Supporting Information for

Direct Delivery of Functional Proteins and Enzymes to the Cytosol Using Nanoparticle-Stabilized Nanocapsules

Rui Tang, Chang Soo Kim[‡], David J. Solfiell[‡], Subinoy Rana[‡], Rubul Mout, Elih M. Velázquez-Delgado, Apiwat Chompoosor, Youngdo Jeong, Bo Yan, Zheng-Jiang Zhu, Chaekyu Kim, Jeanne A. Hardy, and Vincent M. Rotello*

Department of Chemistry, University of Massachusetts-Amherst, 710 North Pleasant Street, Amherst, Massachusetts, 01003, USA

*Address correspondence to rotello@chem.umass.edu.

[‡]These authors contributed equally to this work.

Materials and Methods

GFP expression and purification. Starter cultures from a glycerol stock of BL21(DE3) housing the gene for Enhanced GFP (EGFP) with an *N*-terminal 6-His tag in the pET21b expression vector (Novagen) with or without the peroxisomal-targeting sequence 1 (PTS1) were grown overnight in 50 mL of 2 × YT media with 50 µL of 1000× ampicillin (16 g tryptone, 10 g yeast extract, 5 g NaCl in 1 L water). The cultures were shaken overnight at 250 rpm at 37 °C. The following day, 10 mL of the starter cultures were added to a Fernbach flask containing 1 L of 2 × YT and 1 mL 1000× ampicillin and shaken until OD_{600} = 0.7 was reached. The protein expression was then induced by adding IPTG (1 mM, final concentration) and shaken at 28 °C. After 3 h, the cells were harvested by centrifugation (5000 rpm for 15 min at 4 °C). The pellet was then resuspended in lysis buffer (2 mM imidazole, 50 mM NaH_2PO_4, 0.3 M NaCl) and the cells were lysed using a microfluidizer. Cell lysate was centrifuged at 15,000 rpm for 45 min at 4 °C. The GFP was purified from the supernatant using an imidazole gradient on

a HisPur (Thermo Scientific) Cobalt column. The imidazole used for elution was removed from the GFP sample by dialysis in 5 mM sodium phosphate buffer (pH = 7.4).

Caspase-3 expression and purification. The caspase-3 full-length human gene in the pET23b vector (Addgene) was used for expression of caspase-3. The procedure is similar as GFP expression and purification. The supernatant was loaded onto a 5 mL HiTrap Ni-affinity column (GE Healthcare). The column was washed with a buffer of 50 mM Tris pH 8.5, 300 mM NaCl, 5% glycerol and 50 mM imidazole. The caspase-3 protein was eluted with a buffer containing 50 mM Tris pH 8.5, 300 mM NaCl, 5% glycerol and 250 mM imidazole. The eluted fraction was diluted 9-fold into a buffer containing 20 mM Tris pH 8.0 and 2 mM DTT to reduce the salt concentration. This protein sample was loaded onto a 5 mL Macro-Prep High Q column (Bio-Rad Laboratories, Inc.). The column was developed with a linear NaCl gradient and caspase-3 eluted in a buffer of 20 mM Tris pH 8.0, 120 mM NaCl and 2 mM DTT. The eluted protein was stored at -80 °C in the elution buffer conditions. Caspase-3, analyzed by SDS-PAGE to be ~98% pure was further analyzed by ESI-MS to confirm mass.

Cell culture. HeLa cells were cultured in a humidified atmosphere (5% CO_2) at 37 °C, and grown in Dulbecco's modified eagle's medium (DMEM, low glucose) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin).

Cell transfection and selection. PCDNA3.1(-)-mcherry plasmid was cloned from pCHERRY3 plasmid, purchased from Addgene (ID: 24659). RFP-PTS plasmid was purchased from Origene. HeLa cells were transfected by lipofectamine 2000 according to manufacturer's protocols (Invitrogen). The transfected cells were then selected by

geneticin (Invitrogen) at the concentration of 400 μg/mL in DMEM with 10% FBS until the stably transfected clones were obtained.

