Bioconjugate Chemistry

Communication

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Fluorescent Polyion Complex Nanoparticle That Incorporates an Internal Standard for Quantitative Analysis of Protein Kinase Activity

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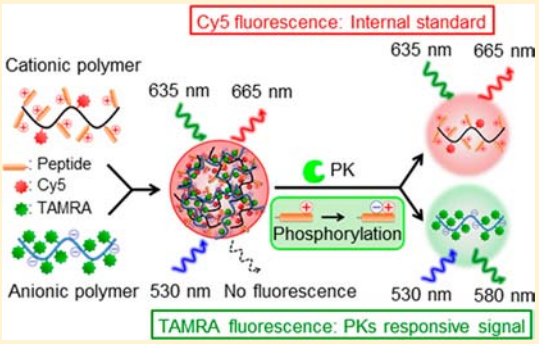
Supporting Information

ABSTRACT: We demonstrate a polyion complex (PIC) nanoparticle that contains both a responsive fluorophore and an "internal standard" fluorophore for quantitative measurement of protein kinase (PK) activity. The PK-responsive fluorophore becomes more fluorescent with PK-catalyzed phosphorylation of substrate peptides incorporated in the PIC, while fluorescence from the internal standard remains unchanged during phosphorylation. This new concept will be useful for quantitative PK assays and the discovery of PK inhibitors.



INTRODUCTION

Protein kinase (PK)-mediated phosphorylation plays a pivotal role in the regulation of cellular processes, and its dysfunction results in various human diseases, notably cancer.[1] Thus, PKs are primary molecular targets for anticancer drugs,[2] and tracking PK activity would be valuable for understanding molecular mechanisms of complex cellular events and subsequent drug development.[3] Measurement of PK activity via fluorescent readout is an attractive alternative to radioactive isotopes.[4−7] Several measurement kits for fluorescence-based PK activity such as LanthaScreen and Z′-LYTE are now commercially available and they are basically end-point assay. To realize real-time kinetic analysis and monitoring of activity in living cells, the fluorescent probes which "turn on" during phosphorylation of substrate peptides by the PKs have been proposed. The turn-on-type probes provide relatively sharp contrast in simple optical readout systems.[8,9] Mechanisms for these fluorescence responses to phosphorylation include metal complex formation,[10−15] protein recognition,[16−19] and concentration quenching.[20,21]

However, quantitative evaluation of PK activity is difficult for probes that turn on, especially for intracellular PKs, because the fluorescence signals depend not only on the PK activity, but also on the probe concentration. This is why fluorescence resonance energy transfer (FRET) is powerful, because it enables quantitative analysis via ratiometric fluorimetry.[5,22,23] However, FRET probes require strict molecular design, and repeated trial and error, to realize dynamic and appropriate distance changes upon phosphorylation.[24,25]

Here, we demonstrate a novel method to quantitatively measure PK activity that is based on a polymeric nanoparticle embedded with both a PK-responsive fluorophore and another fluorophore that is used as an internal standard for quantitative analysis of the PK activity. As shown in Figure 1a, the particle is a polyion complex (PIC) of cationic and anionic polymers. The cationic polymer is a neutral dextran main chain modified with both a cationic peptide substrate for PK and a Cy5 fluorophore (Figure 1b), while the anionic polymer is an anionic poly(L-aspartic acid) main chain modified with tetramethylrhodamine (TAMRA) (Figure 1c). In the PIC, the TAMRA fluorescence is selectively quenched, while the constant Cy5 fluorescence acts as the internal standard. This behavior is achieved by having a high enough concentration of TAMRA in the PIC to induce concentration quenching, and a low enough concentration of Cy5 to avoid it. When the cationic peptides in the cationic polymer are phosphorylated by a target PK, the negatively charged phosphate groups weaken or dissociate the PICs, which in turn reduces the TAMRA concentration quenching and allows more fluorescence. By remaining constant, the Cy5 fluorescence essentially normalizes the TAMRA signal. We demonstrate how the PIC can be used to detect protein kinase Cα (PKCα) activity, which is regarded as an important target for anticancer drugs because it is involved in cancer proliferation signaling.[26,27] Thus, a PKCα-specific substrate

