Intracellular Delivery of a Membrane-Impermeable Enzyme in Active Form using Functionalized Gold Nanoparticles

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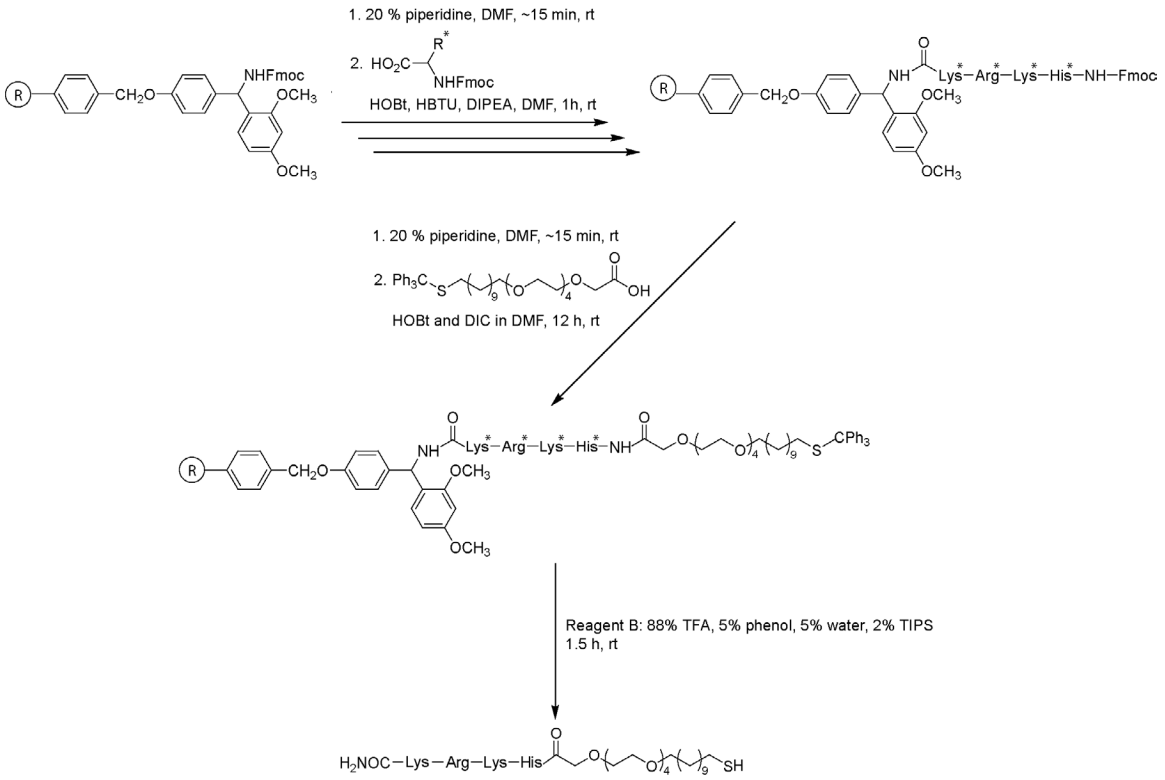
Materials. All chemicals were bought from Aldrich unless otherwise stated. The organic solvents were purchased from EMD and used as received except dichloromethane and toluene which were distilled in presence of calcium hydride. For purification, flash column chromatography was performed using silica gel (SiO_2, particle size 40-63 μm). Amino acids and resin were purchased from Advanced ChemTech (USA). β-Gal, cell culture medium powder and phosphate buffer saline (PBS) were purchased from Sigma. FM 4-64 was purchased from molecular probes (Invitrogen, USA).

Synthesis of peptide-conjugated ligand

The peptide was synthesized manually, using standard Fmoc chemistry with Rink resin (0.7 mg/g) as a solid support. Side-chain protecting groups were as follows: trityl-chloride (Trt) for histidine; 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) for arginine; *tert*-butoxycarbonyl (Boc) for lysine. Fmoc deprotection was carried out using 20% piperidine in DMF for 15 min, and amino acid (5 eq) coupling was performed using HBTU (5 eq), HOBt (5 eq) and DIPEA (10 eq) in DMF for 1 h (Scheme S1).

