

Supplementary Information

Polymeric nanoparticles for protein kinase activity

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Experimental section

Materials. Polyethyleneimine (PEI, linear type, Mw = 25 kDa) was purchased from Polysciences Inc. (Warrington, PA). Poly(aspartic acid) (PAA, Mw = 10 kDa), fluorescein isothiocyanate (FITC, Mw = 389), tetramethylrhodamine isothiocyanate (TRITC, Mw = 444), anhydrous dimethyl sulfoxide (DMSO), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxybenzotriazole (HOBt) were purchased from Aldrich Chemical Co. (Milwaukee, WI). N- α -Fmoc-protected kemptide (Fmoc-Leu-Arg-Arg-Ala-Ser-Leu-Gly) and a scramble peptide (Fmoc-Leu-Arg-Arg-Ala-Ala-Leu-Gly)¹ were obtained from Anygen Co. (Gwangju, Korea). Each peptide was purified to greater than 99% purity by reversed-phase HPLC on a Sim-pack C-18 column (Shimadzu SCL-10A VP, Tokyo, Japan). The molecular mass was determined by MALDI-TOF mass spectrometry (KRATOS Analytical Axima-CFR, Shimzu, Japan). The MS [M+H]⁺ for N- α -Fmoc-protected kemptide and scramble peptide are 977.3 (calculated 977.1) and 961.5 (calculated 961.1), respectively.

Preparation of FITC-PEI-kemptide. Into an aqueous solution of PEI (200 mg, 8 μ mol) in distilled water, FITC (20 mg, 50 μ mol, dissolved in DMSO) was added and the reaction mixture was stirred overnight at room temperature. After the reaction, the polymer solution was dialyzed in distilled water by using dialysis membrane (3.5 kDa molecular weight cutoff (MWCO); Spectrum Laboratory, Inc.,

Rancho Dominguez, CA) and lyophilized. The stoichiometry of fluorophore conjugation (FITC-PEI), calculated by using the extinction coefficients of FITC ($75,000 \text{ M}^{-1} \text{ cm}^{-1}$ at 485 nm), is 5 ± 1.2 mol FITC/mol of PEI, resulting in FITC₅-PEI (Mw = 26.9 kDa). FITC₅-PEI (100 mg, 3.7 μmol) and N- α -Fmoc-protected kemptide (130 mg, 133 μmol) or N- α -Fmoc-protected scramble peptide (127 mg, 132 μmol) were dissolved in distilled water (10 ml, pH 5), followed by the addition of EDC (28.8 mg, 150 μmol) and HOBt (20.3 mg, 150 μmol) and the reaction mixture was stirred overnight at 4 °C. The resulting solution was dialyzed y using dialysis membrane (3.5 kDa MWCO) and lyophilized. On average, each polymer of FITC₅-PEI contained 30 ± 3.2 molecules of N- α -Fmoc-protected kemptide or contained 27 ± 2.2 molecules of N- α -Fmoc-protected scramble peptide, which was determined by TNBS assay.² After the reaction, the deprotection reaction of Fmoc in each peptide was carried out in 20 wt% piperidine solution at 25°C for 30 min³, resulting in FITC₅-PEI-kemptide₃₀ (Mw= 52.9 kDa) and FITC₅-PEI-scramble peptide₂₇ (Mw = 49.8 kDa). Finally, the solution was dialyzed against distilled water (3.5 kDa MWCO) and lyophilized.

Preparation of TRITC-PAA. To a solution of the free acid form of PAA (100 mg, 10 μmol) in DMF (2 ml) was added tBOC-ethylenediamine HCl (8 mg , 50 μmol), triethylamine (70 μl , 500 μmol), EDC (14.3 mg, 75 μmol), and HOBt (10.2 mg, 75 μmol). The reaction mixture was stirred for overnight at 25°C. The resultant solution was precipitated against cold diethyl ether. The precipitate was filtered off and the solvent was removed. The residue was deprotected in methylene chloride/trifluoroacetic acid (1:1, v/v). After the reaction, each PAA polymer contained 5.0 ± 1.2 of amine groups, which was determined by TNBS assay. To a TFA salt form of PAA-ethylenediamine (50 mg, 4.6 μmol), in DMF were added TRITC (33.4 mg, 70.0 μmol , 3-fold higher than the amine groups in PAA-ethylenediamine), DIEA (17.8 μl , 100 μmol), and allowed reaction for 2 h at 25°C. The reaction mixture was precipitated against cold diethyl ether and dried under vacuum. After dissolving of the residual solid in distilled water followed by neutralization with 1 N NaOH, the solution was dialyzed in distilled water by using dialysis membrane (3.5 kDa MWCO) and lyophilized. The stoichiometry of fluorophore conjugation was calculated by using the extinction coefficients (TRITC, $150,000 \text{ M}^{-1} \text{ cm}^{-1}$

at 555 nm) and were 5 ± 0.9 mol TRITC/mol of PAA, in resulting TRITC₅-PAA (M_w = 12.6 kDa). No free amino group on TRITC₅-PAA was detected using TNBS assay, indicating that all the amino groups of ethylenediamine-modified PAA are chemically coupled with TRITC molecules.