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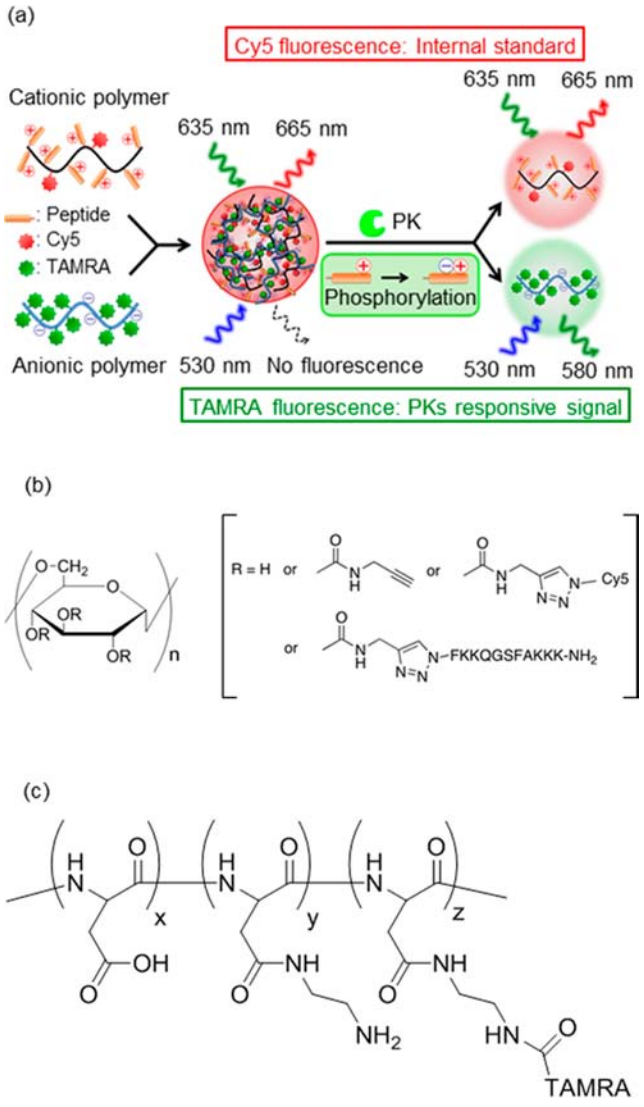

Figure 1. Schematic of a turn-on fluorescent probe, with an internal standard, for quantitative measurement of PK activity (a). Structures of cationic (b) and anionic (c) polymers.

peptide was used here (-FKKQGSFAKKK-NH_2), which changes its net cationic charge from +5 to +3 upon phosphorylation.

RESULTS AND DISCUSSION

The cationic and anionic polymers were synthesized to achieve uneven quenching behavior of the two fluorophores as depicted in Figure 1a. According to our previous report, a high fluorophore content in the polymers does not work for quench-to-recovery-type measurements because the fluorescence can be quenched by hydrophobic intramolecular association among the fluorophores prior to PIC formation.[28] Previously, we found that a few mol % of TAMRA had the best performance;[28] thus, the TAMRA content in the anionic polymer was adjusted to 2.1 mol %. In contrast, the content of Cy5 had to be small enough to avoid concentration quenching in the PIC, but large enough to provide a strong internal standard. In the previous research, we also found that a lower content of a fluorophore (<1.1 mol %) effectively avoids the quenching.[28] Thus, the content of Cy5 in the cationic polymer was lowered to be 0.4 mol %. Meanwhile, the content of the cationic peptide was adjusted to be relatively high (7.8 mol %) to achieve tight PIC formation. The cationic peptide and Cy5 were quantitatively introduced onto the dextran main chain by using "click chemistry".

We compared the reactivity of the substrate peptide that was grafted onto the cationic polymer with a free peptide by using a coupled enzyme assay,[29,30] in which phosphorylation by PK is estimated by absorbance at 340 nm. As shown in Figure 2, the

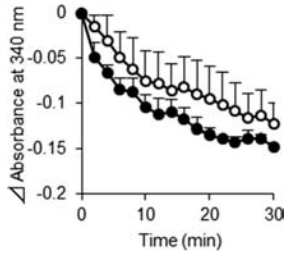

Figure 2. Comparison of the reactivity toward PKCα of free peptides (open circles) and peptides grafted onto the cationic polymer (closed circles). Peptide concentration in both cases is 30 μM; PKCα concentration is 1 U/mL.

substrate peptides on the cationic polymer exhibited almost identical reactivity toward PKCα as that for free peptides, indicating that the effect of substrate immobilization is negligible.

