VIP Cell Imaging

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Protein-Phosphorylation-Responsive Polymeric Nanoparticles for Imaging Protein Kinase Activities in Single Living Cells**

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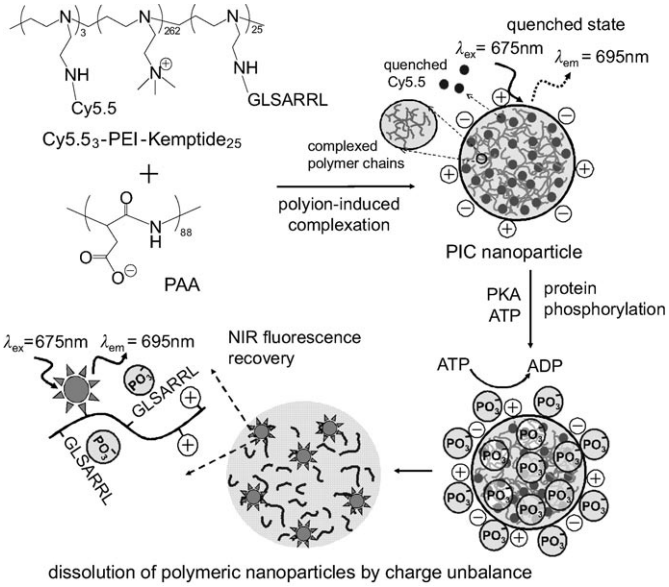
Protein kinases play pivotal regulatory roles in most cell communication and metabolic pathways. Thus, new methods for assaying protein kinase activities are not only valuable in the elucidation of various biological events but are also of profound utility in protein-kinase-targeted drug discovery.[1] Although standard radiometric assays and antibody-based enzyme-linked immunosorbent assays (ELISA) detect protein kinase activities, these methods measure in vitro protein kinase activities discontinuously using a radiolabeling procedure and a phosphopeptide-specific antibody treatment, respectively. These methods cannot be applied to protein kinase assays in living cells. Genetically encoded fluorescent protein reporters capable of fluorescent resonance energy transfer (FRET) have been designed to detect protein kinase activities continuously in single living cells.[2–4] However, these phosphorylation-responsive fluorescent protein reporters exhibit only modest fluorescence changes after protein phosphorylation. Moreover, these methods are limited to cells genetically modified to express fluorescent protein reporters.

Here, we describe protein-phosphorylation-responsive, cell-permeable, and biocompatible polymeric nanoparticles for visualizing protein phosphorylation by protein kinase A (PKA), which is one of the best studied and most important kinases in single cells. The principle of the method is shown in Scheme 1. The polymeric nanoparticles possess a PKA-specific peptide motif (Leu-Arg-Arg-Ala-Ser-Leu-Gly, termed kemptide)[5] and are easily prepared by the self-assembly of a polyion-induced complex (PIC) composed of both positively and negatively charged polyelectrolytes, as previously reported.[6] Upon protein phosphorylation, PIC nanoparticles dissolve because negatively charged phosphate groups are incorporated into the serine residue of kemptide, resulting in polyelectrolyte solubilization.



Scheme 1. Schematic diagram of protein-phosphorylation-responsive polymeric nanoparticles for imaging protein kinase activity in single living cells. The polymeric nanoparticles are prepared by self-assembly of a polyion-induced complex (PIC) composed of positively charged polymer (Cy5.5$_3$-PEI-kemptide$_{25}$) and negatively charged polymer (PAA). In this quenched state, NIR fluorescence intensity will be minimized because of the short distance between Cy5.5 fluorochromes. Upon protein phosphorylation, the negatively charged phosphate groups are incorporated into the serine moieties of Cy5.5$_3$-PEI-kemptide$_{25}$, and the polymeric nanoparticles dissolve owing to charge unbalance, resulting in NIR fluorescence recovery. ATP = adenosine triphosphate, ADP = adenosine diphosphate.