The trityl-protected thiol linker (5 eq) was conjugated to the peptide using 1,3-diisopropylcarbodiimide (DIC, 6 eq) and HOBt (7.5 eq) in a overnight reaction. The thiol linked peptide was cleaved/deprotected for 1.5 h using *Reagent B*: 88% trifluoroacetic



Scheme S1: The procedures for peptide-ligand synthesis (* indicates protected side chains).

acid (TFA), 5% phenol, 5% water and 2% triisopropylsilane (TIPS). TFA was then removed under vacuum, and the ligand was precipitated with cold diethylether. The crude product was purified by reverse phase high pressure liquid chromatography (RP-HPLC) and then analyzed by mass spectrometry (calculated 986.6, obtained 987.6; $M+H^+$) (Figure S1).

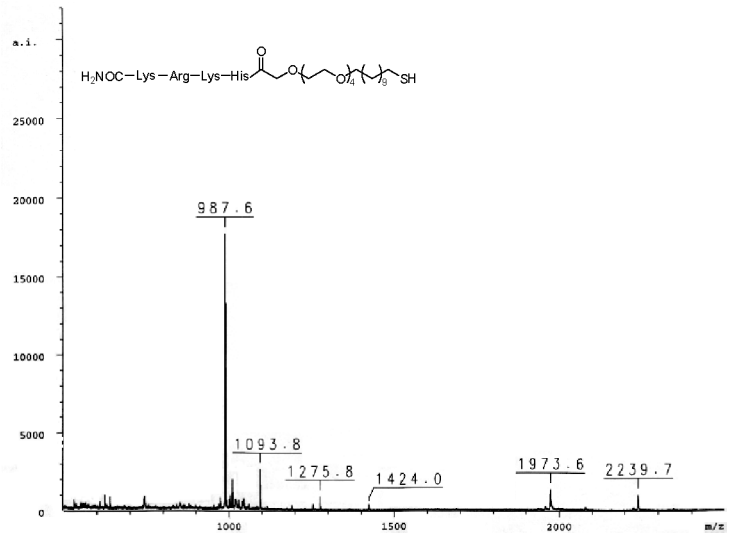
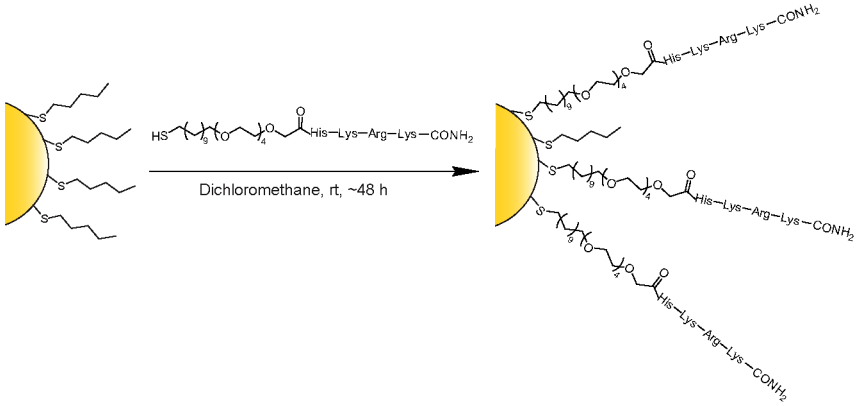


Figure S1: The MALDI-TOF mass spectra of the synthesized peptide ligand.

Synthesis of functionalized gold nanoparticle



Scheme S2: Functionalization of nanopaticles via Murray place-exchange reaction.

1-Pentanethiol-coated gold nanoparticles (10 mg) were dissolved in dichloromethane (DCM), and peptide ligands (20 mg) were taken in methanol. After purging argon into them separately for 30 min, they were mixed together and stirred for ~2 days at room temperature (Scheme S2). Then solvents were rotavaped, and excess ligands were washed away with DCM-methanol (20:1, v/v; 5X)) followed by dialysis (cut off 10 kDa, Pierce) for 2 days. Absence of free ligand was verified by NMR, and particles were characterized by UV, DLS and TEM (Figure S2). We have estimated ~ 100 peptide ligands will be incorporated onto the gold core of a nanoparticle during the place-

exchange reaction. The estimation was based on the data reported by Murray et al, where they observed incorporation of 86 ligands in similar place-exchange condition (mol ratio of reactant thiol: clusture thiol = 2:1).[1]