Preparation of Poly-ion complex (PIC) nanoparticles. Specific amounts of concentrated TRITC₅-PAA solution were added to a solution of FITC₅-PEI-kemptide₃₀ (0.1 mg/ml) in the PKA reaction buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂) to yield different PIC nanoparticles with TRITC₅-PAA/ FITC₅-PEI-kemptide₃₀ molar ratios varying 0 to 3. Dynamic light scattering (DLS, Spectra Physics Laser Model 127-35), fluorescent imaging (12-bit CCD camera, Kodak), and fluorescence spectra (ISS K2 fluorometer) measurements were carried out after each incremental addition of TRITC₅-PAA solution to FITC₅-PEI-kemptide₃₀ solution. The average diameter of PIC nanoparticles was determined using DLS operated at 633 nm and 25 ± 0.1 °C. Also, scattering light intensity of PIC nanoparticles was measured at an angle of 90° and collected with a BI-9000AT autocorrelator and the polydispersity factor, represented as μ_2/Γ^2 , was evaluated from the cumulant method, where μ_2 is the second cumulant of the decay function and Γ is the average characteristic line width.⁴ The fluorescence resonance energy transfer (FRET) efficiency of PIC nanoparticles was calculated by using the following formula: % FRET efficiency = $[1-(F_d'/F_d)] \times 100$, where F_d is the donor fluorescence intensity without an acceptor and F_d' is the donor fluorescence with an acceptor.⁵ The zeta potentials of the PIC nanoparticles were measured using an ELS-8000[®] electrophoretic light scattering spectrophotometer (Otsuka, Electronics Co., Ltd., Japan), wherein each polymer was dissolved in distilled water (pH = 7.5). Transmission electron microscopy (TEM) was performed using a Philips CM 200, operating at an acceleration voltage of 80 kV. To measure the morphology and size distribution of PIC nanoparticles in distilled water, a drop of sample solution was placed onto a 300 mesh copper grid coated with carbon. About 2 min after deposition, the grid was tapped with filter paper to remove surface water, followed by air-drying. Negative staining was performed using a droplet of a 2 wt% uranyl acetate solution.

The average diameter and stability of PIC nanoparticles. The average diameter and stability of PIC nanoparticles were confirmed by DLS measurements. At the TRITC₅-PAA/ FITC₅-PEI-kemptide₃₀ molar ratios of 2.5, the mean diameter of PIC nanoparticles in the PKA reaction buffer was about 57 nm and the polydispersity factors (μ_2/Γ^2) for self-aggregates, estimated by the cumulant method, were fairly low (0.05), implying a narrow size distribution (Fig. SI1a).⁴ The average diameter of PIC nanoparticles was reproducible to repeated experiments. These PIC nanoparticles were completely precipitated by centrifugation at 10,000 g for 10 min and each polymer was not found in the supernatant part, confirmed by UV spectrometer, indicating that all the polymer are self-aggregate to make PIC nanoparticles. When the PIC nanoparticles were stored at 25 °C, the average diameter and polydispersity factors were not changed within 2 days, but the PIC nanoparticles were aggregated and the average diameter and polydispersity factors increased after 2 days (Fig. SI1b). Thus, the freshly prepared PIC nanoparticles should be used to the PKA assay within 2 days.

