Conjugated polymer nanoparticles for biochemical protein kinase assay†

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Sensitive and reliable monitoring of kinase activity was reported by using highly efficient fluorescence resonance energy transfer of conjugated polymer nanoparticles (CPNs) to a rhodamine labelled peptide substrate.

Many diseases such as cancers, diabetes, and inflammation are closely related to uncontrolled functions of certain protein kinases that phosphorylate substrates in the signalling pathways.[1] A structural change resulting from the phosphorylation activates or alters the substrate function in its various signaling pathways. Because of their pivotal roles on the cellular process and diseases consequences, these pathways are prime targets for drug discovery.[2] There have been extensive efforts to screen molecular libraries for inhibitors or activators for a specific kinase. Biochemical assays offer quantitative monitoring of the enzyme activity in the presence of potential inhibitors using high replicate and throughput formats.[3] Among the biochemical assays, fluorescence-based homogeneous assays are widely used because they can detect phosphorylation events with a high degree of sensitivity and because they avoid radioisotopic labels.[4] One common issue associated with fluorescence based detection is interference from the background fluorescence of small molecule compound libraries. These signals can lead to false negatives or positives as a result of their inherent fluorescent or quenching properties, thereby complicating screening results. Time resolved fluorescence (TRF) is an excellent way to minimize the interfering signals by adapting long lifetime (millisecond) lanthanide reagents.[5] Fluorescence signals will be collected at a delayed time to remove fluorescence background from short lifetime (nanosecond) interferents. Fluorescence resonance energy transfer (FRET)-based assay can also reduce the background if the difference between the excitation wavelength of the donor and the emission wavelength of the acceptor, is extremely large (*i.e.*, more than 150 nm). In this case, negligible fluorescence intensity is expected at the long wavelength when the interferents are excited at short wavelength.

Here we exploit the efficient energy transfer of the conjugated polymer nanoparticles (CPNs) for the sensitive detection of rhodamine-labeled peptides commonly used as substrates for kinase enzymes. Highly efficient energy transfer from CPNs to rhodamine allows for the quantitative monitoring of phosphorylation of the peptide substrates by monitoring both fluorescence quenching (FQ) and FRET. We demonstrated a biochemical kinase assay for screening inhibitors in a multiwell format. Combined with the advantage of the large spectral difference between the excitation and emission wavelengths, ratiometric analysis from fluorescence quenching (FQ) of CPN and FRET to rhodamine allows for sensitive and reliable detection of the substrate phosphorylation.

CPNs are easily fabricated by a simple solvent exchange process and their sizes can be controlled by functionality of conjugated polymers. We previously demonstrated a facile formation of polymer particles and sensitive detection of Cy5-labeled oligonucleotide using particles formed by a simple solvent exchange process.[6] The superior sensitivity was attributed to the efficient energy transfer from the three-dimensional particles (donor) to the labeled oligonucleotide (acceptor). While we observed very strong fluorescence quenching of the particles by the Cy-5, a weak FRET peak was observed at 635 nm. Considering the poor spectral overlap between the donor (particle) and acceptor (Cy-5), the FRET signals observed at the Cy-5 emission were somewhat unusual. Despite the weak FRET signal at the Cy-5 emission wavelength (635 nm), the extremely large difference between the excitation wavelength (415 nm) and the emission wavelength (635 nm) is very useful for the sensitive detection of analyte with almost no background signals. This observation lead us to optimize the system to exhibit better FRET by substantially increasing the spectral overlap between the particle and dye, while maintaining the large wavelength difference.

Pentiptycene-containing poly(*p*-phenylene ethynylene (PPE) was synthesized as reported previously (Fig. 1).[6,7] To exploit the outstanding photophysical properties of the pentiptycene-containing polymers for the detection of phosphorylated peptides in aqueous phase, we co-polymerized the pentiptycene with a monomer containing iminodiacetic acids (IDAs) (see ESI†). The IDAs are widely used for metal ion immobilized chromatography to purify various biological substances such as proteins or peptides.[8] The IDA allows the chelation of metal ions onto

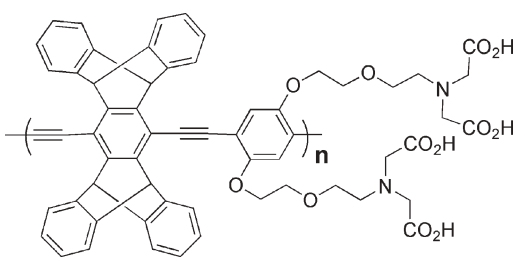

Fig. 1 Chemical structure of poly(*p*-phenylene ethynylene (PPE).