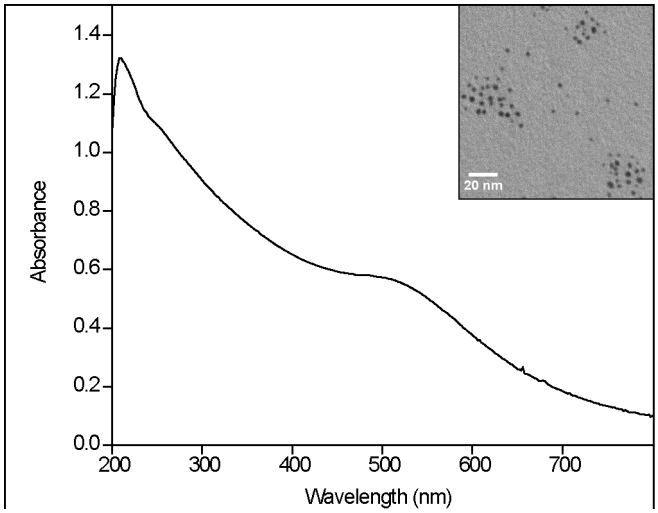


Figure S2: UV spectra and TEM image of the peptide functionalized gold nanoparticles.

Labeling of *β*-gal with FITC

Fluorescein isothiocyanate isomoer I (FITC) was dissolved in dimethyl sulfoxide at a concentration of 4 mg/mL. The *β*-gal (2.5mg) was dissolved in 900 μL of 0.1 M sodium bicarbonate solution (pH 9.0), and mixed with 250 μL freshly prepared FITC solution. The mixture was protected from light and stirred at room temperature for 2 h. The resulting FITC labeled *β*-gal was purified by size exclusion chromatography with Sephadex G-25 as stationary phase and phosphate buffer (5mM, pH 7.4) as mobile phase. Finally, the *β*-gal concentration and labeling efficiency (*β*-gal:FITC=1:16) were measured by ultraviolet-visible absorption spectroscopy.

***β*-Gal-nanoparticle binding experiment**

FITC-*β*-gal (100 nM) was mixed with **NP_Pep** at various molar ratios in 5 mM phosphate buffer (pH 7.4, with and without NaCl of 150 mM) for 10 min at room temperature. The fluorescence was recorded on a Spectro-Max M5 microplate reader (Molecular Device) (Ex 490 nm and Em 520 nm). Absorbance due to nanoparticle was corrected with control TEG-OH nanoparticle (NP_TEG).[2] The corrected intensities were plotted and fitted with a nonlinear equation (eqn 1) using Origin 8, providing K_D value

(11 ± 4 nM and 0.97 ± 0.58 nM, with and without salt respectively) and binding ratio (**NP_Pep**/*β*-gal: ~3.5:1 and ~ 8:1, with and without salt respectively).[3]

$$\frac{I}{I_0} = \frac{\alpha}{2}\{([\beta\text{-gal}]_0 + n[NP]_0 + 1/K_s) - \sqrt{([\beta\text{-gal}]_0 + n[NP]_0 + 1/K_s)^2 - 4n[\beta\text{-gal}]_0[NP]_0}\} \quad \text{eqn 1}$$

Where K_s = association constant, n = binding ratio (considering identical biding site)

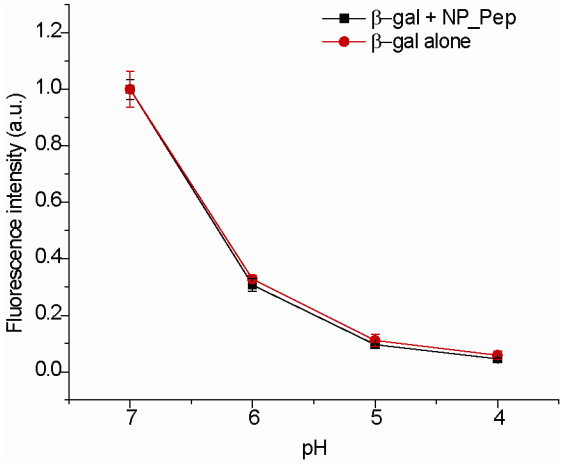


Figure S3: Fluorescence of FITC-β-gal (100 nM) alone or complexed with **NP_Pep** (200 nM) at different pH.