Fluorescence titration. In the fluorescent titration experiment between nanoparticles and GFP, the change of fluorescence intensity at 515 nm was measured with an excitation wavelength of 485 nm at various concentrations of NPs from 0 to 200 nM on a Molecular Devices SpectraMax M3 microplate reader (at 25 °C). Decay of fluorescence intensity arising from 100 nM GFP was observed with increasing NP concentration. Nonlinear least-squares curve fitting analysis was carried out to estimate the binding constant (Ks)[1].

Cell viability assay (Alamar Blue). 7,500 HeLa cells were cultured in a 96-well plate for 24 h prior to the experiment. The cells were washed by cold phosphate buffer saline (PBS) 3 times before the delivery, then different amounts of the GFP-NPSC complex (prepared as mentioned above) were diluted by DMEM and incubated with the cells for 1 h followed by the incubation with DMEM containing 10% FBS and 1% antibiotics for 23 h. After washing with PBS 3 times, the cells were then incubated with 200 μL DMEM containing 10% Alamar Blue for 3 h. Cell viability was calculated by measuring the fluorescence intensity of Alamar Blue at 590 nm, with an excitation of 535 nm.

***In vitro* caspase-3 activity assay.** For kinetic measurements of caspase activity, 20 nM protein with and without 40 nM HKRK AuNPs were assayed over the course of 5 minutes in a caspase-3 activity-assay buffer containing 20 mM Tris (pH 7.5) and 5 mM TCEP. 150 μM substrate (DEVD-AMC [N-acetyl-Asp-Glu-Val-Asp-AMC], Enzo Lifesciences; Ex. 365 nm / Em. 495 nm) was added to initiate the reaction. Assays were performed in triplicate at 25°C in 100 μL volumes in 96-well microplate format using a Molecular Devices Spectramax spectrophotometer.

Live cell imaging. 240 K HeLa cells were cultured in the confocal dish 24 hr prior to the experiment. Before live cell imaging, cells were washed by PBS for three times followed by the incubation with GFP-NPSC in cell culture media. The confocal dish was then placed in the live cell imaging chamber with 5% CO_2 and at 37 °C on the confocal microscope. A series of images were taken at certain time intervals.

Figures

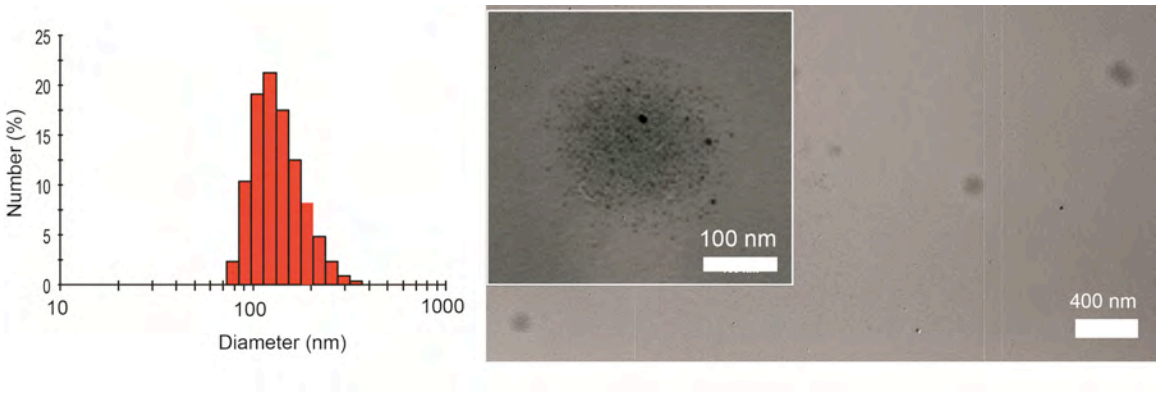

Figure S1. DLS (left) and TEM (right) results of CASP3-NPSCs.