To visualize protein kinase activities in single living cells, we introduced the near-infrared (NIR) fluorochrome Cy5.5 as a fluorescent indicator into protein-phosphorylation-responsive PIC nanoparticles. NIR fluorochromes were chemically coupled to PIC nanoparticles and showed minimal fluorescence intensity in the quenched state because of the short distance between each Cy5.5 in the PIC nanoparticles.[7,8] However, when phosphorylated by PKA, PIC nanoparticles dissolve, resulting in NIR fluorescence dequenching, and

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hence strong NIR fluorescence is recovered. The new system has general applicability for imaging protein kinase activities in living cell as the reagents are cell-permeable and biocompatible.

To produce a phosphorylation-responsive polyelectrolyte, we synthesized a positively charged polymer conjugate by chemical coupling of kemptide and Cy5.5 to poly(ethyleneimine) (PEI, $M_w =$ 25 kDa), which is a cell-permeable and biocompatible polymer. The trinitrobenzenesulfonic acid (TNBS) assay[9] and extinction coefficient measurements showed that each PEI polymer contained 25 ± 1.2 molecules of kemptide and 3 ± 1.4 molecules of Cy5.5, respectively; hence the molecules were designated as Cy5.5$_3$-PEI-kemptide$_{25}$ ($M_w =$ 49.8 kDa). Poly(aspartic acid) (PAA, $M_w = 10$ kDa) was used as a negatively charged polyelectrolyte. The positively charged and negatively charged polymers were dissolved separately in PKA reaction buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$; Tris = tris(hydroxymethyl)aminomethane). The oppositely charged polyelectrolytes Cy5.5$_3$-PEI-kemptide$_{25}$ and PAA should be self-aggregated to make monodispersed nanoparticle structures driven by electrostatic interactions.

To determine the optimal polymer molar ratio required to construct monodispersed PIC nanoparticles maintained by electrostatic interactions, the polydispersity index, the average particle diameter, and the scattering intensity of the polymer mixture were measured using dynamic light scattering (DLS) as a function of the Cy5.5$_3$-PEI-kemptide$_{25}$/PAA molar ratio (Figure 1a). As the molar ratio of Cy5.5$_3$-PEI-kempatide$_{25}$/PAA increased, the scattering intensity also rose to a maximal value at a molar ratio of 0.8. At this optimal molar ratio, PIC nanoparticles were essentially spherical and approximately 50 nm in diameter (see Figure SI1 in the Supporting Information). The polydispersity index was lower than 0.05, suggesting that the PIC nanoparticles were monodisperse.[10] Also, transmission electron microscopy (TEM) showed that freshly prepared PIC nanoparticles were well-dispersed and approximately 50 nm in diameter (Figure 1b). PIC nanoparticles at a molar ratio of 0.8 precipitated completely upon by centrifugation at 10000 g for 10 min; neither polymer was found in the supernatant by UV spectrometry, indicating that all the polymers are self-aggregated to make PIC nanoparticles (Figure 1c). The scattering intensity decreased and the polydispersity index increased dramatically as the Cy5.5$_3$-PEI-kemptide$_{25}$/PAA molar ratio increased above 0.8. This indicated that the presence of an excess amount of negatively charged PAA polymers led to the dissolution of the self-aggregated PIC nanoparticles. As expected, the quenching efficiency of the PIC nanoparticles increased as the molar ratio of Cy5.5$_3$-PEI-kemptide$_{25}$/PAA rose, and the quenching efficiency was maximal (approximately 90%) at a molar ratio of 0.8 (Figure 1d). On the basis of this data, the optimal Cy5.5$_3$-PEI-kemptide$_{25}$/PAA molar ratio in PIC nanoparticles was determined to be 0.8, wherein each polymer combined tightly and formed monodispersed PIC nanoparticles with the highest quenching efficiency.