Circular Dichroism

CD spectra of *β*-gal (100 nM) were taken with or without nanoparticles (200 nM) in 5 mM phosphate buffer after 10 min of complexation. It was performed on a Jasco 720 spectrophotometer using a quartz cuvette of 1-mm path length. After equilibration at 25 ºC for 5 min, the spectra were acquired by scanning from 250 nm to 190 nm. Average of three scans was recorded at a rate of 20 nm/min with 8 sec response and 0.1 nm interval. The final spectra were obtained by subtracting the blank ones (only buffer for the protein and 200 nM particle for the complex) and it was fitted into secondary structure algorithm CDSSTR (protein ref. set 7 comprising of 49 proteins) using DICHROWEB (http://www.cryst.bbk.ac.uk/cdweb/html/home.html).

Tryptophan fluorescence measurement

Fluorescence spectra were measured in a 1 cm quartz cuvette on a Photon Technology International fluorescence spectrometer at 25 °C. Tryptophan fluorescence was monitered at 310~450 nm wavelength by exciting at 295 nm. Spectra of 100 nM of

β-gal solution were collected without/with **NP_Pep** (200 nM) in 5 mM phosphate buffer (pH 7.4) at 25 °C.

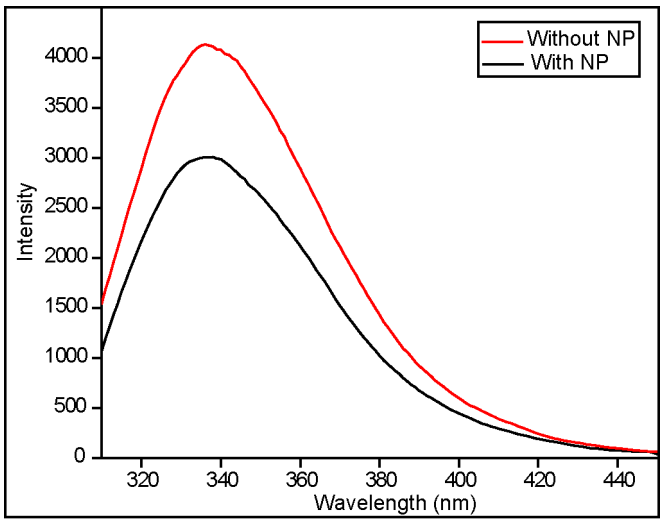


Figure S4: No change in λ_{max} of tryptophan-fluorescence upon addition of nanoparticle.

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). C2C12 and COS-1 cells were cultured in high glucose media (DMEM, high glucose) with 10% FBS. MCF7 cells were cultured in low glucose media (DMEM, low glucose) with 10% FBS.

β-Gal delivery

Cells (HeLa: $3x10^4$, C2C12: 6_10^4, COS-1: $6x10^4$, MCF7: $10x10^4$ per well) were seeded on 24 well plate. Next day β-gal-nanparticle mixture was added onto cells after washing with phosphate buffer saline (PBS). β-Gal was mixed with **NP_Pep** in 5 mM phosphate buffer (pH 7.4) for 10 min, and then diluted 6X with DMEM without serum. The cells were incubated with the transfection medium (protein-nanoparticle complex in DMEM without serum) for 3 h, and then washed with PBS (3X) followed by another 3h of culture in DMEM with 10 % FBS. All delivery experiments (like Figure 3-5 in the manuscript) were done following the above procedure unless otherwise noted.

Fluorescence and confocal microscopy

Cells were washed twice with PBS before capturing images. Fluorescence pictures were taken on an Olympus IX51 inverted microscope keeping all parameters same for different samples.

Confocal pictures were obtained on a Zeiss LSM 510 Meta microscope using 40X objective. For these experiments, HeLa cells ($10x10^4$ per dish) were grown on glass-bottom dishes (MatTek, 14 mm in diameter). For endosomes labeling, FM 4-64 (7.5 μg/mL) was added to the media at the beginning of transfection.

