phostag-TMR** (0.2 mg, 0.2 mol). The purified **Phostag-TMR** was characterized by means of ^{1}H-NMR and MALDI-TOF mass spectrometer. Finally, two equivalent of $Zn(OAc)_2$ was mixed with a aqueous solution containing **Phostag-TMR** to yield the desired probe **1**. ^{1}H-NMR (300 MHz, DMSO-d_6) of **Phostag-TMR**: δ 9.08 (t, J = 6.0 Hz, 1H), 8.96 (d, J = 1.5 Hz, 1H), 8.78-8.70 (m, 2H), 8.62-8.54 (m, 3H), 8.32 (dd, J = 7.5, 1.6 Hz, 1H), 8.20 (dd, J = 7.8, 1.8 Hz, 1H), 8.14 (t, J = 6.1 Hz, 1H), 7.94-7.84 (m, 3H), 7.62 (d, J = 7.8 Hz, 1H), 7.54-7.39 (m, 8H), 7.06-7.02 (m, 4H), 7.00 (s, 2H), 4.38 (d, J = 3.6 Hz, 4H), 4.14 (dd, J = 13.8, 6.0 Hz, 4H), 3.39-3.16 (m, 7H), 3.29 (s, 12H), 2.91-2.73 (m, 4H), 2.59-2.44 (m, 4H), 2.30 (t, J = 2.1 Hz, 2H). MALDI-TOF-MS of **Phostag-TMR**: $[M+H]^+$ = 1085 (obs.), 1085 (calc.).

Determination of V_{max} of the dephosphorylation reaction on p-(dT)$_{20}$ by CIP. For kinetic analysis, fluorescence polarization signals were measured in 3 min for reaction samples (500 μL) with 0.1 U of CIP and **1** (300 nM) in the presence of varying concentrations of p-(dT)$_{20}$ (400, 800, 1200, 1600, and 2000 nM). After evaluation of $1/V$ versus 1/[p-(dT)$_{20}$] in a linear curve fit, V_{max} was determined from the intercept.

Results and Discussion

Previously, much effort has been made to develop phosphate-specific probes. Hamachi group have synthesized anthracene bis(zinc(II)-dipicolylamine) and used it to detect a natural phosphatase catalyzed dephosphorylation. The fluorescent probe bearing two zinc ions coordinated to distinct dipicolylamine (DPA) sites can bind and fluorescently sense phosphate derivatives in aqueous solution.[15] Alternatively, Koike group have developed Phostag, a highly selective Zn(II) chelator, which binds to phosphate or phosphomonoester *via* a charge-based coordination of the chelated Zn^{2+}.[17,18] We have chosen Phostag as the backbone of the probe for phosphatase assays based on fluorescence polarization since it has relatively high binding affinity and specificity for monoalkyl phosphate compared with anthracene-Zn(II)-DPA.[18]

An acrylamide derivative of Phostag (**AA-Phostag**) could be coupled with tetramethylrhodamine (TMR) through two



Scheme 1. Synthesis of the phosphate-specific and fluorescent probe (**1**). Detailed experimental procedures were described in the Experimental Section.

synthetic steps, making the probe fluorescent as well as specific for phosphates (Scheme 1). Treatment of the **AA-Phostag** with 2-aminoethanethiol in aqueous borax solution resulted in the amine-functionalized ligand, which was subsequently coupled with TMR via an amide bond. The resulted fluorescent ligand was then complexed with two molar equivalent of $Zn(OAc)_2$ in water to produce the desired probe **1**.

