Electronic supplementary information

Guanidinium-terminated gold nanoparticles for protein delivery to cell nucleus

Experimental details

Materials. $HAuCl_4.3H_2O$, folate containing Dulbecco's modified Eagle's medium (DMEM) culture medium, lipoic acid, N-hydroxy succinimide, L-arginine methyl ester dihydrochloride, N-(3- dimethylaminopropyl)-N′-ethyl carbodiimide hydrochloride (EDC), fluorescein isothiocyanate (FITC) conjugated bovine serum albumin (BSA) and lysozyme from chicken egg white were purchased from Merck-India. NHS-fluorescein was purchased from Thermo-Fisher Scientific. Fetal bovine serum (FBS) and penicillin-streptomycin antibiotic were purchased from HiMedia.

Preparation of thiol modified–arginine. EDC coupling reaction was performed between lipoic acid and L-arginine methyl ester. In brief, lipoic acid (21 mg, 0.1 mmol), EDC (58 mg, 0.3 mmol) and n-hydroxysuccinimide (23 mg, 0.2 mmol) were mixed in one mL DMF. The mixture was stirred at 4 °C for 30 min. Then 0.2 mL borate buffer (pH 9.0) solution of L-arginine methyl ester (26 mg, 0.1 mmol) was added to the mixture under stirring at 4 °C and kept at this condition for overnight. Then 1.5 mL acetone was added to precipitate the product and to remove the excess reagent.

Synthesis of 1.7 nm Au-arginine: 10 mL aqueous solution of gold salt was prepared by diluting the 0.42 mL aqueous $HAuCl_4$ solution (6 mM) with milli-Q water. Next, 1 mL thiolated arginine (2.5 mM) was mixed. Next, 300 μL of freshly prepared 0.1 mM $NaBH_4$ solution was added under stirring condition and stirring was stopped after 2 min. The solution turns straw yellow immediately after adding $NaBH_4$. Next, Au-arginine was purified and separated from unreacted

reagents using salt-based precipitation. Typically, 1.0 mL of as synthesized nanoparticle solution was mixed with 200 µL of NaCl solution (100 mM) and then centrifuged at 14,000 rpm for 10 min. After that Au-arginine precipitate was collected and supernatant solution was discarded. The Au-arginine precipitate was dispersed in 1.0 mL fresh water. This procedure of precipitation-redispersion was repeated for 2 times and then the purified Au-arginine was finally dispersed in 1.0 mL fresh water. Assuming the complete conversion of gold salt to gold nanoparticle (this is expected as we have used excess borohydride for complete reduction) and no loss during purification, the Au concentration in this stock solution is 0.25 mM.

Using the purified Au-arginine we have separately estimated gold via ICP-MS and guanidinium via phenanthrenequinone test. We found the molar ratio of arginine to gold as 938. Assuming 152 gold atoms present in a 1.7 nm Au the number of arginine per nanoparticle has been calculated as 6.

Preparation of Au-protein conjugate. Lysozyme is conjugated with fluorescein using NHS fluorescein via the reported procedure. FITC conjugated BSA was used as received. Stock solution of proteins were prepared separately with concentration of 0.2 mM. In order to prepare protein-nanoparticle conjugate, about one mL Au-arginine solution was mixed with one mL protein solution (0.2 mM) for 15 min and resultant Au-protein dispersion was used for experiments.

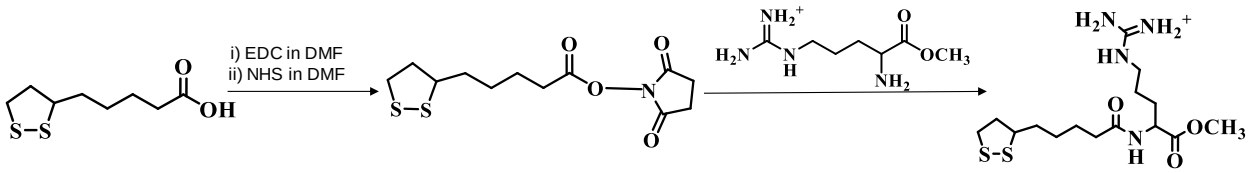
Cell labelling procedure. HT22, KB, HeLa and U87 cells were cultured in DMEM culture media containing low glucose, 10 % fetal bovine serum (FBS) and 1 % penicillin streptomycin (antibiotics), maintaining humidified atmosphere with 5 % CO_2 and temperature of 37 °C. For labelling experiment, cells were cultured in 24-well plate for 24 hrs. Next, cells were washed with buffer solution and serum free media was added and then treated with 55 µL colloidal dispersion of Au-protein for five minutes. Next, cells were washed by PBS buffer solution and used for

fluorescence and dark field imaging. For co-localization study nanoparticle labelled cells were incubated with hoechst (nucleus staining dye) for 10 min and then cells were washed by PBS and used for fluorescence imaging. For high resolution imaging, cells were fixed with 4 % paraformaldehyde solution before imaging.

For cellular uptake mechanism study, cells were incubated with different inhibitors separately for 45 minutes. (methyl β-cyclodextrin - 10 mM , 4 °C incubation, sodium azide - 10 mM, 2-deoxy-D-glucose - 50 mM, cytochalasin-D - 5 µM, ivermectin – 25 µM). After that Au-protein dispersion was added to cells and incubated for 5 min. Next, cells were washed with PBS buffer and used for fluorescence imaging.

Procedure of cell sample preparation for SEM: HT22 cells were cultured in DMEM overnight in a 12-well plate containing 18 mm glass coverslips. Next day, cells were incubated with sample for 5 min and then fixed with 4% paraformaldehyde solution. Next, coverslips were washed by PBS buffer solution for three times. Then cells were post-fixed using 1% (w/v) osmium tetroxide in H_2O for 30 min. Then, those coverslips were washed with water and then dehydrated in an escalating ethanol gradient (50%, 70%, 90% for 5 min each and then three times with 100% ethanol for 10 min per interval). Then, these dehydrated coverslips were treated with a mixture of hexamethylenedisilazane (HMDS)/ethanol (1:2 then 2:1 for 5 min) and 100% HMDS for 5 min each and finally, they were stored under desiccated conditions.

