

Polymeric Nanoparticles as Imaging Probes for Protein Kinase Activity in Cells

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imaging agents · nanomedicine · nanotechnology ·
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Protein kinases are a large family of enzymes that catalyze the transfer of a phosphate group from adenosine triphosphate (ATP) to the OH group of an amino acid side chain in peptides or proteins and generating adenosine diphosphate (ADP) in the process.^[1–3] Most kinases act on serine and threonine; others act on tyrosine, and a number of so-called dual-specificity kinases act on all three. Protein kinases regulate cellular processes by a highly controlled series of phosphorylation reactions, frequently with some kinases acting as substrates for other kinases.^[4] Deregulated kinase activity is a frequent cause of disease, particularly cancer,^[5,6] where kinases regulate many aspects concerning controlling metabolism, cell growth, differentiation, movement, and death (apoptosis).^[7] It has been estimated that approximately 500 protein kinases are encoded within the human genome;^[8] this represents approximately 1.7% of all human genes.^[8] Most of the 30 known tumor suppressor genes and more than 100 dominant oncogenes are protein kinases.^[9]

For the development of useful drugs it is essential to screen a variety of chemical libraries for potent kinase inhibitors and to assess their inhibitory potency toward a particular protein kinase. Therefore, biochemical studies on protein kinases are important not only for basic biology to clarify molecular mechanisms of signal transduction, but also for clinical pharmacology to develop novel anticancer agents.^[5] Targeted therapy will most presumably result in more effective treatment with fewer negative side effects than those currently associated with standard chemotherapy.

Basically two different approaches can be used to study protein kinases.^[10,11] One is to characterize an individual protein kinase in detail by measuring its kinase activity. The second approach is to comprehensively analyze the extent of phosphorylation of substrate proteins or expression profiles of protein kinases themselves. In the past, radiometric assays utilizing radioactive ATP have been used. Owing the environmental impact of waste materials, radiometric methods have largely been replaced by approaches employing fluorescent

measurements. A further factor driving the development of a large number of fluorescent assays has been the rapid growth in the number of available phosphoprotein- and phosphopeptide-specific antibodies.

While biochemical assays for protein phosphorylation are easily carried out, they cannot duplicate the environment of the cell.^[11,12] Cellular phosphorylation cascades are multi-directional pathways rather than single biochemical reactions. Although a particular member of a signaling pathway may be effectively inhibited by a candidate drug, the pathway itself may remain unaffected owing to alternative signaling routes that bypass the targeted kinase. Therefore, the behavior of a drug in a biochemical assay may not correlate with the behavior in either whole cells or in an animal.^[11–13] To more precisely assess the effects of a drug compound on a kinase-mediated pathway, cell-based assay formats must be used to validate the inhibitory effects of a drug candidate on both the target and the targeted pathway. Whole-cell formats also allow a simultaneous assessment of drug penetration and toxicity.^[11–13] For this reason there is the hope that the application of cell-based assays will result in fewer failures further on in clinical development processes.

While most of the cell-based assays access advanced biochemical methods employing classical fluorescent or radio-labeled markers,^[11,12] (bio)luminescent detection,^[11,14] and impedance-based measurements,^[15] a new cell-based assay applying “intelligent” nanoparticles has recently been introduced (Figure 1).^[16] This assay detects protein kinase activities without treatment with phosphopeptide-specific antibodies, without time-consuming washing steps, and without the limitation to cells genetically modified to express fluorescent protein reporters.

Two techniques have been combined making this assay highly interesting: 1) near-infrared (NIR) fluorescence microscopy and 2) smart polymeric nanoparticles that are responsive to protein phosphorylation. Molecules that absorb in the NIR region (700 nm–1000 nm) can be efficiently used to visualize and investigate in vivo molecular targets because most cells generate little NIR fluorescence.^[17] Thus, the NIR fluorescence technology enables extremely sensitive and quantitative analysis of enzyme activity in cultured cells.

In early work a large group of well-known organic nanoparticles, such as liposomes, dendrimers, and polymericosomes, were developed as delivery systems and for therapeutically applications, but these nanostructures have also been

