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Traceless protein delivery with an efficient recyclable nanocarrier

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Experimental

Material and methods

LysoTracker Red DND-99 and DAPI were purchased from Invitrogen. Bovine serum albumin (BSA) and Ribonuclease A from bovine pancreas (RNase) were obtained from Sigma. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) was from J&K Chemical LTD. All other chemicals were from Alfa Aesa. pET28a expression vector containing the gene encoding Asparaginase-eGFP (ASP-eGFP) was provided by Professor Jiahuai Han at Xiamen University. eGFP and TAT-eGFP were cloned according to a reported literature.[1] β-galactosidase (β-GAL) was provided by Professor Xiaomei Yan at Xiamen University. Proteins were overexpressed in BL21(DE3) competent cells (*Escherichia coli*) and then purified to homogenous following Ni-NTA purification protocol from Invitrogen. HeLa, HepG2 and L929 cells were obtained from American Type Culture Collection. The cells were pre-cultured in DMEM for 48 h in cell culture dish 35 mm x 12 mm style Φ20 mm glass bottom at 37 ℃ with 5% CO_2 and then subjected to interactions with protein-nanoparticle composites in the same dish at 37 ℃ with 5% CO_2.

Scanning electron microscopy analysis was performed on HITACHI S-480. Protein concentrations were determined by Bradford assay. Fluorescence spectra of FITC labeled RNase A and FITC labeled BSA were performed on a spectrofluorimeter (Spectamax M5, Molecular Device) using the excitation wavelength (λex) of 490 nm. Cells were analyzed using a fluorescence microscope (Ti-S; Nikon eclipse) equipped with a 100-W mercury lamp (C-SHG1, Nikon) using the following filters: λex@420-490 nm for FITC. Confocal fluorescence microscopic images were obtained on Leica SP5 using the following filters: λex@543 nm and λem@565-625 nm for LysoTracker Red; λex@488 nm and λem@500-530 nm for FITC, ASP-eGFP, eGFP, TAT-eGFP; λex@405 nm and λem@405-480 nm for DAPI. Flow cytometric data were obtained on Beckman Coulter. The fluorescence emission intensity of eGFP, TAT-eGFP were recorded with filter FL1 (510-535 nm). Images of the intracelluair fluorescence of FITC, eGFP, DAPI, LysoTracker Red DND-99 were merged using Photoshop CS 6.0..

Preparation and characterization of calcium phosphate nanoparticles

PLP-CP: $Na_2HPO_4 \cdot 12H_2O$ (120 mg), $CaCl_2$ (90 mg), PLP (100 mg) and hexadecyl trimethyl ammonium bromide (CTAB, 200 mg) were added together to a flask containing aqueous HCl solution (pH 1.0, 50 ml). The mixture was stirred at rt for 2 h followed by dropwise addition of triethylamine until the solution pH reached pH 8.0. The reaction mixture were further stirred vigorously at rt for 24 h and then subjected to centrifugation at 9000 rpm for 5 min. The precipitate was washed with methanol (50 ml x 3) and then distilled water (50 ml x 3) to afford PLP-CP. The nanoparticles were lyophilized fro subsequent studies.

Low-PLP-CP: CP with lower levels of PLP: $CaCl_2$ (90 mg), $Na_2HPO_4 \cdot 12H_2O$ (160 mg), PLP (40 mg) and CTAB (200 mg) were added together to a flask containing aqueous HCl solution (pH 1.0, 50 ml). The mixture was stirred at rt for 2 h followed by dropwise addition of triethylamine until the solution pH reached pH 8.0. The reaction mixture were further stirred vigorously at rt for 24 h and then subjected to centrifugation at 9000 rpm for 5 min. The precipitate was washed with methanol (50 ml x 3) and then distilled water (50 ml x 3) to afford Low-PLP-CP.