Scheme S1. Chemical reactions that are involved in making thiolated arginine.



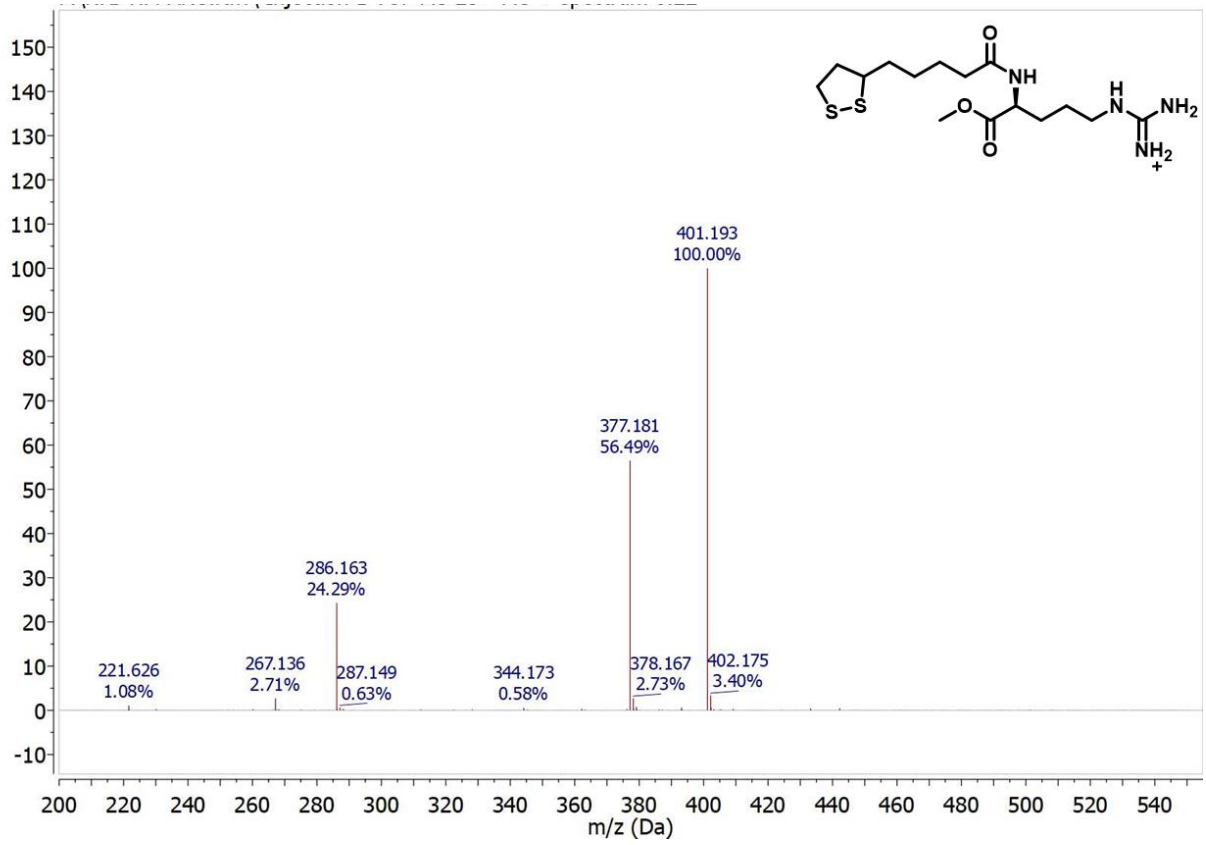


Figure S1. HRMS of thiolated arginine. The peak at 377.181 suggests the formation of lipoic acid conjugated arginine ligand. [calculated for $C_{15}H_{29}N_4O_3S_2^+$ (M^+): 377.1681; found: 377.181]. The peak at 401.193 corresponds $M^+ + Na^+ + H^+$.