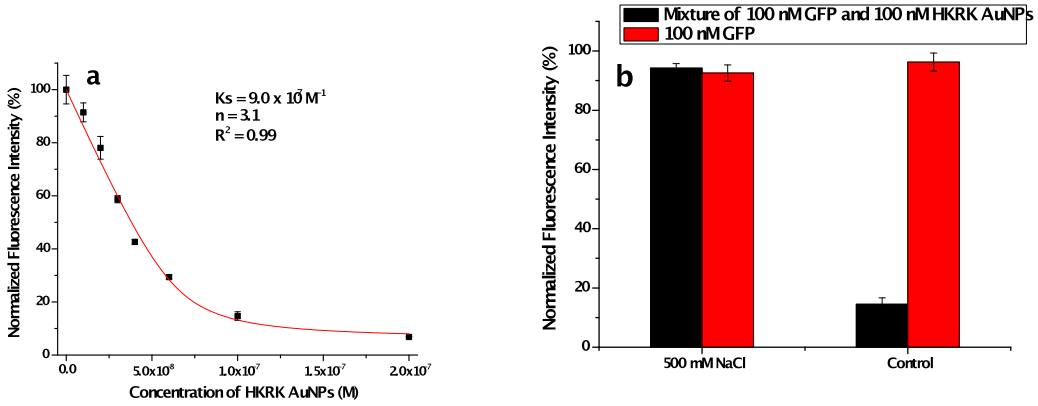

Figure S2. Determination of the interaction between GFP and HKRK AuNPs. (a) Fluorescence titration of HKRK AuNPs in the presence of 100 nM GFP in 5 mM phosphate buffer (pH = 7.4). The binding constant (Ks) and association stoichiometry (n) were calculated through the fitting using the model of single set of identical binding sites[2]. (b) Fluorescence recovery of 100 nM GFP quenched by 100 nM HKRK AuNPs in the presence of 500 mM NaCl or phosphate buffer (control).

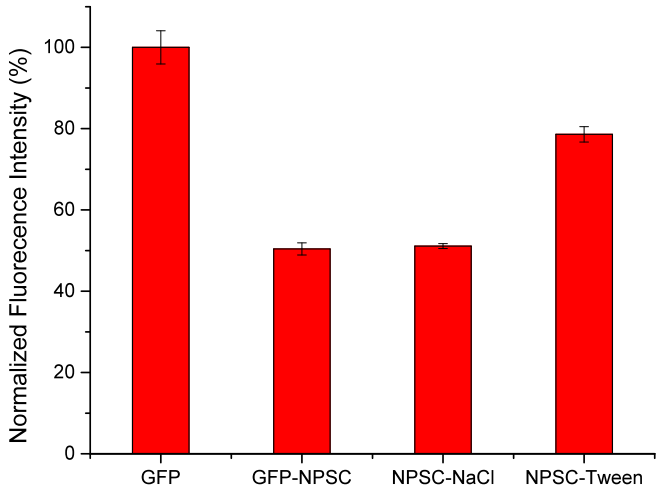

Figure S3. Fluorescence recovery of GFP quenched by NPSC. The concentrations of both GFP and AuNP are 400 nM in 100 μL mixture. The concentration of NaCl is 500 mM and that of Tween-20 is 0.5%. by volume

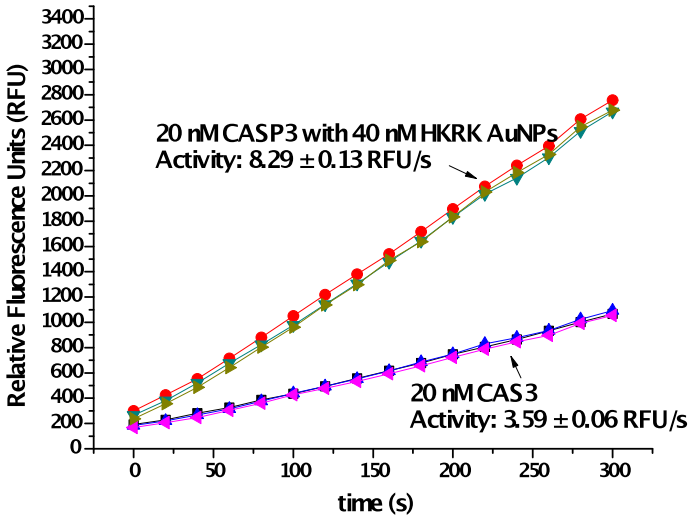

Figure S4. *In vitro* activity assay of CASP3 in the presence or absence of HKRK AuNPs.