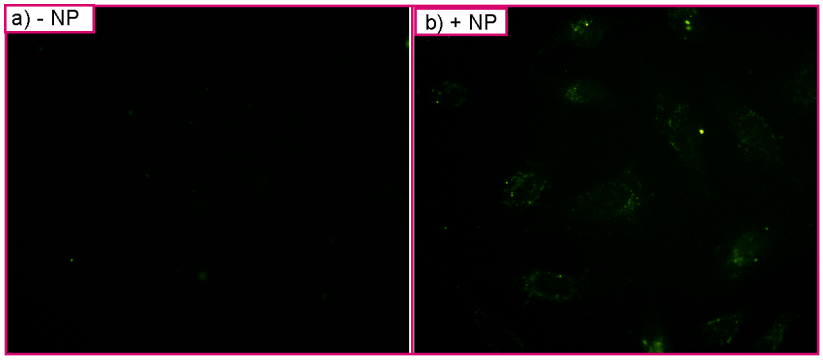


Figure S5: Images captured by fluorescence microscope after protein transfection in presence of serum (a) without and (b) with nanoparticles. Cell were treated with **NP_Pep**/FITC-*β*-gal (100 nM/50 nM) complex in DMEM media with 10% serum for 3 h, then washed with PBS and cultured for another 3 h before taking the images.

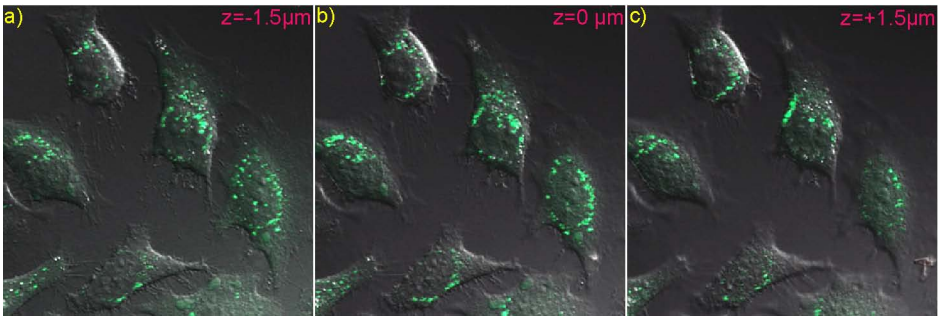


Figure S6: Three consecutive slices at z-axis from confocal experiment indicating localization of protein inside the cells.

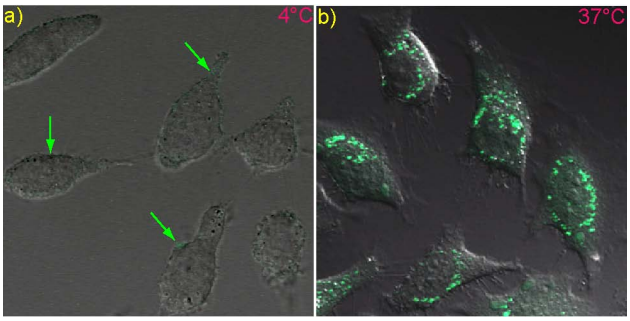


Figure S7: **CLSM** images of HeLa cells treated with **NP_Pep**/FITC-β-gal (100 nM/50 nM) at 4 °C and 37 °C. Protein signal was less at 4 °C, and located mainly on surface as indicate by arrows.

ICP- MS measurements

ICP-MS measurements were performed on a Perkin-Elmer Elan 6100. The operating conditions were as follows: rf power: 1250 W; plasma Ar flow rate: 15 L/min; nebulizer Ar flow rate: 0.96 L/min; dwell time: 50 ms; nebulizer: cross-flow; spray camber: Scott.

After enzyme **(NP_Pep**/β-gal: 100 nM/50 nM**)** delivery, cells were washed twice with PBS and then lysis buffer (250 µL/well, 30 min; Genlantis, USA) was added. Cell lysate was digested overnight with 3 mL nitric acid and 1 ml of hydrogen peroxide. The following morning, 1 ml of freshly prepared aqua regia (caution!) was added and allowed to react with the sample for one more hour. The sample was heated to 100 °C to reduce volume of the above digestion to ~1 mL. It was then diluted to 10 mL using a volumetric flask with ultra pure water and additional aqua regia (5% final concentration). Each sample was measured in quadruplet via ICP-MS as described above. A series of gold standards containing 5% aqua regia was prepared and ran prior to the samples (20, 10, 5, 2, 1, 0.5, 0.2, 0 ppb). The gold uptake in each sample was determined from the resulting calibration line. Deionized water was used to wash the instrument between each sample analysis.

X-gal assay

Cells were stained according to the assay kit (Genlantis, USA). After 12 h of staining, cells were washed once with PBS and observed under an optical microscope

(Nikon/Spot-RT). Experiments were performed in duplicate. For counting, four pictures (each having ~30 cells) were taken randomly from each replica and then averaged.