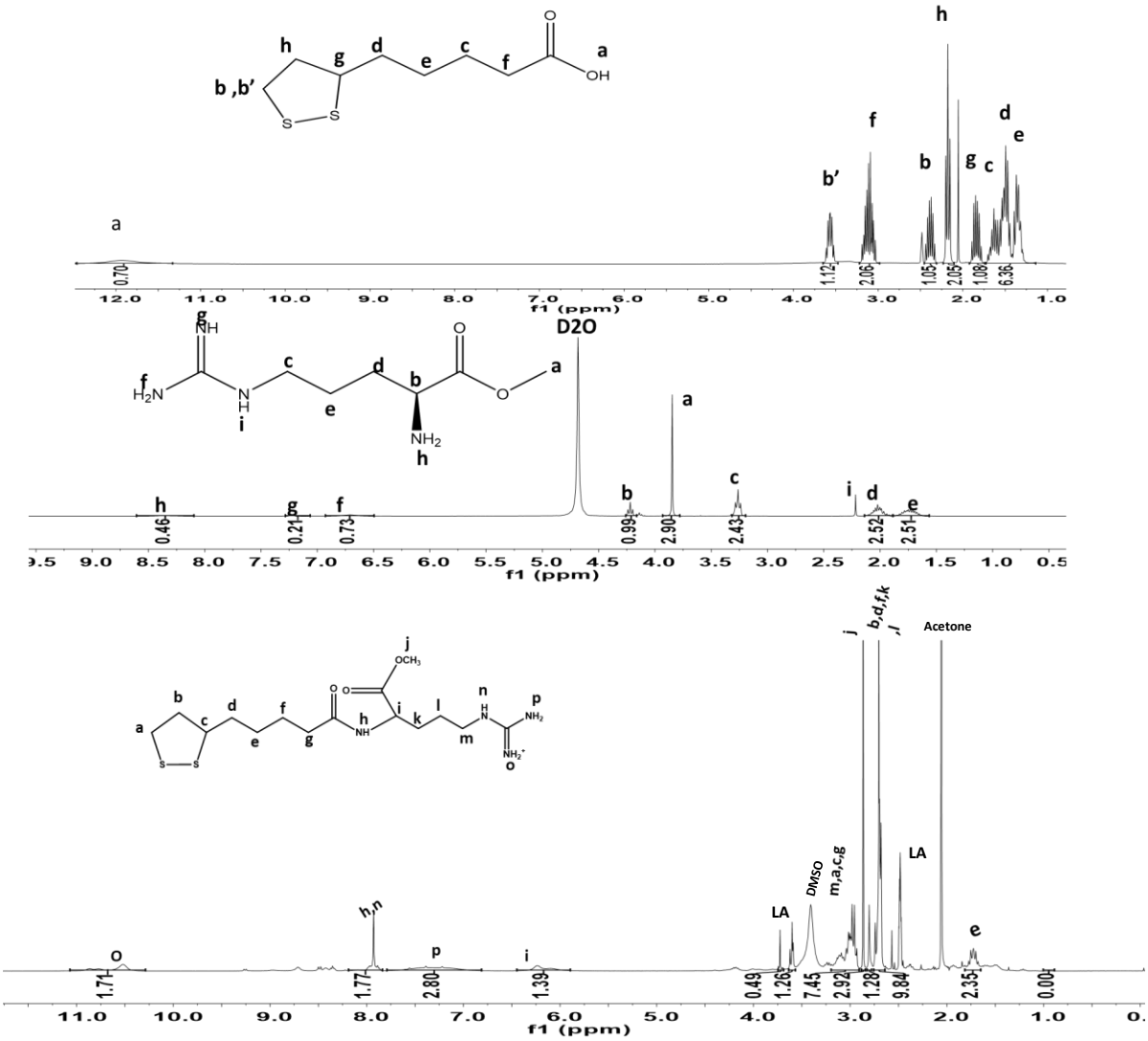


Figure S2. Proton NMR of lipoic acid in DMSO-d_6, arginine methyl ester in D_2O, and thiolated arginine in DMSO-d_6. The appearance of proton signal at 7.9 ppm indicate the covalent linking and amide bond formation. The impurity signals are assigned due to the presence of unreacted lipoic acid (LA) and acetone.

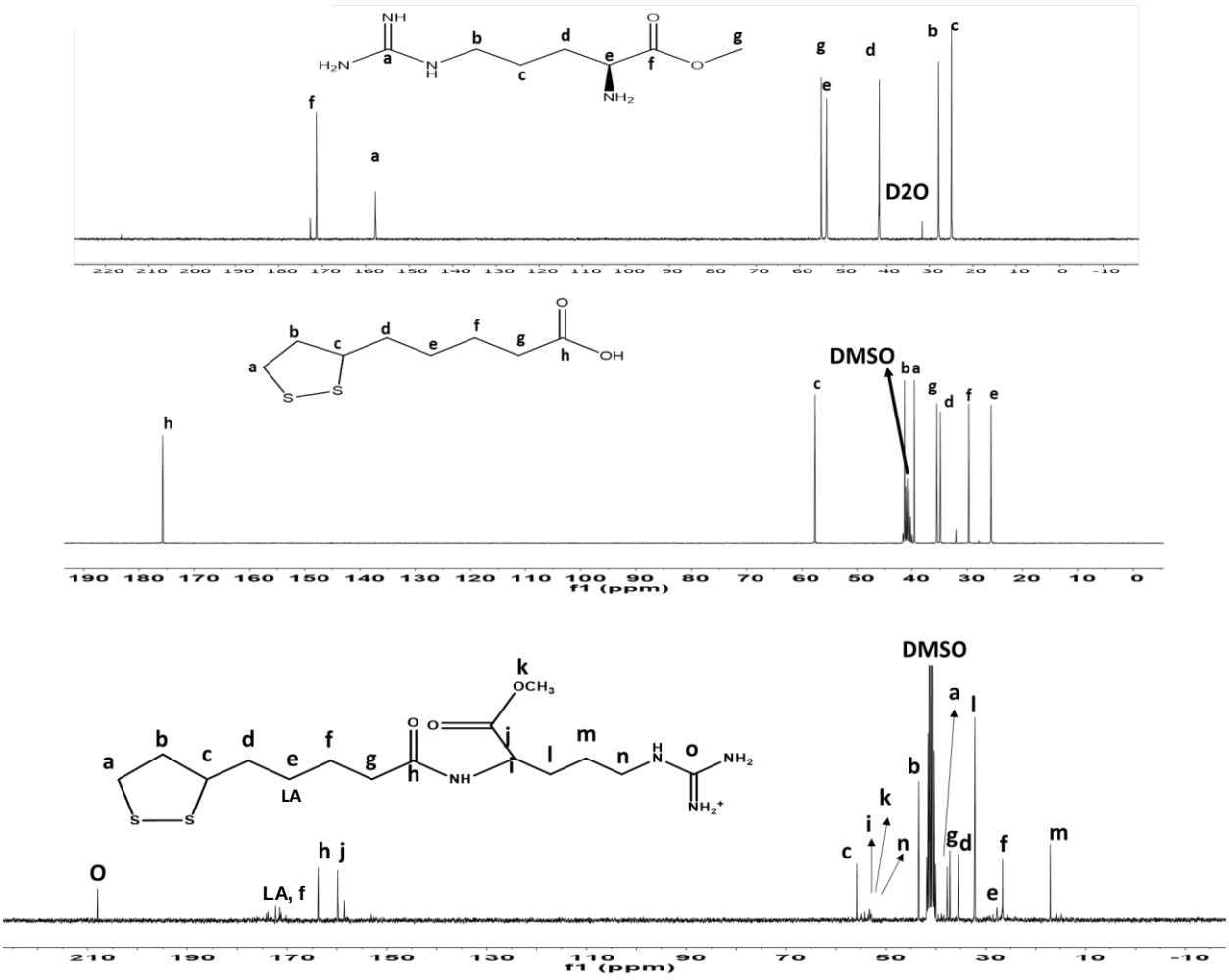


Figure S3. ^{13}C NMR of lipoic acid in DMSO-d_6, arginine methyl ester in D_2O, and thiolated arginine in DMSO-d_6. The shift of "h" labelled C signal of lipoic acid from 175.76 to 163.61 (in thiolated arginine) signifies the change in environment due to the formation of amide bond. The impurity signals are assigned due to the presence of unreacted lipoic acid (LA, f).