First, we evaluated concentration quenching of TAMRA in the anionic polymer by PIC formation, and avoided Cy5 quenching in the cationic polymer. Figure 3a shows

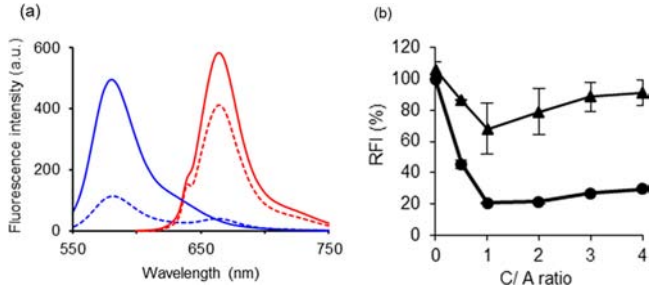

Figure 3. (a) Fluorescence spectra of Cy5 in the cationic polymer and in the PIC (red solid and broken lines, respectively, Ex. at 635 nm), and TAMRA in the anionic polymer and in the PIC (blue solid and broken lines, respectively, Ex. at 530 nm). The C/A ratio was 2. (b) Uneven quenching behavior of TAMRA (closed circles) and Cy5 fluorescence (closed triangles) in PIC prepared with varying C/A ratios. RFI is the relative fluorescence intensity of TAMRA and Cy5. Ex/Em of TAMRA and Cy5 was 530/590 and 635/665, respectively.

fluorescence spectra of the cationic and anionic polymers and the PIC prepared at a cation/anion (C/A) ratio of 2. TAMRA fluorescence in the anionic polymer was quenched to 20% of its original level by PIC formation, while the Cy5 fluorescence intensity was maintained at a high level (>70%). Thus, quenching of each fluorophore can be independently controlled by adjusting its density.

Figure 3b plots the fluorescence of TAMRA and Cy5 as a function of C/A ratios. TAMRA quenching leveled off at 20% at C/A = 1, while Cy5 fluorescence remained at about 70−80%, above C/A = 2. Both TAMRA and Cy5 fluorescence are minimized at C/A = 1, and both slightly increase with C/A > 1. This can be explained by the relaxation of the PIC from the

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electrostatic repulsion caused by overcharging at higher C/A.[31] Hereafter we used PIC prepared at the C/A ratio of 2 for monitoring of PK activity. The size of the PIC at this C/A ratio was determined by dynamic light scattering to be 140 ± 2.3 nm (polydispersity index: 0.32).

Figure 4a,b plots the time dependences of TAMRA and Cy5 fluorescence, respectively, following the addition of various

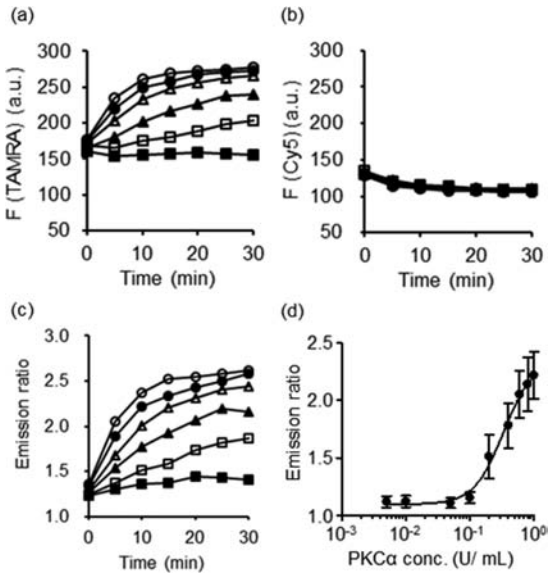

Figure 4. Time dependence of TAMRA (a) and Cy5 (b) fluorescence from PICs triggered by phosphorylation of the substrate peptide grafted onto the cationic polymers at various PKCα concentrations (■ 0, □ 0.2, ▲ 0.4, Δ 0.6, ● 0.8, ○ 1.0 U/mL). (c) TAMRA/Cy5 emission ratio calculated from (a) and (b). (d) PKCα concentration dependence of the emission ratio after 30 min of phosphorylation.