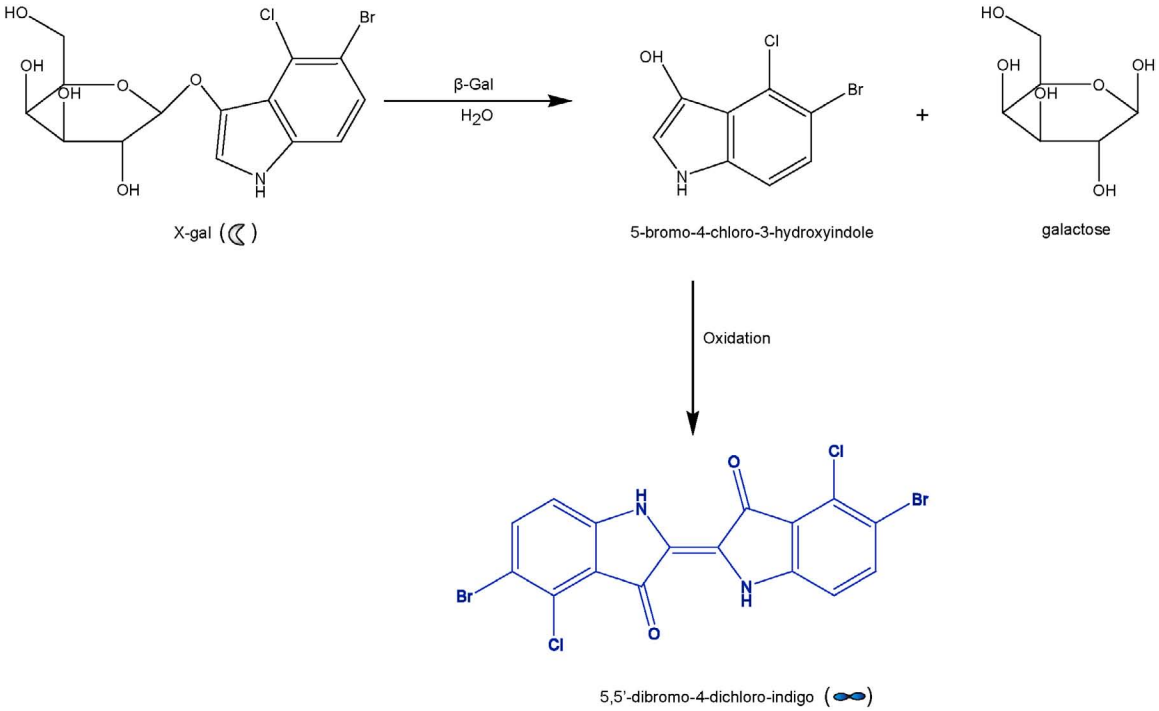


Figure S8: The enzymatic reaction of X-gal in presence of β-gal.[4]

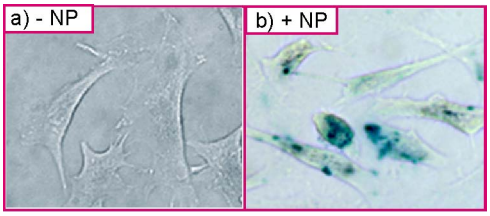


Figure S9: X-gal staining after trypsin digestion. After 3 h of β-gal (50 nM) transfection (a) without and (b) with **NP_Pep** (100 nM), cells were washed, trypsinized and then allowed 4 h more to reattach before X-gal staining..

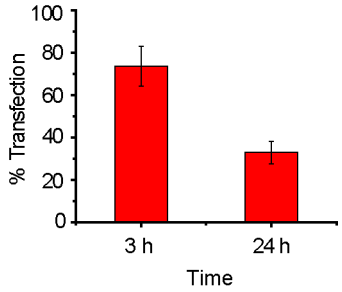


Figure S10: Decrease in the percent of transfection after a day as observed by X-gal staining. HeLa cells were treated with **NP_Pep**/FITC-β-gal (100 nM/50 nM) for 3 h, washed with PBS, and cultured for another 3 h and 24 h before X-gal staining.