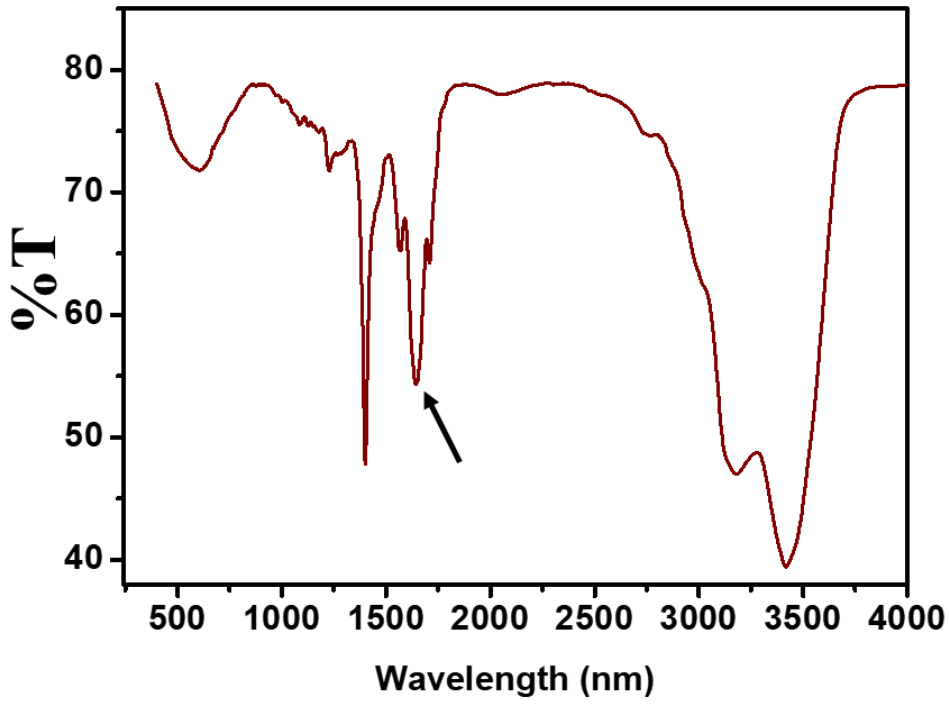


Figure S4. FTIR spectrum of Au-arginine, showing the vibration fingerprint of thiolated arginine. Vibration signal at 1642 cm^{-1} corresponds to carbonyl group of amide bond. Absence of any peak in the range of 2500-2570 cm^{-1} signifies the absence of S-H bond.

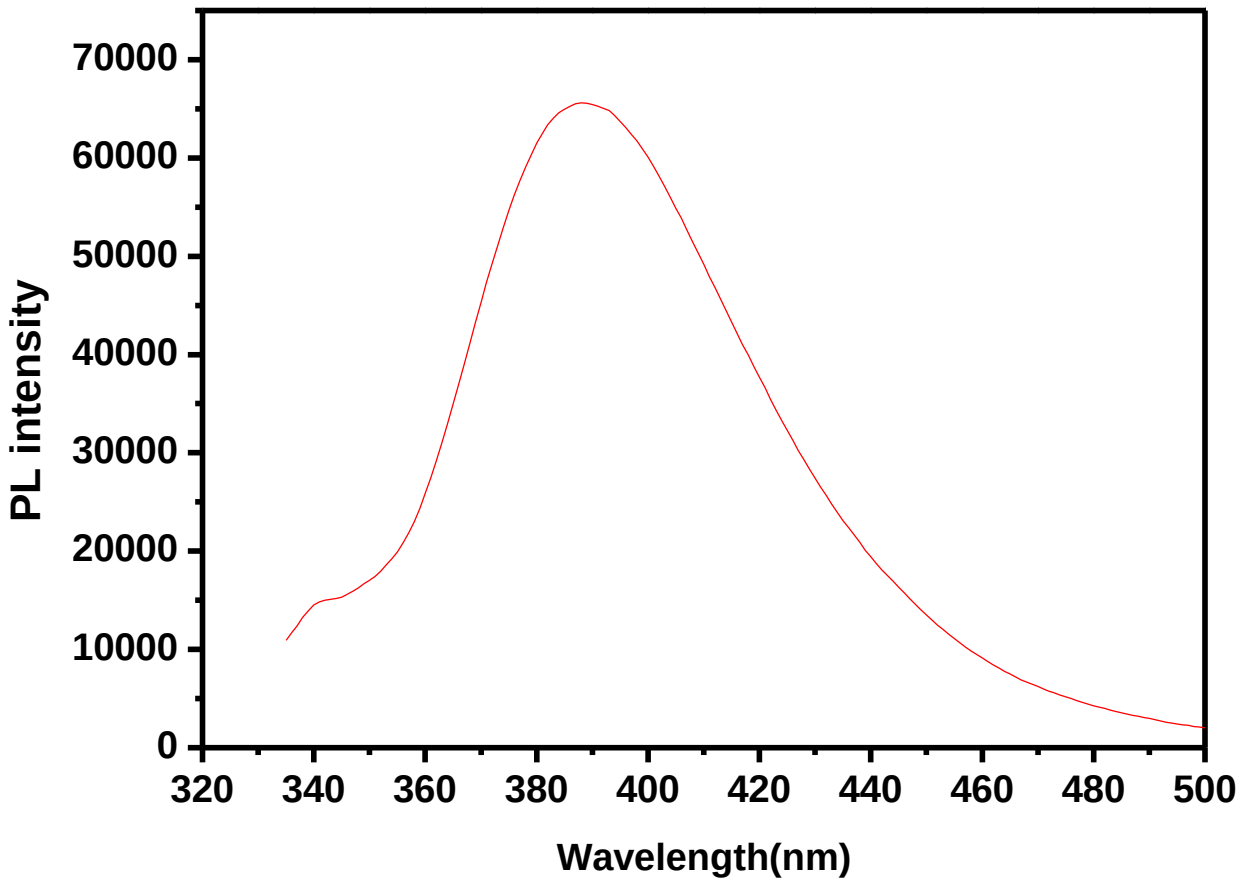


Figure S5. Evidence of the presence of arginine on Au nanoparticle surface by 9, 10-phenanthrenequinone test. Briefly, 0.05 mL Au-arginine solution was mixed with 150 µM ethanolic solution of 9,10-phenanthroquinone, 2 (N) NaOH and then incubated at 60 °C for 3h. Then sample was mixed with 1.2 (N) HCl dropwise until neutralization and the mixture was kept in dark at room temperature for 1h. Fluorescence spectrum was then measured by exciting at 312 nm.