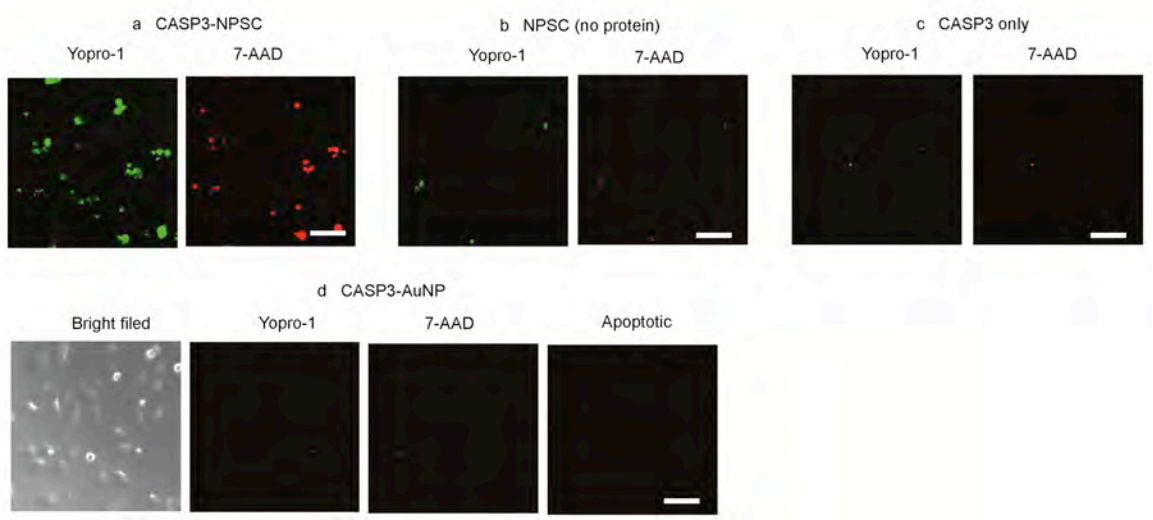

Figure S5. Delivery of caspase-3 into HeLa cells. (a-c) Yopro-1 and 7-AAD channels of Figure 2. (d) HeLa cells were incubated with CASP3-AuNPs for 1 h, and then stained with Yopro-1 and 7-AAD for 30 min. The apoptotic ratio is 4.2 ± 1.0%. Scale bars: 100 μm.

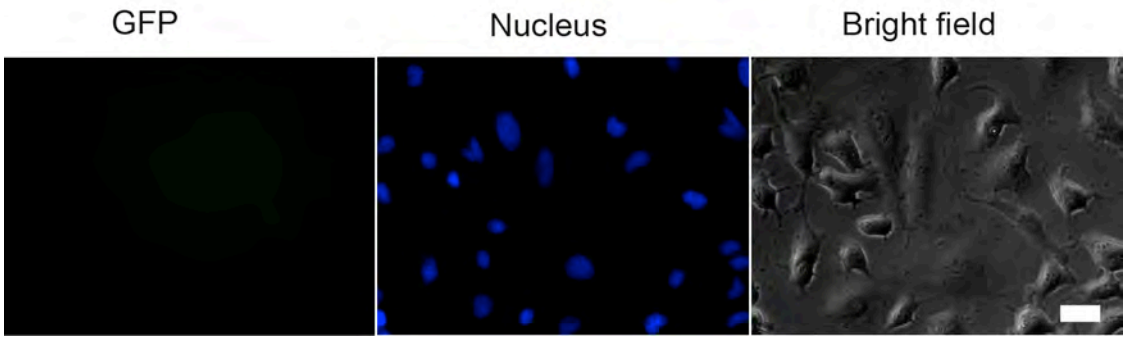

Figure S6. Co-incubation of empty NPSC and GFP with HeLa cells for 1 hr. The bar represent 20 μm.

