Polymeric nanoparticles for protein kinase activity†

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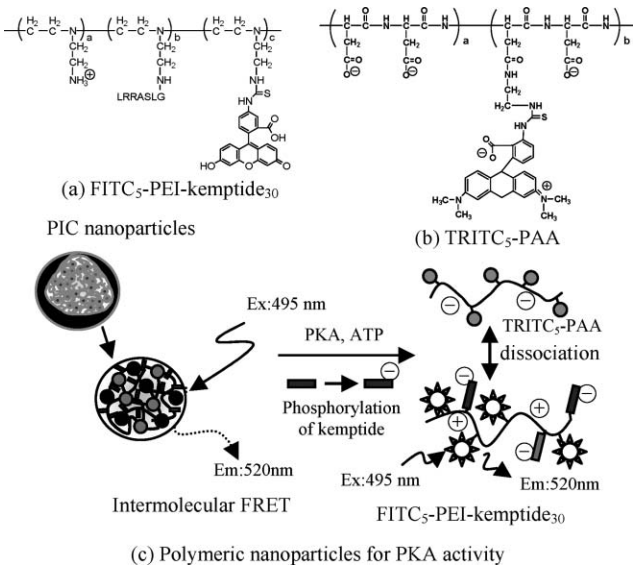
Nanoparticles were prepared from poly-ion complexes, possessing both PEI–FITC–(PKA-specific substrate) (kemptide) and PAA–TRITC, which produce intermolecular FRET; the nanoparticles were dissociated by phosphorylation, presented a strong FITC intensity and can be applied to high-throughput screening for large chemical libraries, for drug discovery of kinase inhibitors.

Protein kinases play pivotal regulatory roles in most cell communication and metabolic pathways.[1] Because of the centrality of protein phosphorylation as a regulatory process, inhibitors of protein kinases not only hold great promise as therapeutic agents for many diseases, especially cancer, but are also of profound utility in the characterization of signalling pathways.[2] Therefore, sensitive and widely applicable high-throughput screening (HTS) of protein kinase activity will provide a valuable tool to monitor protein kinase inhibitors in drug discovery and to understand biological signalling pathways. Standard radiometric assays and antibody-based enzyme-linked immunosorbent assays (ELISA) detect protein kinase activity discontinuously, because both assays require radioactive labelling or expensive and specialized phosphopeptide-specific antibodies, respectively.[3] Genetically encoded fluorescent protein reporters capable of fluorescence resonance energy transfer (FRET) have been designed to detect protein kinase activity continuously in live cells.[4] Unfortunately, these phosphorylation-responsive probes demonstrate modest fluorescence changes. Recently, a non-antibody-based screening method of kinase and phosphatase activities has been developed by using a metal ion-medicated polymer superquenching system in which phosphorylated peptide substrates containing a quencher bind specifically to the metal ion, resulting in the quench of polymer fluorescence.[5] Also, low molecular weight probes with a kinase recognition motif have been chemically synthesized and present fluorescent amplification after exposure to the targeting protein kinase.[6] Based on these fluorescent dequenching and quenching methods, the protein kinase activity could be simply monitored as fluorescent intensity changes.

In this communication we describe polymer-based nanoparticles, which were simply prepared by the self-assembly of poly-ion complexes (PIC) of both positively and negatively charged polyelectrolytes. The nano-sized particles present a significant fluorescent increase specifically responding to the phosphorylation of protein kinase A (PKA), which is one of the well known and important kinases. The polymer-based nanoparticle possesses a PKA-specific peptide motif (Leu–Arg–Arg–Ala–Ser–Leu–Gly, kemptide)[7] and two fluorochromes of fluorescein isothiocyanate (FITC)/tetramethyl rhodamine isothiocyanate (TRITC) that produce intermolecular FRET, due to the short distances between each FITC and TRITC molecule in the nanoparticles. However, when phosphorylated, the PKA-responsive nanoparticles are dissociated by the introduction of negatively charged phosphate groups to the serine moiety of the PKA-specific peptide motif, resulting in a loss of FRET, and hence the dissociated polymers present a strong FITC intensity in response to phosphorylation (Scheme 1).