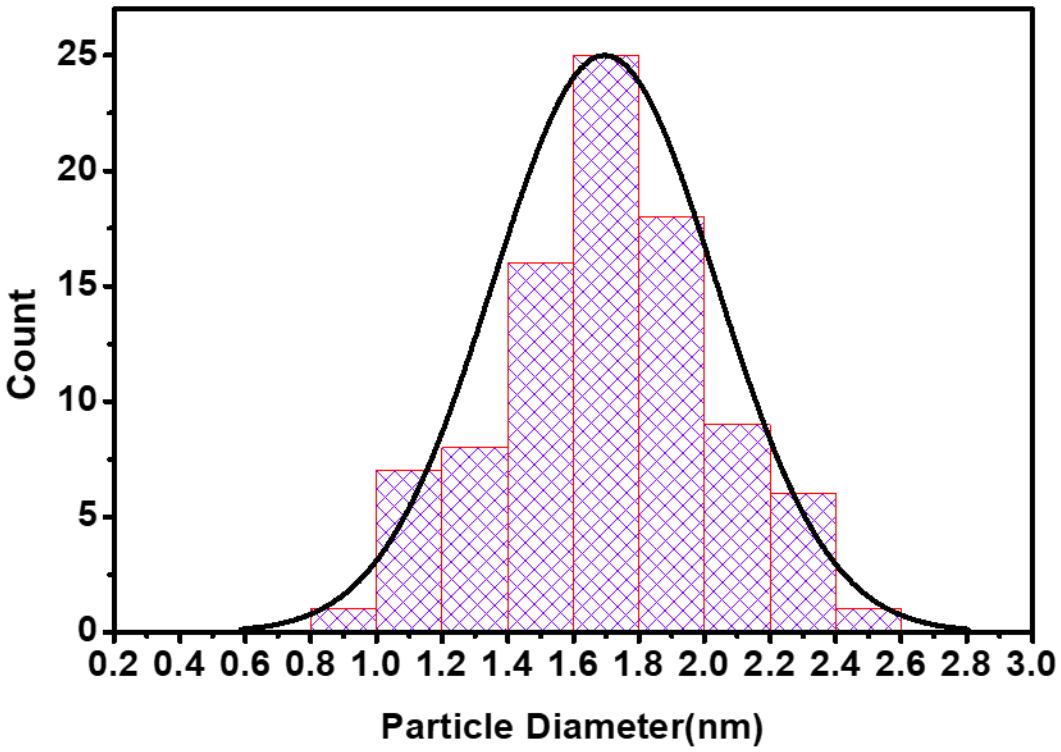


Figure S6. Size distribution of Au nanoparticles in Au-arginine.

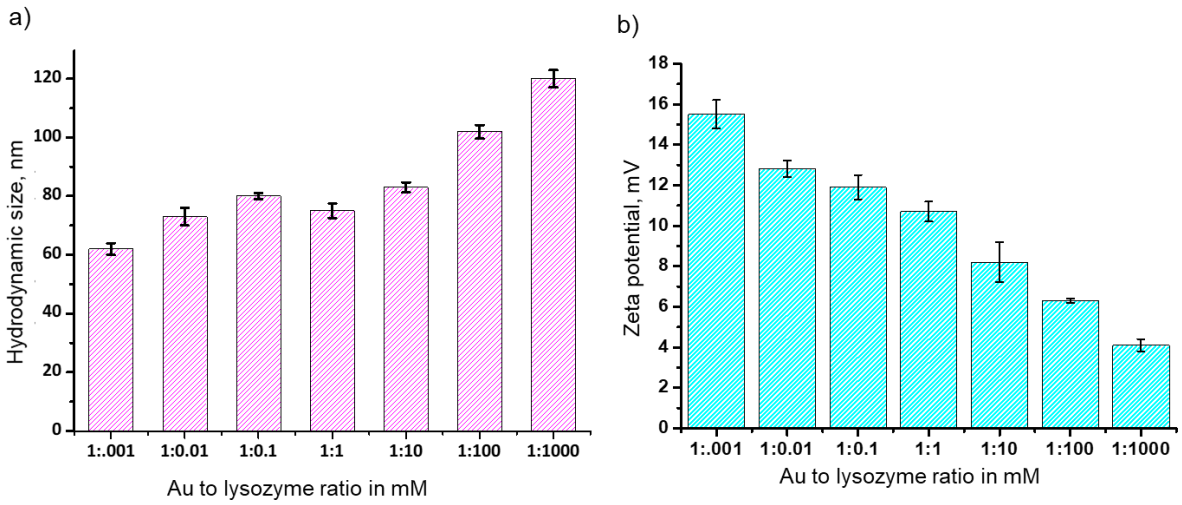


Figure S7. Change of hydrodynamic size and zeta potential of assembly of Au-arginine and lysozyme with their varied ratio.