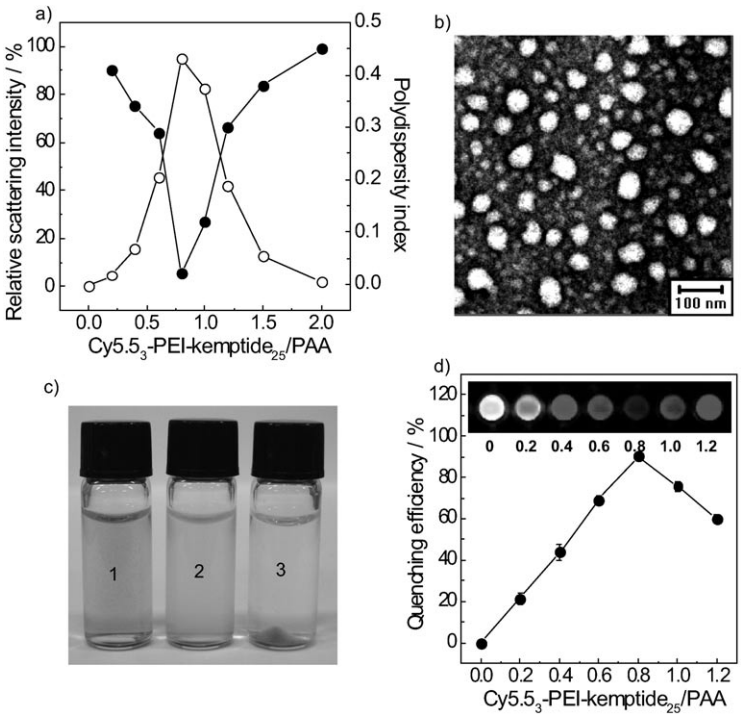

Figure 1. a) Effect of Cy5.5$_3$-PEI-kemptide$_{25}$/PAA molar ratio on the scattering intensity (○) and polydispersity index (●) of PIC nanoparticles in PKA reaction buffer at 25 °C. b) A TEM image of PIC nanoparticles formed in distilled water when the molar ratio was 0.8. c) A photograph of Cy5.5$_3$-PEI-kemptide$_{25}$ and PIC nanoparticle solutions in PKA reaction buffer; 1: Cy5.5$_3$-PEI-kemptide$_{25}$, 2: PIC nanoparticles formed when the Cy5.5$_3$-PEI-kemptide$_{25}$/PAA components were in a molar ratio of 0.8, 3: PIC nanoparticles precipitated after centrifugation at 10000 g. d) Quenching efficiency of PIC nanoparticles as a function of the molar ratio of the polymer mixture. Inset: NIR fluorescence from wells containing polymer mixtures.

The specificity and sensitivity of PIC nanoparticles formed with a Cy5.5$_3$-PEI-kemptide$_{25}$/PAA molar ratio of 0.8 were evaluated in vitro by incubating PIC nanoparticles (10 μg mL^{-1}; kemptide concentration: 5.2 nM) with protein phosphorylation stimulus reagents or inhibitors of protein phosphorylation (PKI(14-22)amide) in PKA reaction buffer at 37 °C for 30 min. In the presence of both excess PKA (10 nM) and ATP (50 nM), the quenched PIC nanoparticle solution yielded an approximately eightfold increase in NIR fluorescence intensity (Figure 2a), confirming that 90% of NIR fluorescence was recovered by protein phosphorylation. The NIR fluorescence amplification of PIC nanoparticles was completely inhibited, however, by 10 nM of PKA inhibitor. In a control experiment, the NIR fluorescence did not change in the absence of PKA or ATP and in the presence of inhibitors alone. Also, control PIC nanoparticles using a scrambled peptide (Leu-Arg-Arg-Ala-Ala-Leu-Gly; LRRAALG) did not yield any change in fluorescence intensity, as the scrambled peptide cannot be phosphorylated by PKA and ATP.[11] In addition, NIR fluorescence imaging showed that quenched PIC nanoparticles produced a strong NIR fluorescence intensity in the presence of both PKA and ATP, but not