CP: PLP-free calcium phosphate nanoparticles: $CaCl_2$ (90 mg), $Na_2HPO_4 \cdot 12H_2O$ (200 mg), and CTAB (200 mg) were added into aqueous HCl solution (50 ml, pH 1.0). The mixture was stirred at rt for 2 h and then neuralized by slow addition of triethylamine until pH of the solution reached pH 8.0. The reaction mixture were further stirred vigorously at rt for 24 h and then subjected to centrifugation at 9000 rpm for 5 min. The precipitate was washed with methanol (50 ml x 3) and then distilled water (50 ml x 3) afford CP. The nanoparticles were lyophilized. The resultant nanoparticles were analyzed by SEM and UV-vis spectrometry.

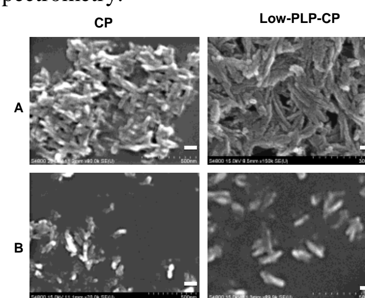


Fig. S1 SEM images of CP and Low-PLP-CP (bar: 100 nm).

Collodial stability of PLP-CP

The newly synthesized PLP-CP and PLP-CP that has been placed

in PBS at room temperature for one month were analyzed by SEM .

PLP Levels on PLP-CP and Low-PLP-CP

Low-PLP-CP (4 mg), PLP-CP (4 mg) or CP (4 mg) were respectively prepared in Na_2HPO_4-H_3PO_4 solution (100 mM, pH 3.0, 4 ml) and subjected to ultrasonication (5 min). To the solution were respectively added ethylenediamine tetraacetic acid (EDTA, 0.4 M, 100 µl). The pH of mixture were adjusted to pH 9.5 with addition of aqueous solution of NaOH (1 M). The solution were analyzed by UV-vis absorption spectrum. Standard concentrations of PLP (0-80 µg ml^{-1}) in Na_2HPO_4-H_3PO_4 solution (100 mM, pH 9.5) were used as the controls. Levels of PLP on PLP-CP were determined by PLP standard curve made by absorbance at 490 nm.

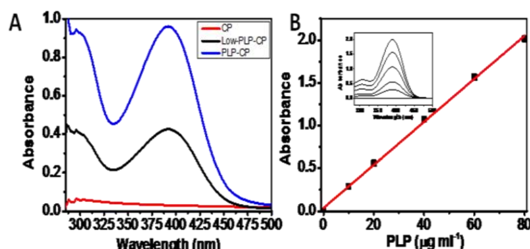


Fig. S2 Levels of PLP on Low-PLP-CP and PLP-CP. (**A**) To Na_2HPO_4-H_3PO_4 solution (100 mM, pH 3.0, 4 ml) was respectively added Low-PLP-CP (4 mg), PLP-CP (4 mg) or CP (4 mg). The solutions were sonicated for 5 min and then to the solution was added EDTA (0.5 M, 100 µl). The solutions were then adjusted to pH 9.5 and then ananlyzed by UV-vis absorption. (**B**) PLP standard curve was plotted by absorbance at 490 nm as a function of PLP concentrations. The insert showed the UV-absorption spectra of Na_2HPO_4-H_3PO_4 solution containing various levels of PLP (0-80 µg ml^{-1}).

Zeta potential analysis

CP, Low-PLP-CP and PLP-CP were respectively dispersed in distilled water by ultrasonication (5 min) to a final concentration of 1 mg ml^{-1}. The solution were analyzed with Zetasizer Nano ZS for zeta potential of the corresponding nanoparticles.

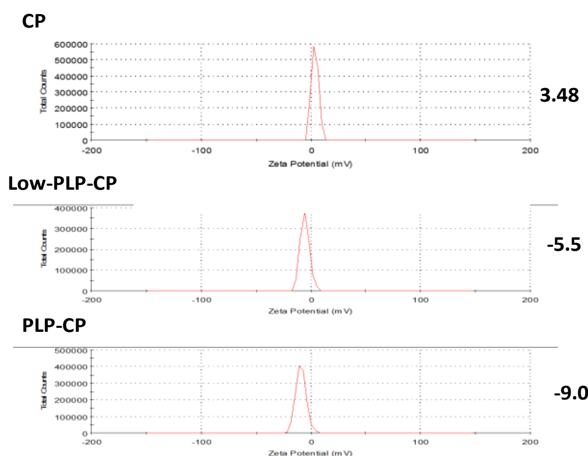


Fig. S3 Zeta potentials of CP-PLP, Low-PLP-CP and CP.