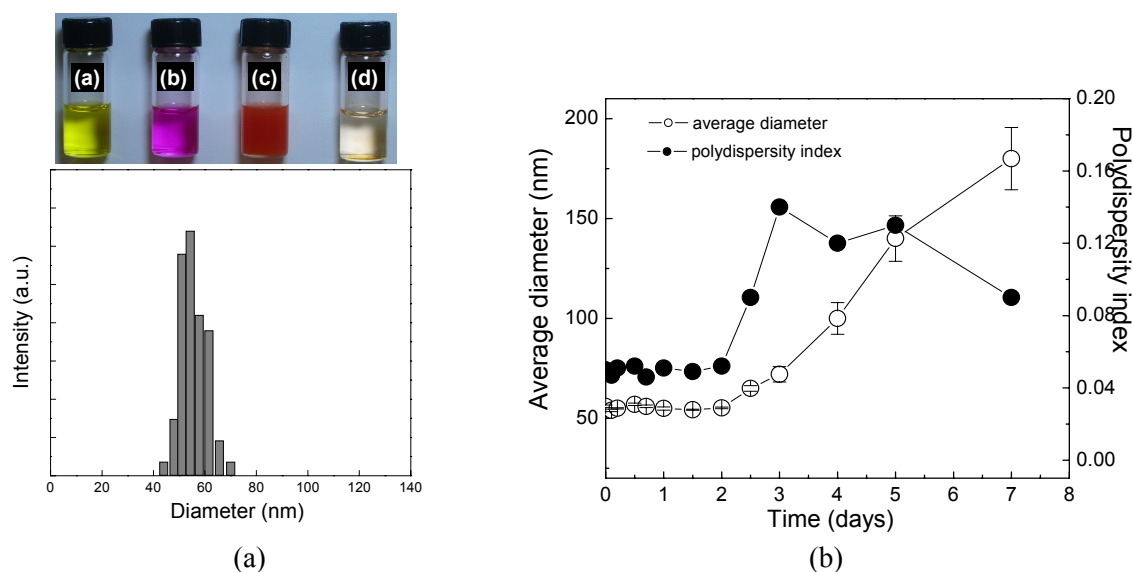


Fig. SI1. (a) The average diameter and (b) the stability of PIC nanoparticles with TRITC₅-PAA/ FITC₅-PEI-kemptide₃₀ molar ratios of 2.5 in the PKA reaction buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂) at 25 °C. The inset image of each polymer solution; (a) FITC₅-PEI-kemptide₃₀, (b) TRITC₅-PAA, (c) PIC nanoparticles, and (d) PIC nanoparticles after centrifugation at 10,000 for 10 min.

Protein kinase assay. The PIC nanoparticles (10 μg), wherein the kemptide concentration is 5.4 nM, were incubated with protein kinase A (PKA) catalytic subunit in the PKA reaction buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl_2) containing 1 mM dithiothreitol and excess ATP (50 nM, 9.25 fold higher than kemptide concentration) at 37 $^\circ\text{C}$. To evaluate the efficacy of the PIC nanoparticles on the PKA assay, the recovery of FITC fluorescence intensity with/without ATP and with/without PKA inhibitor was observed. The PIC nanoparticles were incubated in PKA assay buffer containing PKA (5 nM ~ 50 nM) and inhibitor at 37 $^\circ\text{C}$ for up to 60 min. PKA inhibitor-(14-22)-amide (10 nM) was used for PKA inhibition assay and it should inhibit completely the PKA reaction, because of its highly specific and strong inhibition property ($K_i = 36$ nM) to protein kinase A.⁶ After the different PKA reactions, the efficacy of PIC nanoparticles on detecting the protein kinase activity was confirmed by measuring the changes of FITC fluorescence intensity, monitored by using an ISS K2 multifrequency phase modulation fluorometer (ISS, Champaign, IL) and the relative scattering intensity of PIC nanoparticles, confirmed by DLS. Also, the FITC fluorescent images of the 96-well plate were obtained using a 12-bit CCD camera (Kodak, Japan) equipped with a special C-mount lens and a FITC bandpass emission filter (510 to 600 nm; Omega Optical).

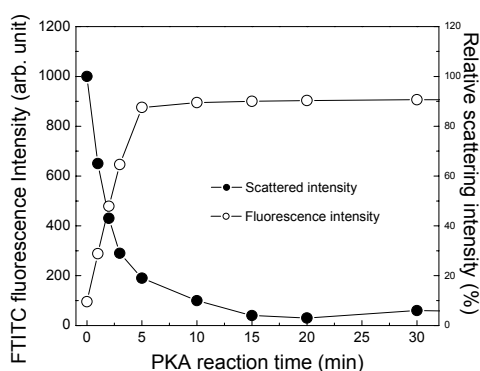


Fig. SI2. The relationship between fluorescence intensity and relative scattering intensity of PIC nanoparticles during the PKA reaction. The PIC nanoparticles (10 μg) were incubated with PKA (50 nM) in the PKA reaction buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl_2) containing 1 mM dithiothreitol and excess ATP (50 nM, 9.25 fold higher than kemptide concentration) at 37 $^\circ\text{C}$.

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