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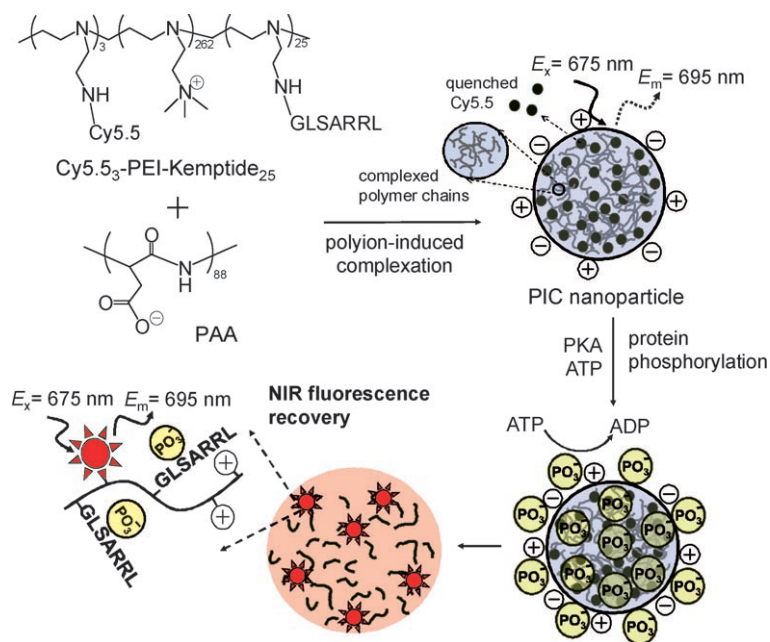


Figure 1. The the imaging of protein kinase activity with polymeric nanoparticles. The nanoparticles are prepared by self-assembly of a polyion-induced complex (PIC) composed of a functionalized positively charged polymer (Cy5.5₃-PEI-kemptide₂₅) and a negatively charged polymer (PAA). A Cy5.5₃-PEI-kemptide₂₅ molecule contains 25 substrate peptides (kemptides) and three NIR fluorochrome units (Cy5.5). In PIC nanoparticles the NIR fluorescence intensity is minimized (quenched) because of the short distance between the NIR fluorochrome molecules. Upon protein phosphorylation, the negatively charged phosphate groups are incorporated into the serine moieties of kemptide. The polymeric nanoparticles dissolve owing to charge imbalance, thus resulting in a strong increase of the NIR fluorescence. E_x = excitation wavelength, E_m = emission wavelength.

applied for in vivo optical imaging.^[18] Along the lines of the well-known liposome technique, polymersomes, for instance, have been synthesized through the cooperative self-assembly of amphiphilic diblock copolymers and conjugated NIR fluorochromes. The latter were located in the hydrophobic inner part of the shell of the polymer vesicles.^[19]

Besides the steady improvement of the NIR fluorescence technology and the development of novel fluorescent NIR probes, a highly innovative nanoparticle-based approach has been introduced by Kwon et al.^[16] This assay utilizes 1) the self-assembly properties of differently charged polyelectrolytes to form nanoparticles, 2) the protein kinase A (PKA)-specific peptide motif, termed kemptide, and 3) the chemically coupled NIR fluorochrome Cy5.5. In Figure 1 the principle of this novel method is shown. One molecule of the positively charged polyelectrolyte poly(ethyleneimine) (PEI) contains 25 molecules of kemptide and three chemically coupled Cy5.5 molecules. This construct is termed Cy5.5₃-PEI-kemptide₂₅.^[16] The polymeric nanoparticles were prepared by the self-assembly of a polyion-induced complex (PIC) composed of both functionalized positively and negatively charged (poly(aspartic acid), PAA) polyelectrolytes by the means of electrostatic interactions.^[20] The resulting monodisperse, essentially spherical PIC nanoparticles with a diameter of approximately 50 nm have been proven to be cell-permeable and biocompatible.^[16]

PKA is one of the best studied and most important kinases in single-cell studies. With this new assay the NIR fluorescence of the phosphorylation-responsive PIC nanoparticles was measured to visualize protein kinase activities in single living Chinese hamster ovary cells overexpressing PKA (CHO-K1). As a result of the short distance between Cy5.5 molecules in the PIC nanoparticles (quenched state), they showed minimal NIR fluorescence intensity. Upon peptide phosphorylation, the PKA-specific substrate kemptide in the PIC nanoparticles becomes phosphorylated, and the negatively charged phosphate groups are incorporated into the serine residue of kemptide (Figure 1). The additional negatively charged phosphate groups cause a charge-unbalanced state, resulting in dissolution of the PIC nanoparticle. As a consequence, the distance of between the Cy5.5 fluorochromes increases, leading to diminished quenching efficiency. Thus, the intensity of the NIR fluorescence increases significantly and reaches levels approximately 8 times greater than those of cells without the PIC nanoparticles (Figure 2). In contrast, PKA-responsive PIC nanoparticles in CHO-K1 cells did not show any significant NIR fluorescence signal in the presence of 10 nm PKA inhibitor (Figure 2).^[16] Thus, this technology enables extremely sensitive and quantitative analysis of PKA activity in cultured cells.