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CPNs for the selective detection of phosphopeptides. The viscous PPE solution in a mixed solvent of DMSO–morpholine was precipitated into an acetone solution and then re-dissolved in DMSO. CPN was fabricated by transferring the PPE in DMSO into an excess amount of water.[6] The CPN solution was purified by ultrafiltration through a membrane filter (molecular weight cut-off: 10 000 Da) in water. Particle size was determined by dynamic light scattering (DLS), indicating a *z*-average size of 28 nm with a polydispersity index (PDI) of 0.254. PDI is a parameter calculated from a cumulative analysis of DLS intensity measured *via* an autocorrelation function. % PDI is the square of the coefficient of variation (CV) and defined as $(PDI)^{1/2} \times 100$. From this relationship, particle size showed 50% of % PDI, indicating very broad size distributions. Transmission electron microscopic study supports formation of spherical nanoparticles (Fig. 2).[9] Although CPN showed poor contrast in the TEM, due to its low density, formation of spherical nanoparticles was confirmed by the image.

For the detection of phosphopeptides, the CPN solution was mixed with trivalent metal ions (*i.e.*, $GaCl_3$) solution in a buffered media (pH 5.5) (see ESI†). The CPN/Ga solution was transferred into rhodamine-labeled kemptide (Rh-kemptide) and rhodamine-labeled phosphokemptide (Rh-p-kemptide).[10] Fig. 3(a) demonstrates the spectra of the CPN mixed with Rh-kemptide (dotted line) and with Rh-p-kemptide (solid line). As shown in Fig. 3(a), CPNs only exhibit efficient energy transfer to Rh-p-kemptides, despite the poor spectral overlap between CPN and rhodamine. Negligible spectral change was observed from the Rh-kemptide, indicating the interaction is phosphopeptide-specific. Zheng and Swager demonstrated that better stacking and orbital interaction between the conjugated polymer backbone and acceptor dyes are the main contributors to efficient energy migration.[11] CPN's efficient energy transfer capability to an acceptor dye with longer absorption wavelength has the direct benefit of reducing unnecessary background signals. The background fluorescence originated from the assay media or fluorescent molecules in the library often interferes with assay readout resulting in uncertainty and complexity in the interpretation of the assay results. The difference between excitation wavelength (400 nm) and emission wavelength (585 nm) was so large that unnecessary background can be minimized.

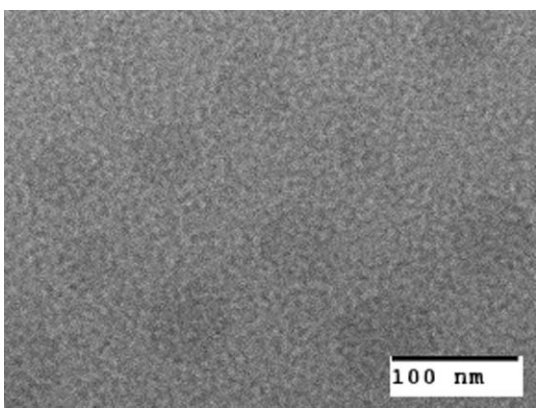

Fig. 2 Transmission electron microscopic image of PPE in water. Spherical shaped nanoparticles are observed. Poor contrast is attributed to the low density of the CPNs.