We first synthesized a positively charged polymer conjugate by chemically coupling FITC and PKA-specific substrate, kemptide, to poly(ethyleneimine) (PEI). Extinction coefficient measurements and a trinitrobenzenesulfonic acid (TNBS) assay showed that each PEI polymer contained 5 ± 1.2 molecules of FITC and 30 ± 3.2 molecules of kemptide, resulting in $FITC_5$–PEI–$kemptide_{30}$. The negatively charged polymer conjugate was prepared by coupling 5 ± 0.9 molecules of TRITC to poly(aspartic acid) (PAA),



Scheme 1 Schematic diagram of a polymeric nanoparticle for protein kinase activity.

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resulting in $TRITC_5$–PAA. The positively and negatively charged polymer conjugates were separately dissolved in the PKA reaction buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$). All the physicochemical and biological properties of the PIC nanoparticles were obtained in the protein kinase reaction buffer without any comment.

In order to make PIC nanoparticles, both oppositely charged polymer conjugates of $FITC_5$–PEI–$kemptide_{30}$ and $TRITC_5$–PAA should be self-aggregated to make a monodispersed nanoparticle structure driven by electrostatic interactions.[8] Hence, to satisfy the optimal polymer molar ratio to make PIC nanoparticles, the average diameter, scattering intensity, and polydispersity index of the polymer mixture were monitored by dynamic light scattering (DLS) as a function of the $TRITC_5$–PAA/$FITC_5$–PEI–$kemptide_{30}$ molar ratio (Fig. 1a). On increasing the $TRITC_5$–PAA/$FITC_5$–PEI–$kemptide_{30}$ molar ratio of the polymer mixture, the scattering intensity slowly increased and its value maximized at the molar ratio of 2.5. The average diameter of the PIC nanoparticles was about 60 nm and their polydispersity index lower than 0.05, suggesting that the PIC nanoparticles were evenly dispersed and monodispersed (Fig. SI1a†).[9] The PIC nanoparticles were completely precipitated by centrifugation at 10 000*g* for 10 min and neither polymer was found in the supernatant part, confirmed by UV spectrometry, indicating that all the polymers had self-aggregated to make PIC nanoparticles (Fig. SI1a†). When the molar ratio increased above 2.5, the scattering intensity decreased, whereas the polydispersity index increased dramatically, indicating that the neutralized PIC nanoparticles were dissociated by the additional negative $TRITC_5$–PAA polymers. As predicted, at the molar ratio of 2.5, the surface charge of the nanoparticles is almost zero in distilled water, due to the self-aggregation of the charged polymers through electrostatic interactions (Fig. 1b) and the inset TEM showed that the freshly prepared PIC nanoparticles were essentially spherical and approximately 50 nm in diameter (inset image in Fig. 1b), indicating that both $FITC_5$–PEI–$kemptide_{30}$ and $TRITC_5$–PAA conjugates tightly combine to form a nanoparticle structure.

The FRET efficiency of the PIC nanoparticles was maximum at a molar ratio of 2.5 and was approximately 90% (Fig. 1c), because the distances between the FITC and TRITC molecules in the PIC nanoparticles were short enough to produce a significant FRET effect. However, above a molar ratio of 2.5, the FRET efficiency decreased, because the additional negatively charged polymers could dissociate the tight PIC nanoparticle structure. Also, FITC fluorescence imaging indicated that the minimal FITC intensity was found at the optimal molar ratio of 2.5 (Fig. 1d). The freshly prepared PIC nanoparticles were stable within 2 days at room temperature in the PKA reaction buffer, wherein the average diameter and the polydispersity were not changed (Fig SI1b†).