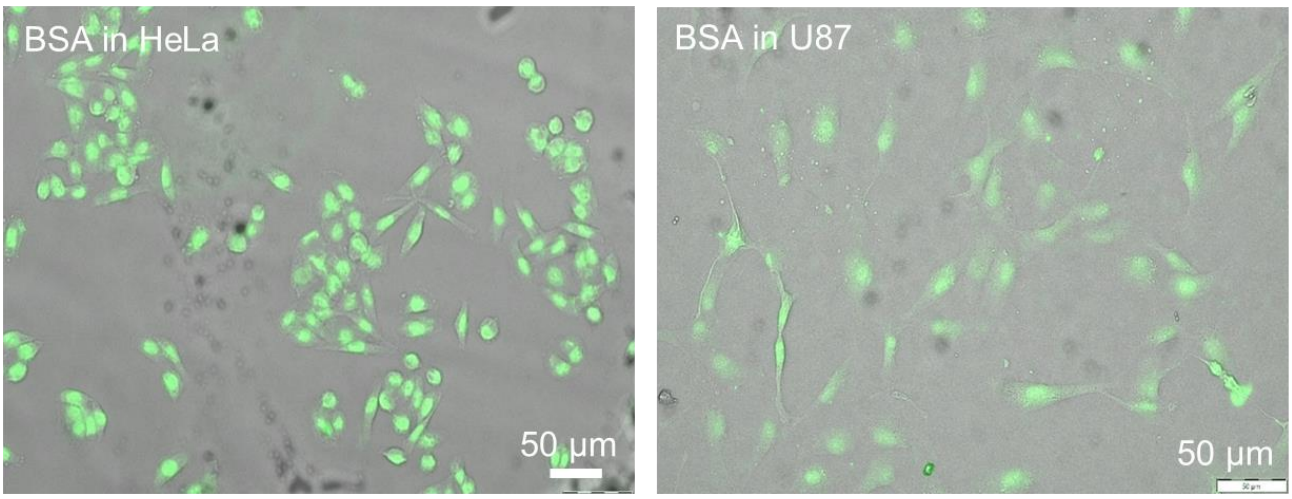


Figure S8. Nuclear delivery of BSA to HeLa and U87 cells using 1.7 nm Au-arginine. Typically, BSA is mixed with Au-arginine, added to cell culture media and incubated for 5 min. Next, washed cells are imaged under bright field or fluorescence mode and then merged.

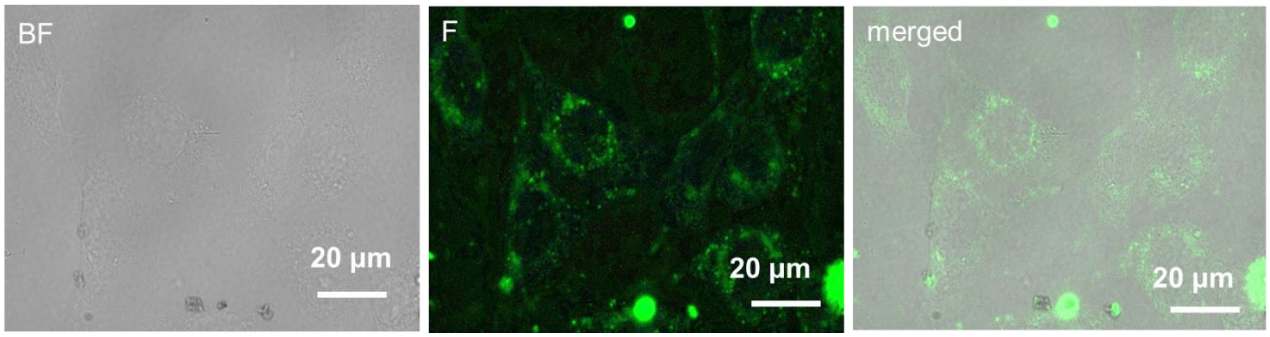


Figure S9. Inefficient nuclear delivery of lysozyme to HT22 cells when higher amount of Au-arginine is used. Typically, lysozyme is mixed with higher amount of Au-arginine (100 times than the optimum condition as described in Figure 3), added to cell culture media and incubated for 5 min. Next, washed cells are incubated in fresh culture media for 2 hrs and then imaged under bright field (BF), fluorescence (F) and merged. Results show that lysozymes are primarily aggregated inside cell.

