Electronic Supplementary Information

Polymer coated Gold Nanoparticle-Protein Agglomerates as Nanocarriers for Hydrophobic Drug Delivery

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Experimental Section

Materials: Hydrogen tetrachloroaurate trihydrate, $HAuCl_4.3H_2O$ (Sigma, USA), (S)-(+)-camptothecin (Sigma, China), poly(D,L-lactide-*co*-glycolide), acid terminated, 50:50, M_W: 7000-17000 Da (Sigma, Germany), hen egg white lysozyme (SRL, India), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT (Himedia, India), Proteinase K (Bioline, UK), calcein AM (Fluka, USA) and trisodium citrate dihydrate (Merck, India) were purchased and used as received without any further purification. Milli-Q ultrapure water (>18 MΩ cm, Millipore) was used in all the experiments.

Synthesis of Cit-Au NPs: The dispersion of Cit-Au NPs was obtained via citrate reduction of $HAuCl_4$.[15] Briefly, Milli Q grade water (100 mL) containing trisodium citrate dihydrate (0.0034 M) was refluxed under stirring condition. When the solution started boiling highly, $HAuCl_4$ (1 mL of 1.73×10^{-2} M) was added and the reaction was allowed to continue for another 30 min. This resulted in the formation of dispersion of Cit-Au NPs. The concentration of Cit-Au NPs formed at this stage was calculated to be 1.51 nM, assuming that complete reduction of $HAuCl_4$ has occurred. In order to separate out the larger particles, the above dispersion was centrifuged at 2000 rcf and 10°C for 15 min. The supernatant (referred as Cit-Au NPs in the manuscript) was collected and used in all the experiments, as applicable.

Development of PLGA coated NCs: The dispersion of Cit-Au NPs (15 mL) was mixed with Lyz (1.2 mL of 0.01 mg mL^{-1}) and incubated for 30 min. The Lyz solution was prepared in sodium phosphate buffer (0.01 M) of pH 7.4. The pH of the resulting Au NP-Lyz

dispersion was found to be ~6.9. To this, the solution of CPT in DMSO (0.04 mL of 0.5 mg mL^{-1}) was added and incubated in dark for another 1 h. After this, the above solution was emulsified with PLGA (pKa 3.85) in DCM (1 mL of 5 mg mL^{-1}), using the microtip probe sonicator (UP200S ultrasonic processor, hielscher), set at cycle 0.5 and amplitude 40 % for 2 min, to form oil in water single emulsion. The emulsified solution was allowed to mix gently in a rocker for 4 h and then the DCM was evaporated using the water bath maintained at 40°C for 2 h. Finally, the dispersion was centrifuged (20000 rcf, 4°C for 15 min) and the pellet was washed twice. The resulting pellet was redispersed in water using an ultrasonic bath.

Characterization: The characterization of the NCs were done using the double beam UV-vis spectrophotometer (Hitachi U-2900), DLS based particle size analyzer (Zetasizer Nano ZS90, Model No. ZEN3690, Malvern) and TEM (JEOL JEM 2100, maximum accelerating voltage 200 kV).

Stability tests against proteases: The stability of the NCs against protease degradation was tested via SDS-PAGE and TEM analysis. PLGA coated NCs were prepared in duplicate, centrifuged, washed and both the pellets were redispersed together in 60 μL of water. As a control, Au NP-Lyz agglomerates (without PLGA coating) and albumin coated NCs (as mentioned in our previous study)[15] were also prepared and the pellet after centrifugation and several washings were redispersed in 30 μL of water separately. The solutions of PLGA coated NCs, albumin coated NCs, Lyz and albumin were mixed individually with Proteinase K and incubated at 37°C in a shaking water bath for 1h. After this, the solutions were immediately boiled for 10 min to stop the reaction. The above solutions along with the solutions which were not subjected to protease treatment were boiled again with loading buffer for 5 min and then loaded in polyacrylamide gel (16%). After the completion of electrophoresis, the gel was stained (using coomassie brilliant blue R-250), destained and

then photographed (using Gel Logic 212 PRO Imaging System, Carestream). The details of the samples used in different lanes of Fig. 2b, Fig. S3† and Fig. S4† are given in Table S1, S2 and S3 respectively. The PLGA coated NCs or albumin coated NCs with (500 ng) and without protease treatment were also observed under TEM.