Fig. 2a shows the whole range of emission of the donor (FITC) and acceptor (TRITC) in the nanoparticles when excited at 495 nm (λ_{max} of FITC). After preparing PIC nanoparticles with a molar ratio of 2.5, FITC fluorescence is immediately quenched and TRITC fluorescence is increased, indicating a FRET effect between FITC and TRITC molecules in the PIC nanoparticles. In the presence of both PKA and ATP, the FITC fluorescence of the PIC nanoparticles is 7.3-fold enhanced, whereas the TRITC florescence decreases. It is deduced that the PIC nanoparticles are dissociated by the introduction of negatively charged phosphate groups to kemptide molecules in the PIC nanoparticles, resulting in a loss of FRET. The dissociated polymer presents a strong FITC fluorescence and 70% of the FITC fluorescence is recovered by comparing with the positively charged polymer of $FITC_5$–PEI–$kemptide_{30}$ which shows a single peak at 520 nm. The emission ratio of PIC nanoparticles (FITC/TRITC; 520 nm/572 nm) increased from 0.14 to 3.02 in the presence of both PKA and ATP, which is a 21.5-fold increase in emission ratio. It is a dramatic change of emission ratio when compared to the previously reported data which show about a 1.5–2.5-fold increase

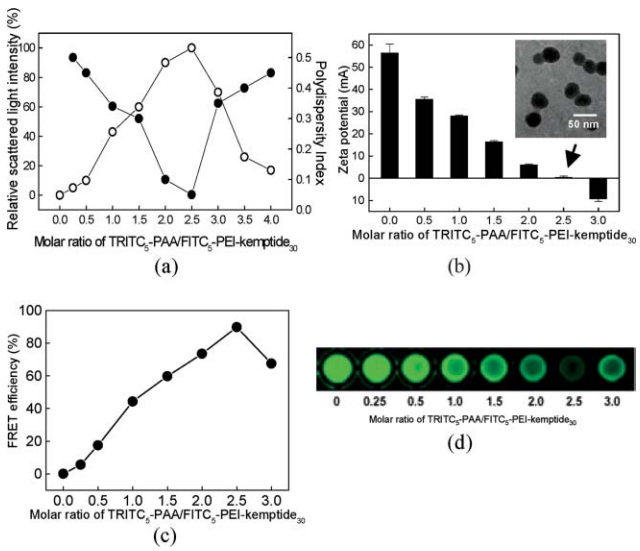

Fig. 1 (a) Effect of $TRITC_5$–PAA/$FITC_5$–PEI–$kemptide_{30}$ molar ratio on the scattering intensity (○) and polydispersity index (●) of PIC nanoparticles prepared in the PKA reaction buffer (50 mM Tris-HCl buffer, pH 7.5, 10 mM $MgCl_2$) at 25 °C. (b) Zeta-potentials of polymer mixtures prepared in distilled water (pH 7.5). Inset TEM image shows the PIC nanoparticles with a molar ratio of 2.5. (c) FRET efficiency and (d) FITC fluorescence image of a 96-well containing polymer mixtures in the PKA reaction buffer at 25 °C.

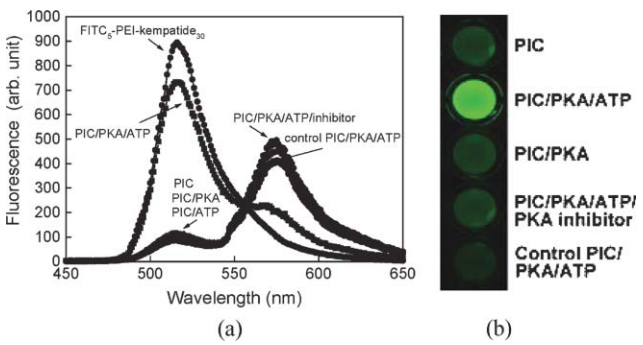

Fig. 2 PKA specificity of PIC nanoparticles (10 μg; kemptide concentration is 5.4 nM) with a $TRITC_5$–PAA/$FITC_5$–PEI–$kemptide_{30}$ molar ratio of 2.5 studied by fluorescence energy transfer. (a) Excitation wavelength was set at 495 nm and the whole range of PIC nanoparticles monitored with/without PKA (10 nM), ATP (50 nmol), or PKA inhibitor (10 nM) in the PKA reaction buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$) containing 1 mM dithiothreitol at 37 °C for 1 h. (b) Fluorescence image of a 96-well containing PIC nanoparticles with/without PKA and ATP, or with PKA inhibitor.