First of all, we analyzed binding specificity of the probe to a DNA substrate in order to determine proper concentrations of the probe to be used for the phosphatase assay. The fluorescent probe was incubated at 200 nM with increasing amounts of either deoxythimidine 20-mer with 5'-end phosphorylated (p-dT_{20}) or unphosphorylated (dT_{20}). When the unphosphorylated DNA (dT_{20}) was added to the buffer solution containing **1**, no significant binding was observed. In contrast, when we added the phosphorylated DNA, p-$(dT)_{20}$ to the buffer solution containing the probe, we observed a drastic increase in the fluorescence polarization of the probe with a K_d of 128 nM (Figure 1). The discriminative association of **1** with the phosphorylated DNA over the unphosphorylated DNA is due to the specificity of Phostag for monophosphate dianions in the presence of phosphodiester groups according to the previous report.[19]

Next, we performed phosphatase assays using p-$(dT)_{20}$ as a phosphorylated DNA substrate. The DNA substrate was mixed with calf intestinal phosphatase (CIP) in a reaction buffer at room temperature. The probe **1** was added to the reaction mixture after 10 min, and fluorescence polarization was measured. Compared with the fluorescence polarization value of the same mixture without CIP, the polarization value after the phosphatase reaction was relatively low, thereby suggesting that dephosphorylation could easily be detected (Figure 2a). To test this assay method for protein substrates, a phosphatase reaction was carried out with α-casein, a phosphoprotein, in the same condition except with increased amount of CIP. The fluorescence polarization signal of the phosphatase reaction mixture was again significantly decreased from the signal of the same mixture without CIP (Figure 2b). An increased difference in the fluorescence polarization signal was observed compared with that in the case of the assay using p-$(dT)_{20}$ as the substrate. This is in accordance with our expectation since the molecular weight of α-casein (23 kd) is far bigger than the DNA

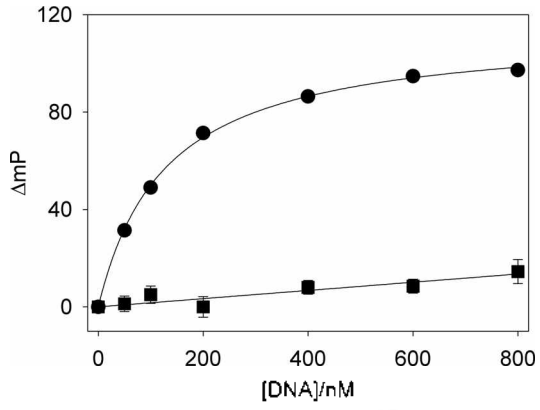


Figure 1. Binding affinity measurements for **1** to p-$(dT)_{20}$ and $(dT)_{20}$. The probe **1** (200 nM) was mixed with increasing concentrations of p-$(dT)_{20}$ (circle) or $(dT)_{20}$ (square) (0, 50, 100, 200, 400, and 800 nM) in a buffer (500 μL, 50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH = 7.9), and their fluorescence polarization changes were measured. ΔmP denotes the net difference of fluorescence polarization values of a sample with and without DNA at the specified concentration. Each data point represents the average value of two independent experiments.

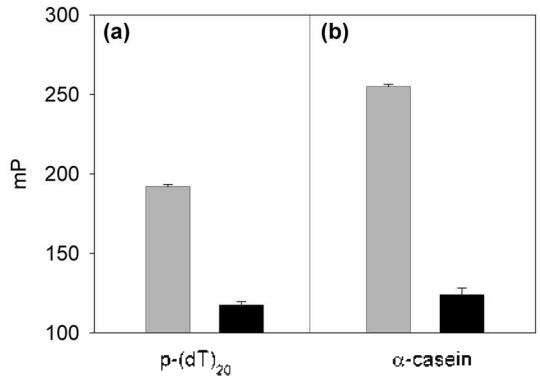


Figure 2. Phosphatase reactions on (a) p-$(dT)_{20}$ (300 nM) and (b) α-casein (300 nM). The concentration of **1** used was 300 nM. The amount of CIP used was 0.1 U and 1 U for p-$(dT)_{20}$ and a-casein, respectively. Reactions were performed in the buffer (500 μL, the same buffer as in Figure 1) at room temperature for 10 min. Black and gray bars denote fluorescence polarization values of a reaction mixture with and without CIP, respectively. Each data point represents the average value of two independent experiments.