Stability of the PLGA coated NCs in human blood serum and in water: The concentrated dispersion of PLGA coated NCs were equally diluted with human blood serum as well as with water and their extinction spectra were recorded at different time intervals. It is important to mention here that the NCs diluted with serum were stored at 37 °C and NCs diluted with water were stored at 4 °C.

Encapsulation efficiency (E.E.) and in-vitro cumulative release (C.R.) study: The PLGA coated NCs were prepared, centrifuged (at 20000 rcf and 4 °C for 15 min) and the pellet was washed twice. The supernatant of the initial step was mixed with the supernatants of the washing steps and its emission spectrum was recorded in a HORIBA Jobin Yvon FluoroMax-4 spectrofluorimeter by exciting at 370 nm. The emission at 434 nm was noted and the concentration of the CPT in the supernatant was calculated using the standard curve of CPT. Then the E.E. was calculated using the formula

$$E.E.\ \% = \frac{CPT_i - CPT_s}{CPT_i} \text{x}100$$

where, $[CPT]_i$ is the initial concentration of CPTand $[CPT]_s$ is the concentration of CPT in the supernatant.

Generation of standard curve of CPT for E.E. study: Considering that the supernatant may contain moieties which may have influence on the fluorescence profile of CPT, the standard curve for calculating $[CPT]_s$ (the concentration of CPT in the supernatant) was made by dissolving different concentrations of CPT in the supernatant (obtained after the centrifugation and washing of non-loaded NCs) and recording their emission spectrum by

excitation at 370 nm. The intensity at 434 nm was noted and the standard curve of emission intensity vs concentration was obtained.

For *in vitro* cumulative release study, the PLGA coated NCs were prepared, centrifuged and washed. Finally the pellet was redispersed in acetate buffer (0.02 M, pH 4.0), phosphate buffer (0.02 M, pH 7.4) and DMSO, separately respectively. Then the respective dispersion of NCs were equally distributed in different centrifuge tubes and incubated in a shaking water bath maintained at 37 °C for 24 h. At different intervals of time, the centrifuge tubes were taken out, centrifuged and the supernatants were collected whose emission intensities were measured at 434 nm, by exciting the samples at 370 nm in a fluorimeter. By knowing the emission intensities, the concentrations of CPT released in the supernatants were calculated using the standard plot of CPT. Then, the cumulative release were obtained using the formula

$$C.R.\ \% = \frac{CPT_s}{CPT_e} \text{X}100$$

where, $[CPT]_s$ is the concentration of CPT released in the supernatant and $[CPT]_e$ is the concentration of CPT encapsulated in the NCs. Similarly the cumulative releases in phosphate buffer and in DMSO were calculated.

Generation of standard curve of CPT for C.R. study: The non-loaded NCs were centrifuged and washed, similar to CPT loaded NCs. The pellet was resuspended in acetate buffer (0.02 M, pH 4.0) and again centrifuged. The supernatant obtained was used for preparing CPT solutions of different concentrations. The emission spectrum of the respective solutions were recorded and the intensities at 434 nm were noted. These intensities were plotted against CPT concentrations to have a CPT standard curve in acetate buffer. The standard curve of CPT in phosphate buffer and DMSO were made by dissolving the non-loaded NC pellet in phosphate buffer (0.02 M, pH 7.4) and DMSO respectively and then following the same procedure as above.

TEM and FESEM analyses of the treated HeLa cells: For TEM and FESEM analyses, the HeLa cells were seeded in a 35 mm cell culture plate and were then allowed to grow overnight. Then the serum medium was removed, washed with phosphate buffer saline (PBS) and the fresh serum medium containing CPT loaded NCs was added to the cells. The cells were incubated in a humidified atmosphere containing 5% CO_2 at 37°C for 5 h. Then the medium was removed, washed with PBS for two times, trypsinized and centrifuged. The cell pellet obtained was resuspended in 2 % glutaraldehyde and incubated at room temperature for 3 h. The fixed cells were then dehydrated with graded ethanol solutions and finally resuspended in absolute ethanol. Similarly the suspension of HeLa cells without treatment was also prepared. These cell suspensions were used for TEM and FESEM analysis. The cell suspension was drop-cast on a carbon-coated copper TEM grid for TEM analysis and on a glass slide (wrapped with an aluminium foil) for FESEM analysis.