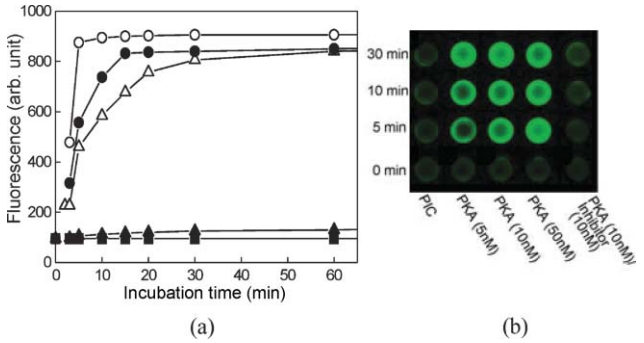

Fig. 3 PKA assay with PIC nanoparticles (10 μg; kemptide concentration is 5.4 nM) with a $TRITC_5$–PAA/$FITC_5$–PEI–$kemptide_{30}$ molar ratio of 2.5 in the PKA reaction buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$) containing 1 mM dithiothreitol and 50 nM ATP at 37 °C for 1 h (a) FITC fluorescence intensity measurements of PIC nanoparticles without PKA (■) or with PKA (5 nM; △, 10 nM; ●, 50 nM; ○) at various PKA concentrations and excess PKA inhibitor (10 nM; ▲) as a function of time. (b) Fluorescence image of a 96-well containing PIC nanoparticles with/without PKA (5–50 nM), or with PKA inhibitor (10 nM).

in emission ratio based on the genetically encoded protein reporters.[4]

As a control experiment, the FITC fluorescence did not change in the absence of PKA or ATP because phosphorylation reaction occurs in the presence of both PKA and ATP. In addition, control PIC nanoparticles with a scramble peptide (Leu–Arg–Arg–Ala–Ala–Leu–Gly; LRRAALG) did not show any change of FITC fluorescence in the presence of both PKA and ATP because the scramble peptide cannot be phosphorylated by PKA.[10] Fluorescence imaging showed that the PIC nanoparticles only presented a strong FITC signal in the presence of PKA and ATP, implying specificity to the phosphorylation reaction.

Fig. 3a shows that the change of FITC fluorescence intensity of PIC nanoparticles was time- and PKA concentration-dependent. FITC fluorescence intensity increased proportionally to PKA concentrations and PKA reaction time, but the control sample with scramble peptide or PKA inhibitor maintained its fluorescence intensity up to 1 h. During the PKA reaction, the scattering intensity of PIC nanoparticles decreased proportionally to the observed increase in fluorescent signal (Fig. SI2†). It means that the PIC nanoparticles were dissociated by the introduction of negatively charged phosphate groups to the serine moiety of PKA-specific peptide motif, resulting in a strong FITC intensity in response to phosphorylation. Therefore, PIC nanoparticles could detect the PKA activity at the level of nanomolar concentration. In addition, the PKA activity was simply measured with a color image, which was obtained by CCD camera (Fig. 3b). It reveals that the PIC nanoparticles have potential for screening of protein kinase inhibitors.

In summary, we have developed a polymer-based nanoparticle that uses intermolecular FRET between FITC and TRITC to detect and image PKA activity. Based on our results, polymeric nanoparticles enable the detection of PKA activity and screening of PKA inhibitors more simply. The PIC nanoparticles can be applied to HTS for large chemical libraries in the drug discovery of kinase inhibitors, because they do not require radioactive labelling, phosphopeptide-specific antibodies, or genetically encoded fluorescent protein reporters.

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