Research and pharmaceutical laboratories require advanced cell-based screening and analysis technologies in order to understand complex

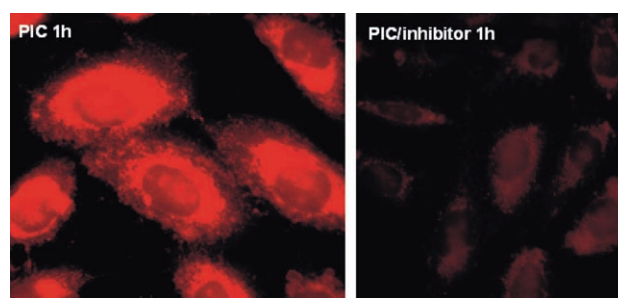


Figure 2. NIR fluorescence microscopic image of PIC nanoparticles (10 µg mL⁻¹) in CHO-K1 cells overexpressing protein kinase A incubated for one hour without (left) or with (right) PKA inhibitor.

disease mechanisms, as well as to develop the next generation of clinical drugs and therapies. This new nanotechnological cell-based assay employing PIC nanoparticles and the NIR fluorescence technology continuously reflects protein kinase activity. The homogeneous assay does not require labeling or washing steps, and there is no need for cells genetically equipped with fluorescent protein reporters. Furthermore, it also reduces the cost associated with expensive antibody and peptide labeling, as well as the cost associated with antibody development and procurement.

However, the ability to entrap native full-length proteins in PIC nanoparticles should be investigated, particularly as some kinases may fail to demonstrate measurable activity unless a full-length protein substrate is provided. Nevertheless, the present PIC nanoparticle-based assay provides a comprehensive solution to kinase inhibitor screening, compound profiling, and substrate identification in single living cells and thus, constitutes an interesting example for nanomedicine and cancer nanotechnology. In summary, the PIC nanoparticle-based technique is a highly versatile and sensitive assay platform for the investigation of a variety of protein kinase activities in various single living cells and may be applied for high-throughput cell-based drug screening seeking for drugs targeting protein kinases.

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- [1] T. Pawson, J. D. Scott, *Trends Biochem. Sci.* **2005**, *30*, 286–290.
 - [2] J. Villén, S. A. Beausoleil, S. A. Gerber, S. P. Gygi, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 1488–1493.
 - [3] Z. Songyang, S. Blechner, N. Hoagland, M. F. Hoakstra, H. Piwnica-Worms, L. C. Cantley, *Curr. Biol.* **1994**, *4*, 973–982.
 - [4] E. Faryard, L. A. Tintignac, A. Baudry, B. A. Hemmings, *J. Cell Sci.* **2005**, *118*, 5675–5678.
 - [5] T. Pawson, N. Warner, *Oncogene* **2007**, *26*, 1268–1275.
 - [6] P. Blume-Jensen, T. Hunter, *Nature* **2001**, *411*, 355–365.
 - [7] L. Liotta, E. Petricoin, *Nat. Rev. Genet.* **2000**, *1*, 48–56.
 - [8] G. Manning, D. B. Whyte, R. Martinez, T. Hunter, S. Sudarsanam, *Science* **2002**, *298*, 1912–1934.
 - [9] P. A. Futreal, A. Kasprzyk, E. Birney, J. C. Mullikin, R. Wooster, M. R. Stratton, *Nature* **2001**, *409*, 850–852.
 - [10] A. Ishida, I. Kameshita, N. Sueyoshi, T. Taniguchi, Y. Shigeri, *J. Pharmacol. Sci.* **2007**, *103*, 5–11.
 - [11] M. D. Olive, *Expert Rev. Proteomics* **2004**, *1*, 89–103.
 - [12] C. Zhao, M. Cai, Y. Zhang, Y. Liu, R. Sun, N. Zhang, *Anal. Biochem.* **2007**, *362*, 8–15.
 - [13] T. Worzella, A. Gallagher, *JALA* **2007**, *12*, 99–103.
 - [14] J. M. Atienza, N. Yu, X. Wang, X. Xu, Y. Abassi, *J. Biomol. Screening* **2006**, *11*, 634–643.
 - [15] L. K. Minor, *Expert Rev. Mol. Diagn.* **2005**, *5*, 561–571.
 - [16] J. H. Kim, S. Lee, K. Park, H. Y. Nam, S. Y. Jang, I. Youn, K. Kim, H. Jeon, R. W. Park, I. S. Kim, K. Choi, I. C. Kwon, *Angew. Chem.* **2007**, *119*, 5881–5884; *Angew. Chem. Int. Ed.* **2007**, *46*, 5779–5782.
 - [17] B. Ballou, L. A. Ernst, A. S. Waggoner, *Curr. Med. Chem.* **2005**, *12*, 795–805.
 - [18] J. Rao, A. Dragulescu-Andrasi, H. Yao, *Curr. Opin. Biotechnol.* **2007**, *18*, 17–25.
 - [19] P. P. Ghoroghchian, P. R. Frail, K. Susumu, D. Blessington, A. K. Brannan, F. S. Bates, B. Chance, D. A. Hammer, M. J. Therien, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 2922–2927.
 - [20] J. H. Kim, S. Lee, K. Kim, H. Jeon, R. W. Park, I. S. Kim, K. Choi, I. C. Kwon, *Chem. Commun.* **2007**, DOI: 10.1039/b612773h.
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