Labeling of prtoeins with FITC

To PBS (10 ml, pH 7.4) containing RNase (50 mg) was added the solution of FITC in DMF (25 µl, 6 mg ml^{-1}). The mixture was gently stirred at 4 °C for 1 h and then extensively dialyzed against PBS (pH 7.4) to afford FITC-labeled RNase (RNase-FITC). FITC-labeled BSA (BSA-FITC) was prepared using the same procedure as that of RNase-FITC.

Complexation of proteins with PLP-CP

BSA (5 mg), BSA-FITC (5 mg), RNase (5 mg), RNase-FITC (5 mg), ASP-eGFP (5 mg), eGFP (5 mg) or β-Galactosidase (5 mg) were respectively incubated with PLP-CP (10 mg) in PBS (10 ml, pH 7.4) were at 4 °C for 12 h with gentle stirring. The mixtures were centrifuged, washed with PBS (10 ml) and then resupended in PBS (4 ml, pH 7.4) to afford PLP-CP/BSA, PLP-CP/BSA-FITC, PLP-CP/RNase, PLP-CP/RNase-FITC, PLP-CP/ASP-eGFP, PLP-CP/eGFP or PLP-CP/β-Galactosidase. The morohology of synthesized PLP-CP/RNase-FITC was analyzed by SEM.

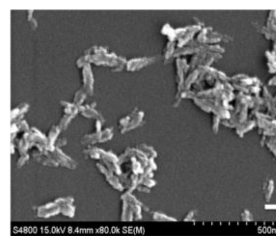


Fig. S4 SEM image of PLP-CP/RNase-FITC (bar: 100 nm).

Levels of proteins on calcium phosphate nanoparticles

BSA (3 mg), BSA-FITC (3 mg), RNase (3 mg), RNase-FITC (3 mg), were respectively incubated with CP (3 mg), Low-PLP-CP (3 mg) or PLP-CP (3 mg) in PBS (3 ml, pH 7.4) at rt for 12 h with gentle stirring. The mixtures were centrifuged, washed with Na_2HPO_4-H_3PO_4 (100 mM, pH 7.4). The levels of proteins on CP, Low-PLP-CP, or PLP-CP were determined by Bradford assay.

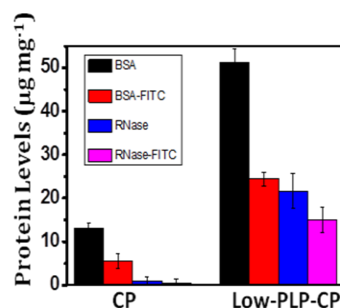


Fig. S5 Levels of protein loaded on Low-PLP-CP and CP.

pH dependent release of protein from PLP-CP

PLP-CP/RNase-FITC (2.5 mg) was respectively added to Na_2HPO_4-H_3PO_4 buffer (100 mM, 1 ml) of various pH values (4.0, 5.0, 6.5 or 7.5). The solutions were maintained at 37 °C with stirring at 100 rpm in a shaker. At fixed time points (0-30 min), a potion of the solutions was aliquoted and centrifuged at 9000 rpm for 5 min, the supernatant free of nanoparticles was decanted. The pellets were resuspended in distilled water (1 ml)

and then anaylzed for fluorescence emission intensities using λex@490 nm.

Stabililty of PLP-CP/eGFP in serum

PLP-CP/eGFP (1 mg) was respectively added to a serial of tubes containing fetal bovine serum (FBS, 1 ml). The mixtures were incubated at 4 °C for 0, 2, 4, or 6 h. The mixtures were centrifuged at 9000 rpm for 10 min at the indicated incubation time and the supernatant was decanted. The pellets were resuspended in PBS (1 ml) and recorded at fluorescence spectrophotometer using λex@490 nm.