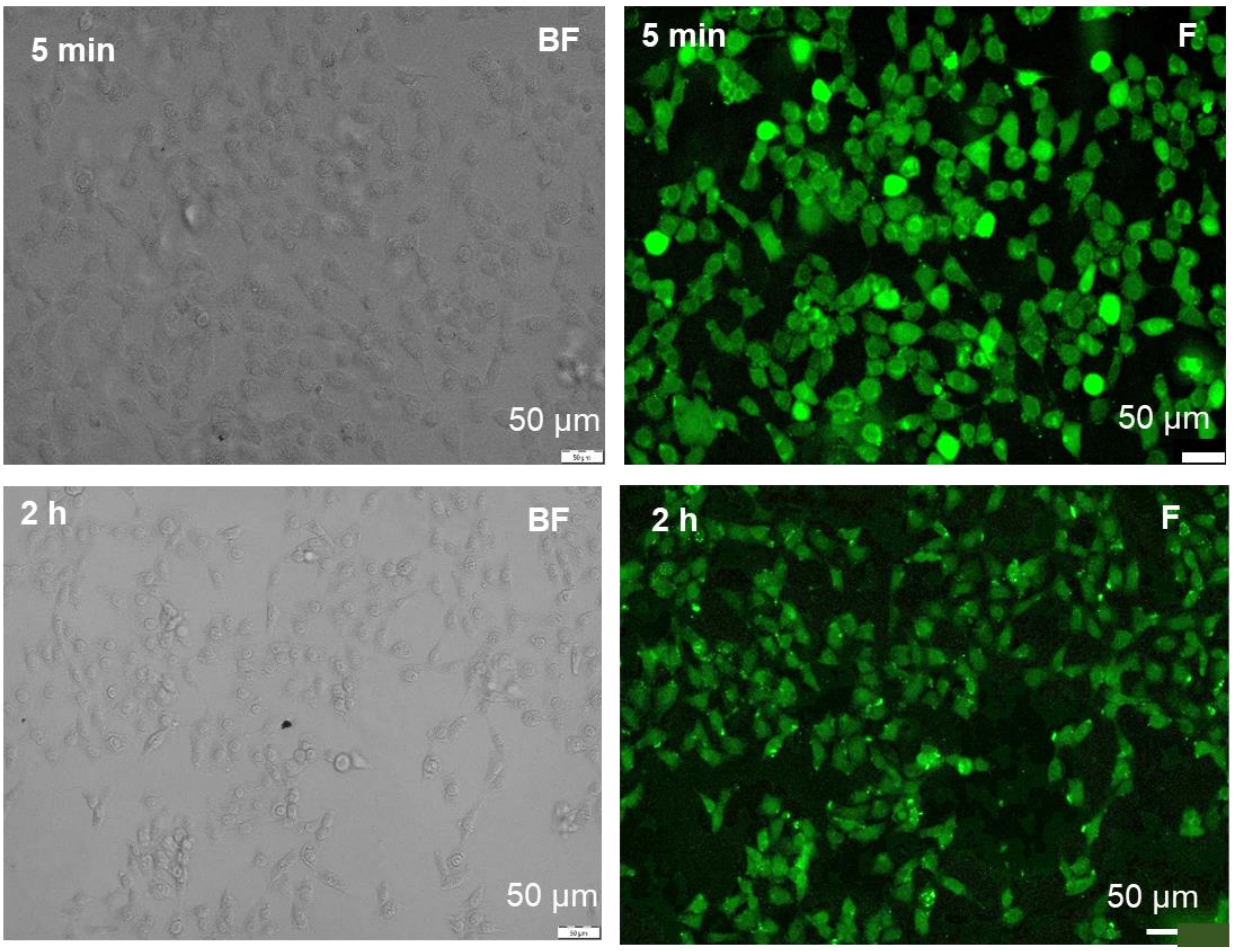


Figure S10. Delivery of beta-galactosidase to HT22 cells using 1.7 nm Au-arginine. Typically, beta-galactosidase is mixed with Au-arginine, added to cell culture media and incubated for 5 min. Next, washed cells are further incubated with fresh media for 2 h. Next, washed cells are imaged under bright field (BF) or fluorescence (F) mode. Results show cytosolic delivery of beta-galactosidase without any nuclear localization.

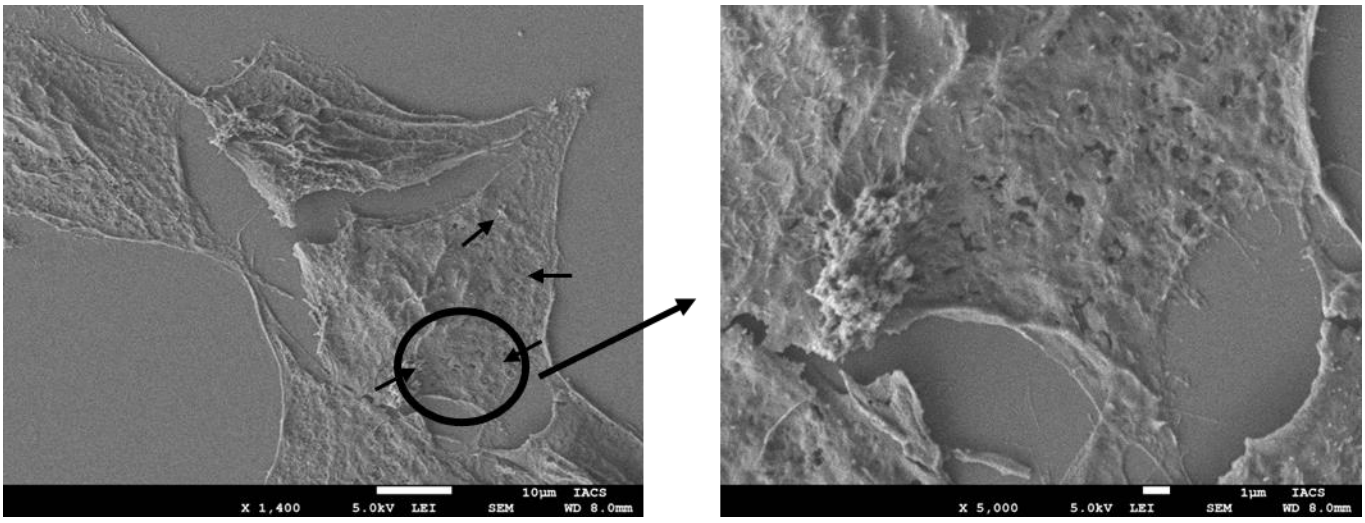


Figure S11. SEM images of HT22 cells at two different magnifications after incubating with Au-arginine for 5 min. Black arrows indicate the pores that are 50−200 nm in size.

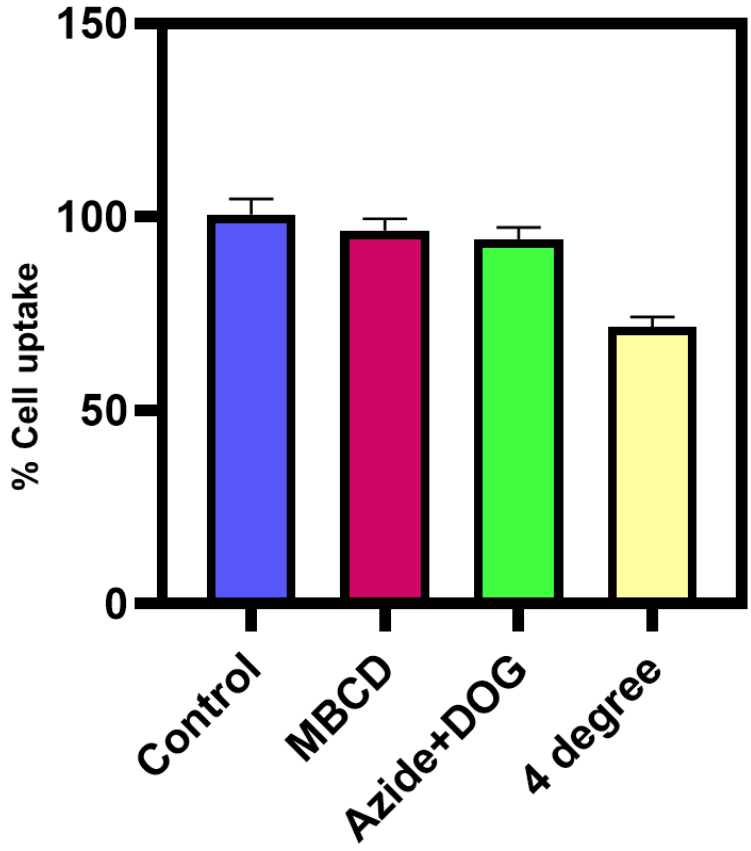


Figure S12. Evidence of direct uptake for lysozyme via assembly with Au-arginine. HT22 cells are incubated with a mixture of FITC-conjugated lysozyme and Au-arginine for 5 min in the presence or absence (control) of different inhibitors or at different conditions. Next, washed cells are analysed via flow cytometry. Results show insignificant uptake inhibition under any of those conditions. Error bars signify average of three measurements.