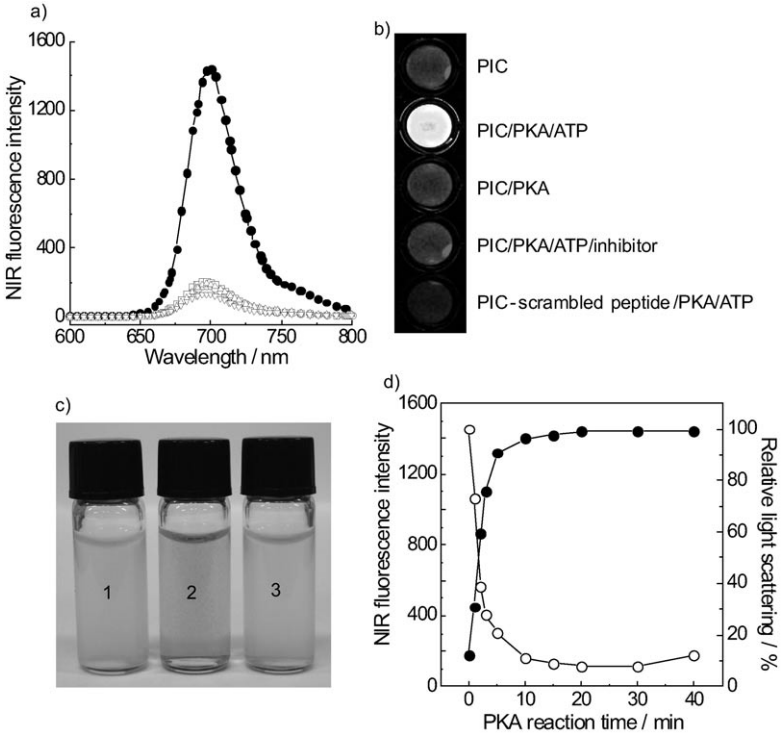

Figure 2. a) The fluorescence spectra ($\lambda_{ex} = 675$ nm) of PIC nanoparticles with a Cy5.5$_3$-PEI-kemptide$_{25}$/PAA molar ratio of 0.8 in PKA reaction buffer with 1 mM dithiothreitol at 37 °C for 30 min; ○ PIC, ● PIC/PKA/ATP, □ PIC/PKA, □ PIC/PKA/ATP/PKA inhibitor, ▽ PIC-scrambled peptide/PKA/ATP. b) NIR fluorescence image from wells containing PIC nanoparticles incubated with PKA stimuli or inhibitors. c) Photograph of PIC nanoparticles in the PKA reaction; 1: PIC nanoparticles, 2: PIC nanoparticles/PKA/ATP, 3: PIC nanoparticles with scrambled peptide/PKA/ATP. d) The relationship between fluorescence intensity and relative scattering intensity of PIC nanoparticles in the presence of PKA and ATP at 37 °C.

when PKA only was present, and not in the presence of PKA inhibitor (Figure 2b). Visual inspection of solutions in glass vials yielded valuable information on phosphorylation-responsive PIC nanoparticles. During an active PKA reaction, an opaque PIC nanoparticle solution changed to clear blue in color only in the presence of PKA and ATP (Figure 2c). The scattering intensity of PIC nanoparticles decreased in proportion to the PKA reaction time, and 90% of PIC nanoparticles were solubilized within 10 min with a concomitant increase in NIR fluorescence intensity. These data support our suggestion that dissolution of PIC nanoparticles occurs only after protein phosphorylation (Figure 2d).

To image protein phosphorylation in single living cells, the imaging probe should be cell-permeable and biocompatible, as the PKA reaction occurs in the cytoplasm. We therefore tracked fluorescein isothiocyanate (FITC)-labeled PIC nanoparticles (10 μg mL^{-1}) in PKA-overexpressing CHO-K1 cells (prepared by transient transfection with RSV CHO PKA C-β; see Figure SI2 in the Supporting Information)[12] (Figure 3). As the incubation time increased, FITC fluorescence increased in the cytoplasm and reached a maximum after 1 h. Moreover, most of the internalized PIC nanoparticles were localized in the cytoplasm, while a few PIC nanoparticles were observed in the perinuclear compartment. We next examined whether PIC nanoparticles showed biological toxicity. We found that a PIC nanoparticle concentration of ≤ 50 μg mL^{-1} was non-toxic. On this basis, a level of 10 μg mL^{-1} of imaging probe was chosen for cellular PKA imaging tests, and cell viability was 100% for 2 days (see Supporting Information; Figure SI3).