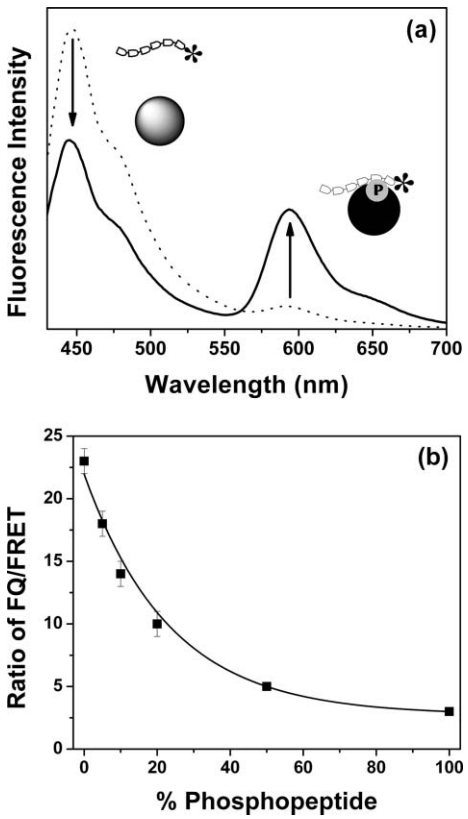

Fig. 3 (a) Emission spectra of CPN mixed with rhodamine-labeled kemptide (Rh-kemptide, dotted line) and with rhodamine-labeled phosphokemptide (Rh-p-kemptide, solid line). Strong FRET occurred from metal ion-assisted adsorption of phosphokemptides, while no significant FRET was observed from nonphosphorylated kemptide. (b) Ratiometric (FQ/FRET) calibration curve generated from standard mixtures of Rh-kemptide and Rh-p-kemptide.

Fig. 3(b) indicates that the fluorescence quenching and FRET intensity are proportional to the amount of phosphorylation. Standard Rh-kemptide/Rh-p-kemptide solutions were prepared by proportionally mixing Rh-p-kemptide and Rh-kemptide (*i.e.* 0, 5, 10, 25, 50 and 100% of Rh-p-kemptide). By taking the ratio of fluorescence quenching to FRET signal, an accurate and quantitative measurement at the low percent conversion of phosphorylation was achieved. The ratiometric method can eliminate fluorescence variations caused by the fluctuation of excitation source or different assay concentrations.[12] As seen in Fig. 3(b), a significantly different signal change (5%) was observed from the low phosphopeptide conversion.

To demonstrate the kinase assay, we monitored kinase activity as a function of the inhibitor. The enzymatic reaction was performed by incubating Rh-kemptide with protein kinase A (PKA) for 60 min at room temperature in the presence of adenosine triphosphate (ATP). PKA, is an important enzyme involved in several key second-messenger signalling pathways, and is implicated in a variety of cellular processes including cell cycle progression, apoptosis, transcription, and cellular metabolism.[13] After the enzymatic reaction, CPN/Ga solution was added and incubated for 30 min. The amount of phosphorylated substrate was measured by calculating the ratio between the FQ of the CPNs at 455 nm and the FRET signal at 585 nm. Using the

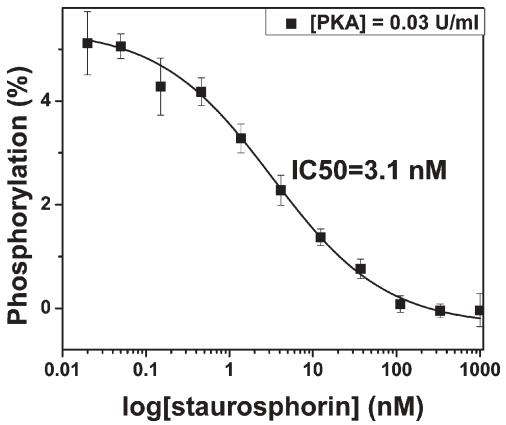

Fig. 4 Inhibition curves of PKA in the presence of staurosporin. IC50 was determined by sigmoidal curve fitting.