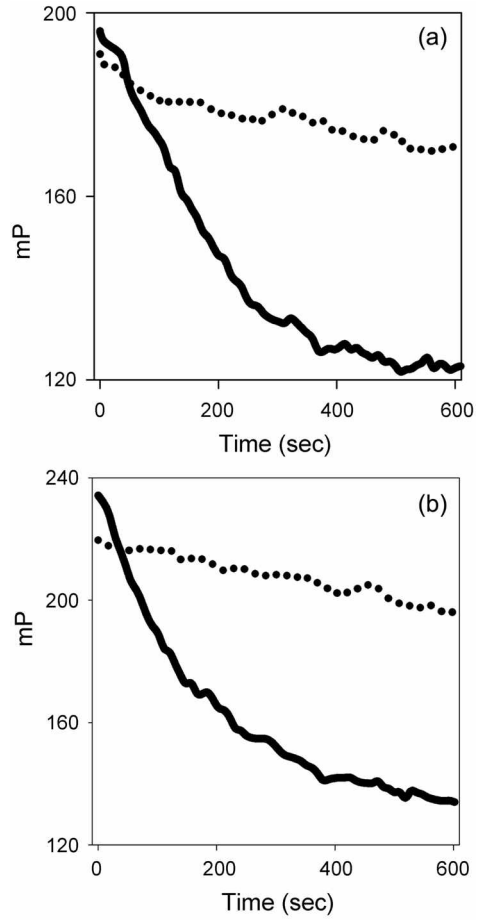


Figure 3. Real-time detection of phosphatase reactions by fluorescence polarization. Dephosphorylation reactions were performed on (a) p-$(dT)_{20}$ (300 nM) or (b) α-casein (300 nM) in the presence (solid traces) or absence (dotted traces) of sodium orthovanadate (20 μM). The amount of CIP was 0.1 U and 1 U for p-$(dT)_{20}$ and α-casein, respectively. Fluorescence polarization was continuously measured at room temperature for 10 min.

substrate (6 kd). Overall, this result demonstrated that the idea to detect dephosphorylation reaction based on change of the fluorescence polarization value of **1** was feasible.

We then explored the possibility of performing the enzymatic reaction in the presence of **1** and monitoring the progress of the reactions in real time by continuous measurements of the fluorescence polarization signal. Reactions were performed at the same condition as before except the probe added from the beginning of the reaction, and the fluorescence polarization signal was monitored for 10 min. Continuous decreases of the fluorescence polarization signal over time were clearly observed as the dephosphorylation reaction proceeded (Figure 3), demonstrating that the phosphatase assay could be monitored in real time. However, in the presence of excess of sodium orthovanadate (20 μM), a non-specific phosphatase inhibitor, a very sluggish decrease of fluorescence polarization signals was observed compared with that of the reaction without orthovanadate, thereby confirming that measurable decreases in fluorescence polarization were due to dephosphorylation of substrates by CIP.

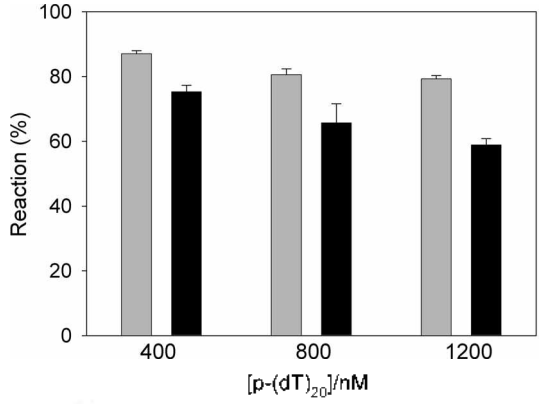


Figure 4. Effect of **1** on the rate of enzymatic dephosphorylation of p-$(dT)_{20}$ by CIP. Dephosphorylation reactions were carried out in the presence (black) or absence (gray) of **1** (300 nM). The probe was added to the reaction mixtures after quenching in the latter case. Reactions proceeded for 3 minutes and then were quenched by addition of sodium orthovanadate (20 μM).