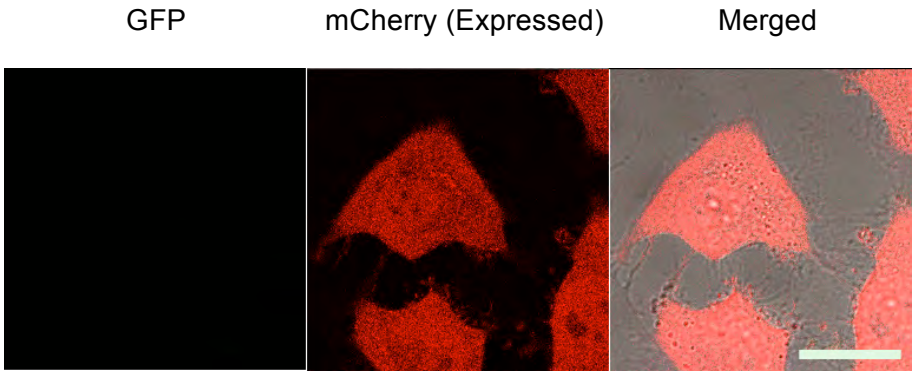

Figure S7. HeLa cells stably express mCherry (control group for Figure 2d in context). The scale bar represents 20 μm.

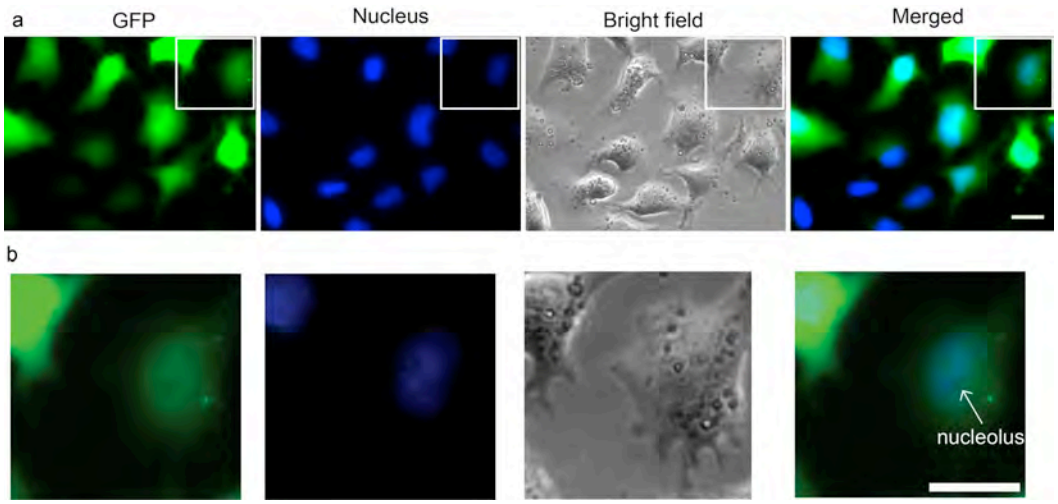

Figure S8. (a) Colocalization of GFP and Hoechst 33342 in a HeLa cell. (b) Magnified from boxes of Figure S6a. The scale bars represent 20 μm.

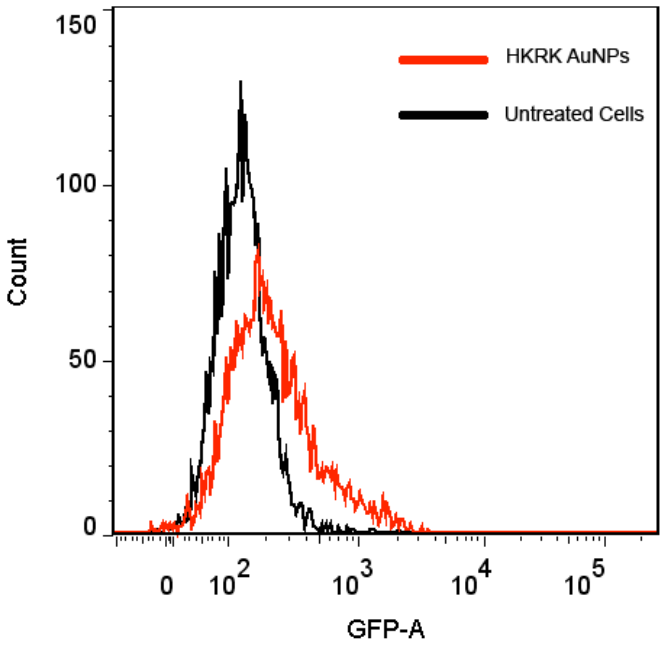

Figure S9. Flow cytometry results of HeLa cells cultured with GFP-HKRK AuNPs for 2 h, using untreated HeLa cells as the control.