For the control assay, PLP-CP (1 mg) was dispersed into FBS (1 ml) and the mixture was incubated at 4 °C for 6 h. The mixture was centrifuged at 9000 rpm for 10 min and the supernatant was decanted. The pellets were resuspended in PBS (1 ml) and analyzed for fluorescence emission using λex@490 nm.

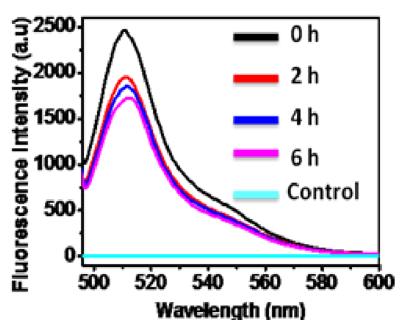


Fig. S6 Stability on PLP-CP/eGFP in serum. PLP-CP/eGFP (1 mg) were respectively dispersed in FBS (1 ml) and then incubated for 0-6 h at 4 °C. The nanoparticles were havrvested by centrifugation and then dispersed in PBS. The fluorescence emission of the resultant nanoparticles were recorded using λex@490 nm. PLP-CP free of GFP was processed under idential conditions and used as the control.

PLP-CP for intracellular protein delivery as compared to CP

HeLa cells were respectively cultured for 24 h in DMEM medium in cell culture dish. To the cell lines were respectively added fresh DMEM (1 ml) spiked with RNase-FITC (10 μg), PLP-CP/RNase-FITC (25 μg), or RNase-FITC (25 μg) and CP. The particles were centrifuged, washed with Na_2HPO_4-H_3PO_4 (100 mM, pH 7.4) before used. The cells were further incubated for 4 h then analyzed with a Leica SP5 confocal fluorescence microscope.

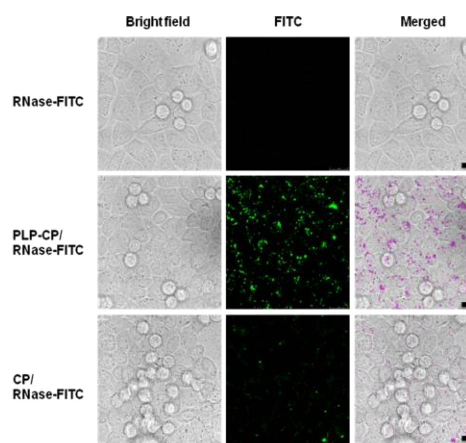


Fig. S7 PLP-CP mediated intracellular RNase-FITC as compared to CP in HeLa cell lines. The protein was shown in green, Bar: 10 μm.

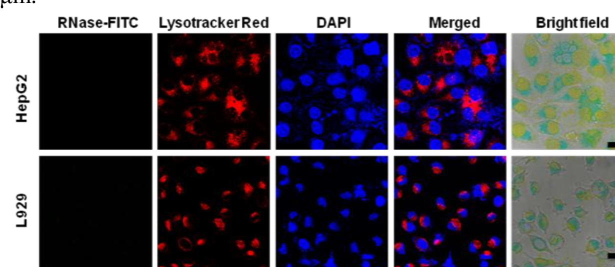
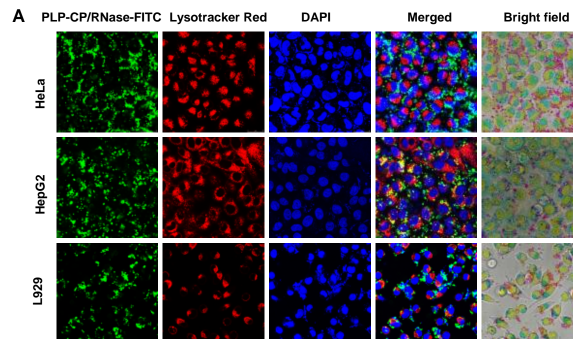


Fig. S8 Intracellular delivery of RNase-FITC. HepG2 or L929 cells were respectively incubated with RNase-FITC (10 μg) in DMEM (1 ml) for 4 h, and then analyzed by confocal fluorescence microscope. The location of lysosomes were shown in red and that of nucleus were shown in blue. The proteins was shown in green. Bars, 20 μm.