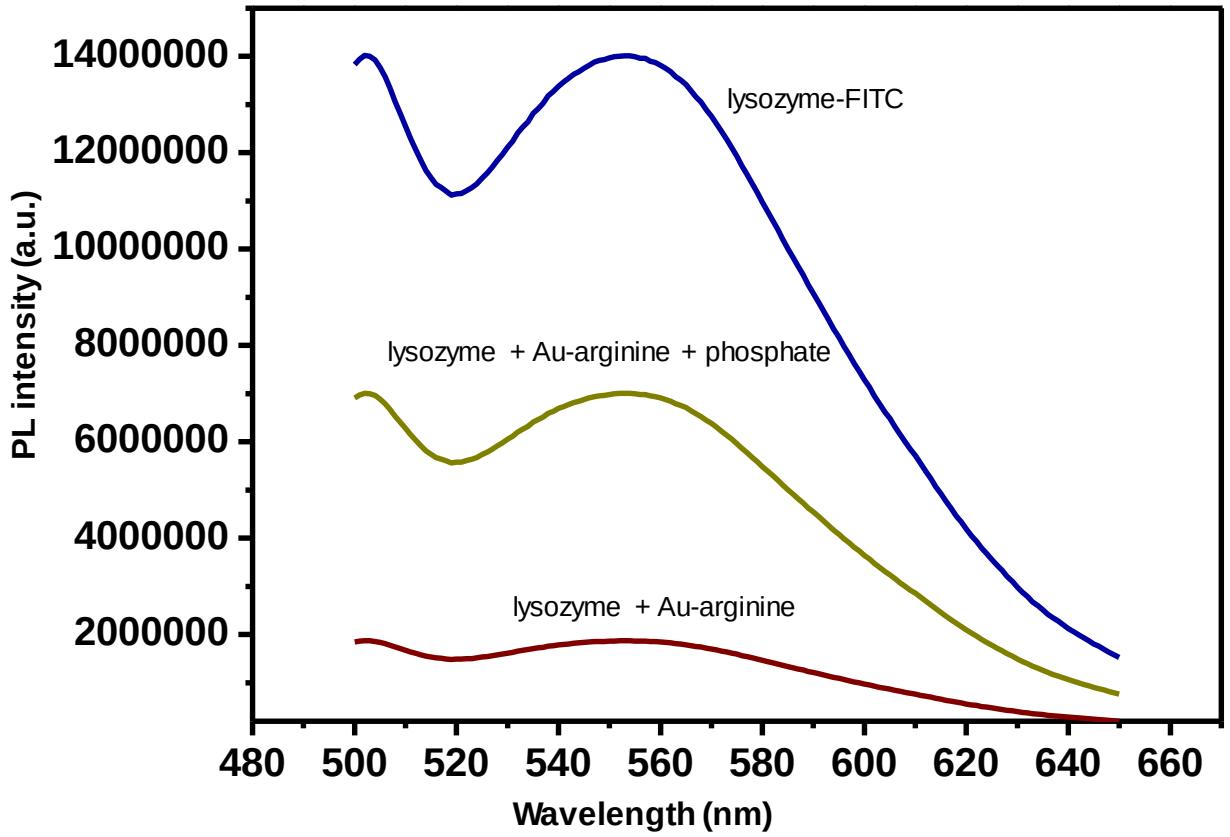


Figure S13. Evidence of partial quenching of FITC fluorescence of FITC conjugated lysozyme by Au-arginine and recovery of quenched fluorescence by phosphate.

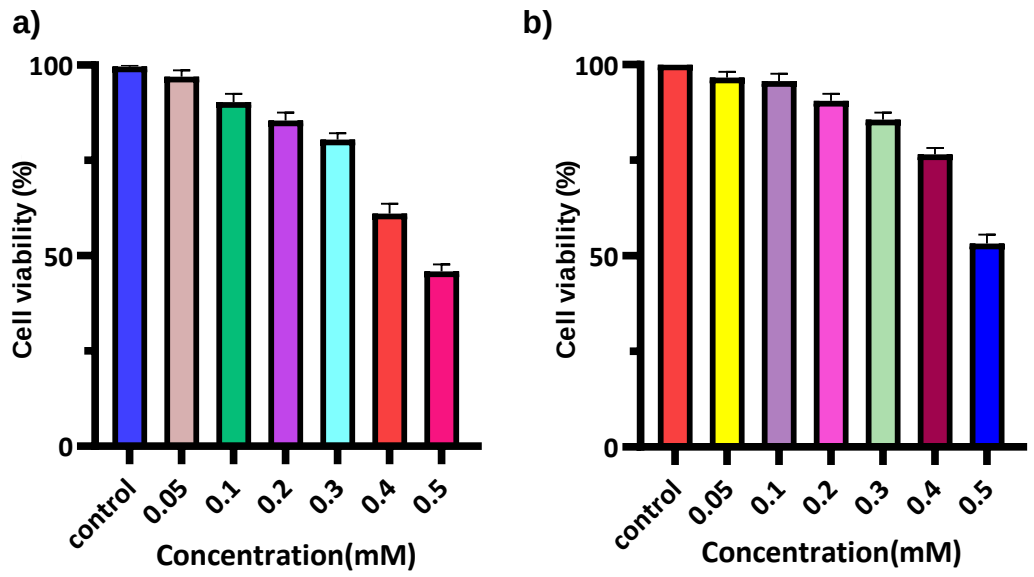


Figure S14. MTT-based **c**ell viability assay in presence of Au-arginine (a) and mixture of Au-arginine and lysozyme (b). HT22 cells are incubated with different dose of sample for 24 hrs for this assay. The % viability is calculated assuming 100 % viability for control cells without any sample treatment.