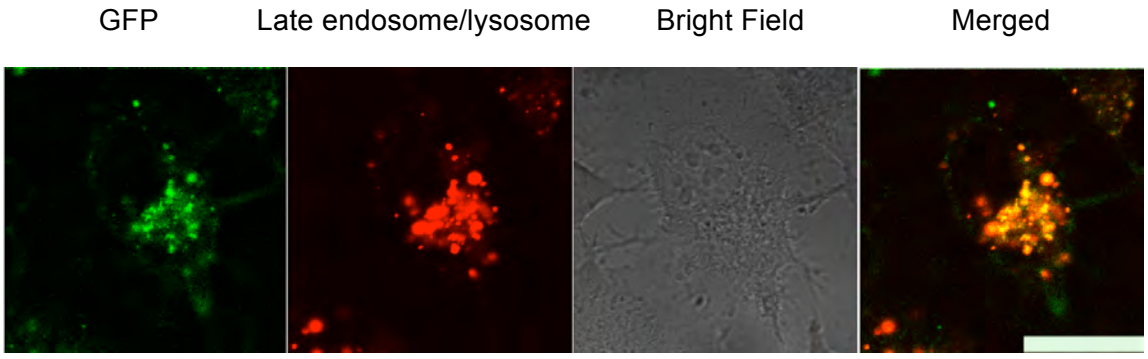

Figure S10. Confocal image of the colocalization of GFP with late endosomes and lysosomes after 1 hr of delivery by HKRK AuNPs. The scale bar represents 20 μm.

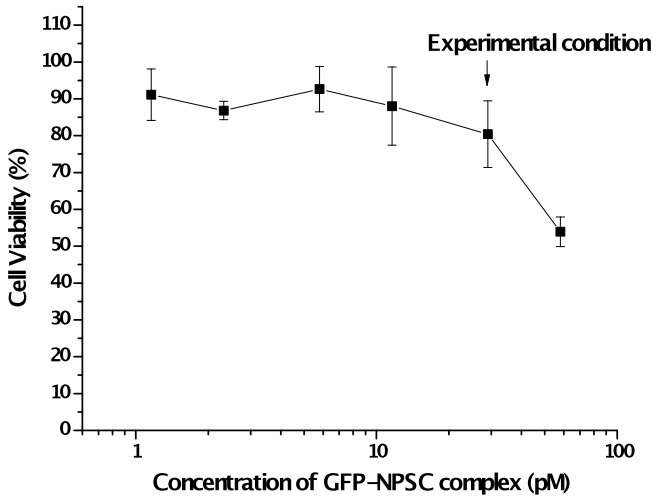

Figure S11. Viability of HeLa cells at different concentrations of GFP-NPSC complexes measured by Alamar Blue assay.

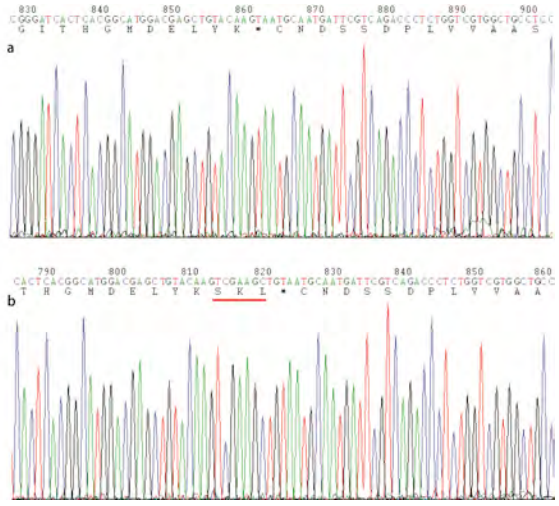

Figure S12. GFP fused with PTS1 motif.