Having achieved monitoring the phosphatase reaction in real time by fluorescence polarization in the presence of **1**, we compared the reaction rates in the presence and absence

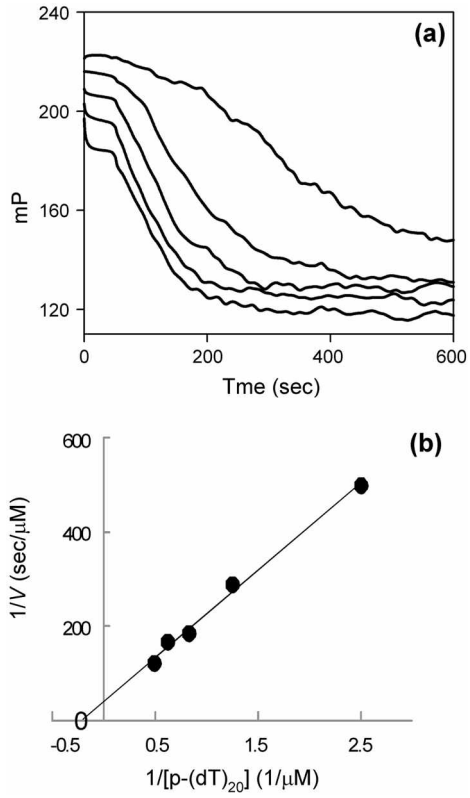


Figure 5. Real-time detection of phosphatase reactions by CIP in the presence of 1. (a) Fluorescence polarization signals were measured at 0.1 U of CIP with varying concentration of p-$(dT)_{20}$ (400, 800, 1200, 1600, and 2000 nM from down to up, respectively). (b) Lineweaver-Burk plot was obtained for the dephosphorylation reaction of p-$(dT)_{20}$ by CIP in the prescence of 1 (300 nM). Rates were determined with varying concentration of p-$(dT)_{20}$ (400, 800, 1200, 1600, and 2000 nM). Each data point represents the average value of two independent experiments. Assays were performed as described in the Experimental section.

of **1** in order to test whether the probe in the reaction mixture gives any influence on the enzymatic reaction rate. Phosphatase reactions using p-$(dT)_{20}$ as the substrate were carried out in the absence or presence of the probe, and the reactions were stopped by addition of sodium orthovanadate (20 μM) after 3 min. The presence of **1** resulted in slight retardation on the reaction rate (Figure 4). We speculated that the reason for this rate reduction might be that the access of the phosphatase to phosphate groups of substrates could be slightly interfered by phosphate binding capacity of the probe.

We also performed the phosphatase reactions at several different concentrations of the DNA substrate. Both the initial and the final fluorescence polarization values of reaction mixtures were slightly elevated at high concentrations of the substrate (Figure 5a). This might result from increased affinity of **1** with both phosphorylated and unphosphorylated DNA molecules when the DNA substrate was used at high concentrations as shown in Figure 1. We could also obtain V_{max} (260 ± 16 nM·sec^{-1}·U^{-1}) of the reaction in the presence of **1** by Lineweaver-Burk plot as shown in Figure 5b.

Conclusion

In summary, we have synthesized a phosphate-specific and fluorescent probe, and using this probe, we could successfully perform homogeneous phosphatase assays based on fluorescence polarization without any additional process such as labeling on substrates and adding antibodies specific for phosphopeptide before or after the measurement of polarization. Moreover, using the probe **1** *in situ*, we could successfully monitor phosphatase reactions in real time without severely disturbing the rate of reactions. We expect that the phosphatase assay method presented here can generally be used when the substrate of phosphatase assays are biopolymers with high molecular weight (> 5 kd) including single stranded DNAs, double stranded DNAs, polysaccharides, and proteins. Therefore, the label-free, homogeneous, and real-time monitoring method described here is expected to be very useful in high-throughput screening applications particularly for developing inhibitors of various phosphatases.

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