Intracellular delivery of proteins mediated by PLP-CP

HeLa, HepG2 and L929 cells were respectively cultured for 24 h in DMEM medium in cell culture dish. To the three cell lines were respectively added fresh DMEM (1 ml) spiked with RNase-FITC (10 μg), ASP-eGFP (10 μg), PLP-CP/RNase-FITC (25 μg), or PLP-CP/ASP-eGFP (25 μg). The cells were further incubated for 2-10 h. At fixed time points, the cells were analyzed with a Leica SP5 confocal fluorescence microscope.



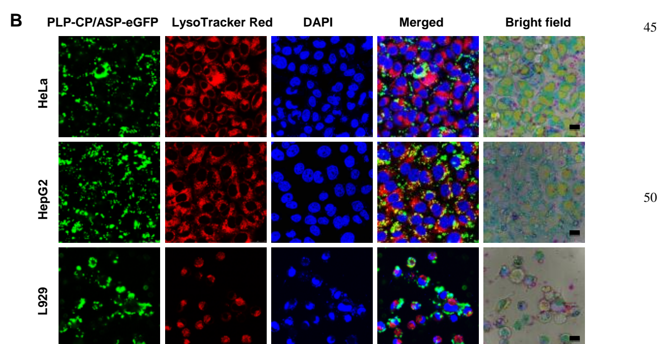


Fig. S9 PLP-CP mediated transduction of RNase (A) and Asp-eGFP (B) into cells. HeLa, HepG2 and L929 cells were respectively incubated with PLP-CP/RNase-FITC (25 μg) or PLP-CP/ASP-eGFP (25 μg) in DMEM (1 ml) for 4 h, and then analyzed by confocal fluorescence microscopy. The location of lysosomes were shown in red and that of nucleus were shown in blue. The proteins was shown in green. Bars, 10 μm.

Enzymatic activity of β-galactosidase delivered into cells by PLP-CP

HeLa, HepG2 and L929 cells were respectively incubated for 4 h in DMEM (1 ml) containing β-galactosidase (25 μg) or PLP-CP/β-galactosidase (25 μg). The resultant cells were cultured in fresh DMEM supplemented with X-gal (100 μM) for 12 h and then visualized with a fluorescence microscope.

TAT mediated intracelluar delivery of eGFP

HepG2 cells were cultured in DMEM (1 ml) for 4 h spiked with eGFP (5 μg), TAT-eGFP (5 μg) or PLP-CP/eGFP (50 μg) that levels of eGFP on PLP-CP was about 5 μg determined by Bradford Assay. The cells were washed with PBS (1 ml x 3), suspended in fresh DMEM (1 ml), and then examined with a Leica SP5 confocal fluorescence microscope.

Flow cytometry analysis of protein transduction mediated by TAT and PLP-CP

HeLa, HepG2 and L929 cells were respectively cultured for 4 h in DMEM (1 ml) containing no addition, eGFP (5 μg), TAT-eGFP (5 μg), or eGFP (5 μg) preloaded on PLP-CP/eGFP. The cells were washed with PBS (1 ml x 3), suspended in PBS (1 ml), and then analyzed by flow cytometry.

Cytotoxicity of PLP-CP

HepG2 cells were cultured in DMEM containing various levels of PLP-CP (0, 10, 25, 50, 100 μg ml^{-1}) for 0-24 h. The cells before and after incubations were stained with trypan blue. Cell number and cell viability were determined using the trypan blue exclusion test.

References

[1] N. J. Caron, Y. Torrente, G. Camirand, M. Bujold, P. Chapdelaine, K. Leriche, N. Bresolin, J. P. Tremblay, *Molecular Therapy,* 2001, **3**, 310.