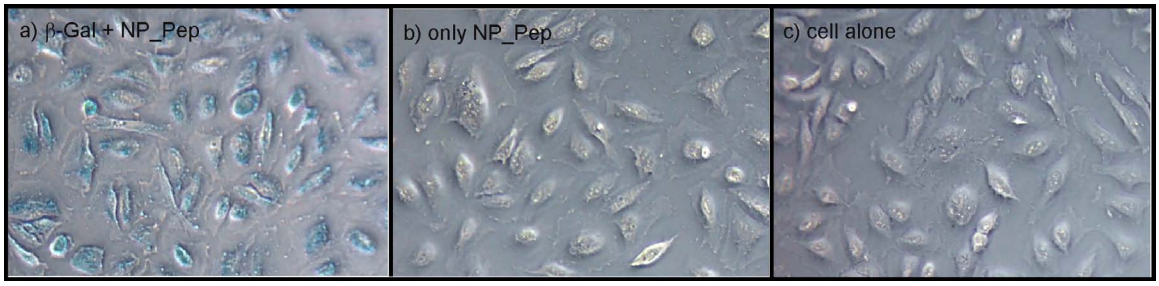


Figure S11: No staining observed upon treating cells with **NP_Pep** alone. HeLa cells were treated with (a) **NP_Pep**/β-gal (100 nM/50 nM), (b) **NP_Pep** (100 nM) and (c) without **NP_Pep** or β-gal for 3 h, washed with PBS, and cultured for another 3 h before X-gal staining.

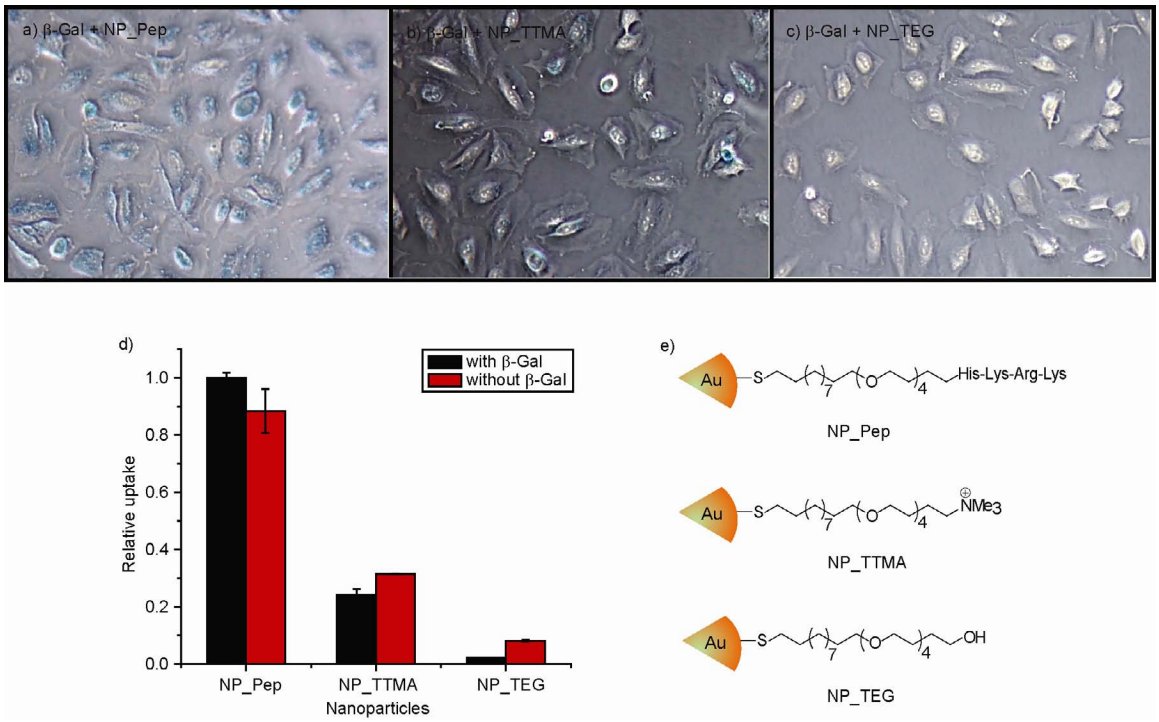


Figure S12: HeLa cells transfected with **NPs**/β-gal (100 nM/50 nM) and transfection efficiency analyzed by (a-c) X-gal assay and (d) ICP-MS. X-gal staining after β-Gal delivery using (a) **NP_Pep**, (b) **NP_TTMA** and (c) **NP_TEG**. (d) NP uptake measured by ICP-MS after transfection with **NP**-β-gal complexes (black bars) or **NPs** alone (red bars). (e) Structure of the particles used.