concentrations of PKCα (0−1.0 U/mL) to the PIC dispersion. The TAMRA fluorescence increased rapidly with increasing PKCα concentration because of the weakened PICs. Meanwhile, the Cy5 fluorescence was almost constant over the same time span irrespective of PKCα concentration. These results clearly show that Cy5 fluorescence can be used as the internal standard to monitor the PKCα activity using the turning-on fluorescence of TAMRA. Thus, TAMRA fluorescence was normalized by Cy5 fluorescence and the normalized values (TAMRA/Cy5 emission ratio) are plotted in Figure 4c. At the highest PKCα concentration (1.0 U/mL), the ratio levels off at 2.5 after 15 min. Figure 4d plots the PKCα concentration dependence of the ratio at 30 min from the start of the phosphorylation. From this curve, the EC_{50} (50% effective concentration) of PKCα was calculated and found to be 0.34 U/mL, and the detection limit of PKCα was determined to be 0.29 U/mL (=182 ng/mL), which is comparable or more sensitive than that for other PK assays.[9,28,32]

Finally, we demonstrated determination of the IC_{50} (half maximal inhibitory concentration) of PKCα inhibitors using the PIC probe. Here we used two kinds of PKCα inhibitors, Gö6983 and Ro-31-8425, which have similar IC_{50} values; 7 nM for Gö6983[33] and 8 nM for Ro-31-8425.[34] Phosphorylation of PICs by 0.6 U/mL PKCα was performed for 30 min in the presence of the inhibitors at various concentrations. After the reactions, the TAMRA and Cy5 fluorescence intensities were measured to calculate the emission ratio, with Cy5 fluorescence being the internal standard. As shown in Figure 5a and b, the

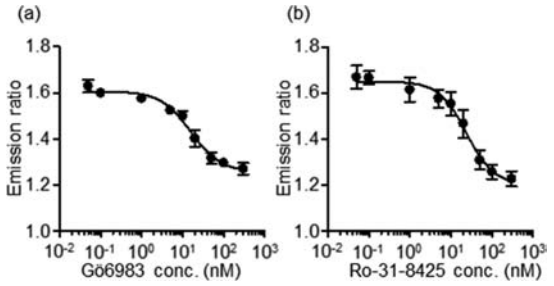

Figure 5. Inhibition of PKCα activity by specific inhibitors, Gö6983 (a) and Ro-31-8425 (b) as monitored by the TAMRA/Cy5 emission ratio of the PIC.

ratios decreased with increasing concentrations of the inhibitors. Thus, the inhibitors suppressed the PKCα phosphorylation and the subsequent weakening of the PIC. From these curves, the IC_{50} values of Gö6983 and Ro-31-8425 were determined to be 16 and 24 nM, respectively, which are consistent with the reported values.[33,34]

■ CONCLUSION

We have demonstrated the detection of PKCα activity using a PIC fluorescence probe. This probe was designed to include two fluorescent signals, i.e., an increasing TAMRA signal corresponding to PKCα activity, and a constant Cy5 signal acting as an internal standard. The difference in fluorescence intensities was achieved by adjusting the modification ratio of each fluorophore on the cationic and anionic polymers, respectively. The PIC probe enables quantitative detection of PKCα activity and was successfully applied to the evaluation of PKCα-specific inhibitors. The concept of "internal-signal-embedded probes" will be especially useful for designing probes for intracellular PK activity measurements, where the fluorescence depends on the number of probes transferred into the cell. The fluorescent probe proposed here has difficulty to apply to intracellular activity measurement of PK *in vitro* due to the weakness of the PIC in culture medium and intracellular condition and low cellular uptake. These issues would be overcome by strengthening of the PIC and enhancing the endocytotic uptake by a ligand modification.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental details on the preparation and use of cationic and anionic polymers in coupled enzyme assays, measurements of fluorescence intensity, PKCα assays, and inhibitor screening assays.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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■ ABBREVIATIONS

PK, protein kinase; PIC, polyion complex; PKCα, protein kinase C α

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