(a) Sequence of C-terminal of GFP from the plasmid (sequence: MASHHHHHHMVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFIC TTGKLPVPWPTLVTTFTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGN YKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNF KIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFV TAAGITHGMDELYK).

(b) Sequence of C-terminal of GFP fused with PTS1 motif from the plasmid (sequence: MASHHHHHHMVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFIC

TTGKLPVPWPTLVTTFTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGN
YKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNF
KIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFV
TAAGITHGMDELYKSKL).

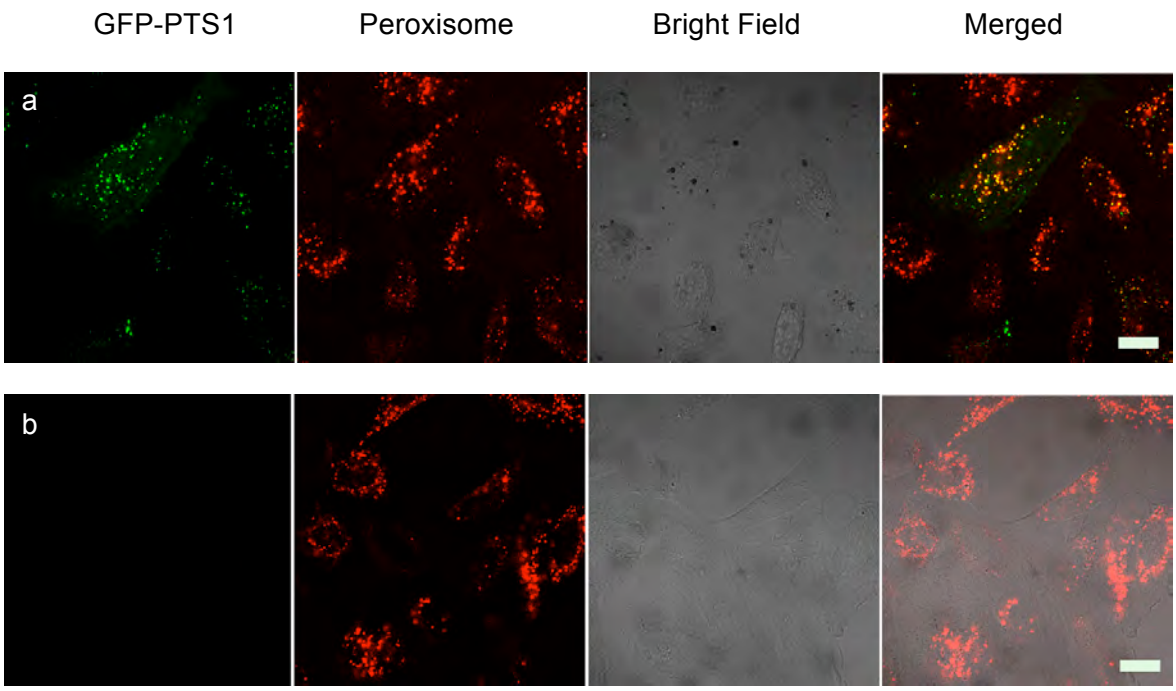

Figure S13. (a) Delivery of GFP-PTS1 into HeLa cells by NPSCs. (b) Untreated cells under the same exposure conditions. The scale bars represent 20 μm.

Movies S1 and S2. Live cell imaging for rapid GFP release into the cytosol of HeLa cell by NPSC.

Reference

1. You, C. C., De, M., Han, G. & Rotello, V.M. Tunable inhibition and denaturation of α-chymotrypsin with amino acid-functionalized gold nanoparticles. *J. Am. Chem. Soc.* **127**, 12873-12881 (2005).

2. De, M. *et al*. Sensing of proteins in human serum using conjugates of nanoparticles and green fluorescent protein. *Nat. Chem.* **1**, 461-465 (2009).