Cytotoxicity assays

Trypan blue exclusion test was performed to count live cells. After transfction with mentioned concentration of β-gal at molar ratio of 2, cells were cultured for some period (3 h or 24 h) and then they were trypsinized. Then cells were centrifuged, redispersed in PBS, mixed with 1:1 trypan blue (Fluka) and counted (duplicate, more than 100 cells for each replicate) on hemocytometer.

Live/dead cells were also assayed using **calcein AM** (Viability/Cytotoxicity Kit, Invitroge, USA) after 3 h of transfection with **NP_Pep**/ β-gal (200 nM/100 nM) according to the manufacturer's protocol.

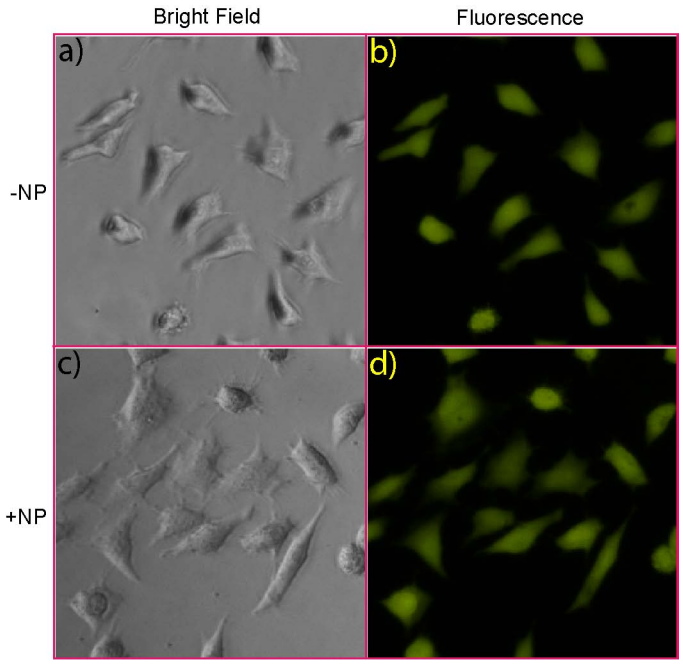


Figure S13: Green fluorescence from cells after calcein AM assay indicating they are alive.

Alamar blue assay was carried out following the manufacturer's protocol (Invitrogen Biosource, 11SA). After delivery, cells were treated with 10% alamar blue solution and kept at 37 °C for another 2 h. Red fluorescence, resulting from the reduction of alamar blue, was monitored (Ex: 560 nm, Em: 590 nm) on a SpectroMax M5 microplate reader (Molecular Device).

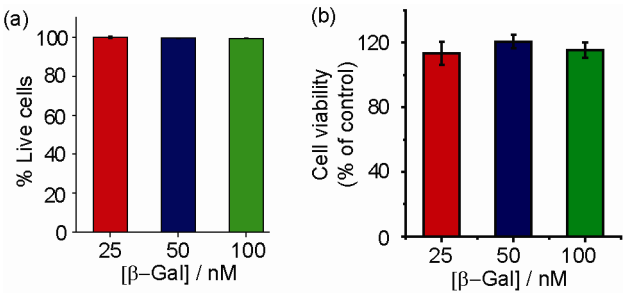


Figure S14: No toxicity from particles after 3 h of protein transfection as measured by (a) trypan blue exclusion test and (b) alamar blue assay.

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1. (a) Hostetler, M. J.; Templeton, A. C.; Murray, R. W. *Langmuir* **1999,** *15,* 3782. (b) Hostetler, M. J.; Wingate, J. E.; Zhong, C. J.; Harris, J. E.; Vachet, R. W.; Clark, M. R.; Londono, J. D.; Green, S. J.; Stokes, J. J.; Wignall, G. D.; Glish, G. L.; Porter, M. D.; Evans, N. D.; Murray, R. W. *Langmuir* **1998,** *14,* 17.

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3. You, C. C.; De, M.; Han, G.; Rotello, V. M. *J. Am. Chem. Soc.* **2005**, *127*, 12873- 12881.

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