Finally, we used NIR fluorescence microscopy to examine the utility of PIC nanoparticles in the visualization of PKA activities in CHO-K1 cells overexpressing PKA (Figure 4). Cells were incubated with PIC nanoparticles formed using a Cy5.5$_3$-PEI-kemptide$_{25}$/PAA molar ratio of 0.8 (10 μg mL^{-1}), and NIR fluorescence intensities were measured over a range of PKA reaction times at 37 °C. The NIR fluorescence signals in PKA-overexpressing CHO-K1 cells increased within 5 min and increased continuously. After 1 h of incubation, the NIR fluorescence intensity had increased significantly, achieving levels approximately eight times those of background cells (see Figure SI4 in the Supporting Information). In contrast, control PIC nanoparticles containing a scrambled peptide sequence and PKA-responsive PIC nanoparticles with 10 nM PKA inhibitor did not show any significant NIR fluorescence signal even after 1 h of incubation, reflecting the high specificity and sensitivity of the PIC nanoparticles. Based on cell-imaging results, our PIC nanoparticles did not show any

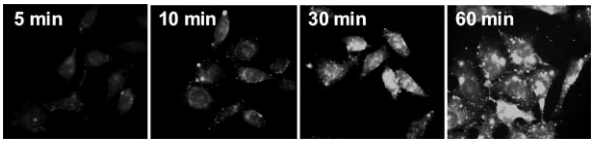

Figure 3. Cellular uptake of PIC nanoparticles with a Cy5.5$_3$-PEI-kemptide$_{25}$/PAA molar ratio of 0.8 as a function of incubation time. CHO-K1 cells (5×10^3) were incubated with FITC-labeled Cy5.5$_3$-PEI-kemptide$_{25}$/PAA (10 μg mL^{-1}).

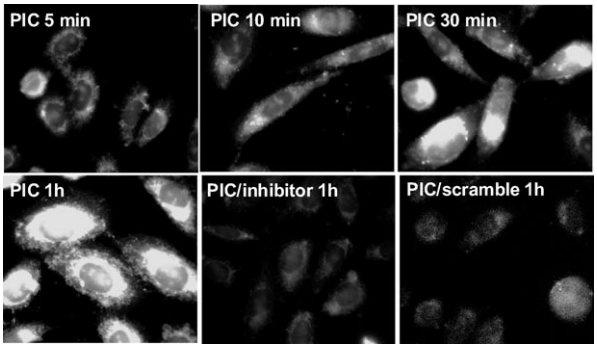

Figure 4. NIR fluorescence microscopy of PIC nanoparticles (10 μg mL^{-1}) incubated in CHO-K1 cells overexpressing PKA for 5 min, 10 min, 30 min, and 1 h with or without PKA inhibitor. The control PIC nanoparticles with a scrambled peptide were also incubated in PKA-overexpressing CHO-K1 cells for 1 h.

NIR fluorescence in the quenched state. After protein phosphorylation, however, the PIC nanoparticles showed strong NIR fluorescence intensity in single living cells.

In summary, our PIC nanoparticles continuously reflected protein kinase activities. There is no need for repeated washing and labeling steps. The PIC nanoparticles also do not require the use of cells genetically equipped with fluorescent protein reporters for FRET imaging prior to measurement of protein kinase activities. This new cellular imaging probe using PIC nanoparticles is simple and can be used to explore protein kinase activities in various single living cells that express protein kinase activities. The technique may also be applied to high-throughput cell-based drug-screening systems targeting protein kinases.

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