calibration curve [Fig. 3(b)], ratios from the assay were converted to the amount of phosphorylation in the substrate (percent phosphorylation). Therefore, the enzyme efficiency can be measured as a function of percent phosphorylation. Enzyme reactions were conducted in the presence of 10 μM ATP, 0.5 μM Rh-kemptide substrate, and various amounts of inhibitor (staurosporin). By plotting enzyme efficiency (percent phosphorylation) as a function of inhibitor concentration, 50% inhibitory concentration (IC50) was calculated. Fig. 4 demonstrates that CPNs can reliably and robustly detect ~5% phosphorylation even at the low enzyme concentration (0.03 U PKA mL^{-1}). Sigmoidal dose response with acceptable error range indicates the sensitivity and reliability of CPN assay. The IC50 value is well matched with published values (3.6 nM).[14]

In conclusion, we have fabricated a CPN with highly efficient energy transfer to an acceptor dye and used it for sensitive detection of kinase activity. Biochemical assay results show promise for high throughput screening of small molecules with improved sensitivity and reliability.

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Notes and references

1 For comprehensive reviews, see, *Chem. Rev.*, 2001, **101.**
2 M. E. M. Noble, J. A. Endicott and L. N. Johnson, *Science*, 2004, **303**, 1800.
3 D. M. Olive, *Expert Rev. Proteomics*, 2004, **1**, 327.
4 D. S. Lawrence and Q. Z. Wang, *ChemBioChem*, 2007, **8**, 373; F. Rininsland, W. S. Xia, S. Wittenburg, X. B. Shi, C. Stankewicz, K. Achyuthan, D. McBranch and D. Whitten, *Proc. Natl. Acad. Sci. U. S. A.*, 2004, **101**, 15295; J. H. Kim, S. Lee, K. Kim, H. Jeon, R. W. Park, I. S. Kim, K. Choi and I. C. Kwon, *Chem. Commun.*, 2007, 1346; M. D. Shults and B. Imperiali, *J. Am. Chem. Soc.*, 2003, **125**, 14248.
5 Y. Jia, C. M. Quinn, A. I. Gagnon and R. Talanian, *Anal. Biochem.*, 2006, **356**, 273; Y. W. Park, R. T. Cummings, L. Wu, S. Zheng, P. M. Cameron, A. Woods, D. M. Zaller, A. I. Marcy and J. D. Hermes, *Anal. Biochem.*, 1999, **269**, 94.
6 J. H. Moon, R. Deans, E. Krueger and L. F. Hancock, *Chem. Commun.*, 2003, 104.
7 T. M. Swager, *Acc. Chem. Res.*, 1998, **31**, 201.
8 Z. Elrassi and C. Horvath, *J. Chromatogr., A*, 1986, **359**, 241.
9 The average particle size in the TEM image was about 70 nm. It was very difficult under the microscopic conditions to observe smaller particles than 70 nm that are believed as a major population.
10 Kemptide is a synthetic heptapeptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) specific substrate for protein kinase A (PKA): B. E. Kemp, D. J. Graves, E. Benjamini and E. G. Krebs, *J. Biol. Chem.*, 1977, **252**, 4888.
11 J. Zheng and T. M. Swager, *Chem. Commun.*, 2004, 2798.
12 H. Sun, A. M. Scharff-Poulsen, H. Gu and K. Almdal, *Chem. Mater.*, 2006, **18**, 3381; A. E. Albers, V. S. Okreglak and C. J. Chang, *J. Am. Chem. Soc.*, 2006, **128**, 9640.
13 J. I. Huggenvik, M. W. Collard, R. E. Stofko, A. F. Seasholtz and M. D. Uhler, *Mol. Endocrinol.*, 1991, **5**, 921; W. Matten, I. Daar and G. F. V. Woude, *Mol. Cell. Biol.*, 1994, **14**, 4419; B. T. Gjertsen and S. O. Doskeland, *Biochim. Biophys. Acta*, 1995, **1269**, 187.
14 Q. Li, K. W. Woods, S. Thomas, G. D. Zhu, G. Packard, J. Fisher, T. M. Li, J. C. Gong, J. Dinges, X. H. Song, J. Abrams, Y. Luo, E. F. Johnson, Y. Shi, X. S. Liu, V. Klinghofer, R. D. Jong, T. Oltersdorf, V. S. Stoll, C. G. Jakob, S. H. Rosenberg and V. L. Giranda, *Bioorg. Med. Chem. Lett.*, 2006, **16**, 2000.