Confocal Laser Scanning Microscopic Analysis: For confocal analysis, 200000 cells/well were seeded onto coverslips in a 6 well plate and allowed to grow overnight. Then the medium was removed, washed with PBS and fresh media containing CPT loaded NCs (0.45 mg mL^{-1}), CPT (1.3 μg mL^{-1}) or non-loaded NCs (0.45 mg mL^{-1}) were added to separate wells. The cells were incubated for 5 h and then following washing with PBS, the cells were counterstained with calcein AM at 37 °C for 15 min. The cells were again washed with PBS and fixed using freshly prepared 4 % paraformaldehyde solution at room temperature for 15 min. Finally the cells were washed and the cover slips were mounted on glass microscopic slide with a drop of mounting agent. The coverslips were sealed using nail polish and then analysed under Leica TCS-SP8 confocal microscope. The calcein and CPT were excited by 488 nm and 404 nm lasers respectively.

Cell viability assay: 10000 cells/well were seeded in a 96 well microplate and grown overnight. Then the medium was removed and fresh media containing varying concentrations

of non-loaded NCs (0.05-0.45 mg mL^{-1}) or CPT loaded NCs (0.05-0.45 mg mL^{-1}) or CPT (0.15-1.3 μg mL^{-1}) were added to the cells. After 24 h incubation, MTT based cell viability assay was carried out and the absorbance of the formed formazan in DMSO was recorded at 550 nm using a Bio-Rad 680 microplate reader.

Table. S1 Compositions of the samples for SDS-PAGE analysis corresponding to Fig. 2b.

Lane No.	Composition of SDS-PAGE Samples				
	Amount/ Sample	Water	Proteinase K	Loading Dye	Total Volume
1	5.0 µL of Protein Marker	11.0 µL	-	5.0 µL	21.0 µL
2	15.0 µL of 0.1 mg mL^{-1} Lyz	1.0 µL	-	5.0 µL	21.0 µL
3	15.0 µL of 0.1 mg mL^{-1}Lyz	-	1.0 µL of 250.0 µg mL^{-1}	5.0 µL	21.0 µL
4	15.0 µL of PLGA coated NCs	1.0 µL	-	5.0 µL	21.0 µL
5	15.0 µL of PLGA coated NCs	-	1.0 µL of 250.0 µg mL^{-1}	5.0 µL	21.0 µL
6	15.0 µL of PLGA coated NCs	-	1.0 µL of 500.0 µg mL^{-1}	5.0 µL	21.0 µL
7	-	15.0 µL	1.0 µL of 250.0 µg mL^{-1}	5.0 µL	21.0 µL

Table. S2 Compositions of the samples for SDS-PAGE analysis corresponding to Fig. S3†.

Lane No.	Composition of SDS-PAGE Samples				
	Amount/ Sample	Water	Proteinase K	Loading Dye	Total Volume
1	5.0 µL of Protein Marker	11.0 µL	-	5.0 µL	21.0 µL
2	15.0 µL of Au NP-Lyz agglomerates	1.0 µL	-	5.0 µL	21.0 µL
3	15.0 µL of Au NP-Lyz agglomerates	-	1.0 µL of 250.0 µg mL^{-1}	5.0 µL	21.0 µL

Table. S3 Compositions of the samples for SDS-PAGE analysis corresponding to Fig. S4†.

Lane No.	Composition of SDS-PAGE Samples				
	Amount/ Sample	Water	Proteinase K	Loading Dye	Total Volume
1	5.0 µL of Protein Marker	11.0 µL	-	5.0 µL	21.0 µL
2	15.0 µL of 0.1 mg mL^{-1} Lyz	1.0 µL	-	5.0 µL	21.0 µL
3	15.0 µL of 0.1 mg mL^{-1} Lyz	-	1.0 µL of 500.0 µg mL^{-1}	5.0 µL	21.0 µL
4	-	15.0 µL	1.0 µL of 500.0 µg mL^{-1}	5.0 µL	21.0 µL
5	15.0 µL of 0.1 mg mL^{-1}albumin	1.0 µL	-	5.0 µL	21.0 µL
6	15.0 µL of 0.1 mg mL^{-1}albumin	-	1.0 µL of 500.0 µg mL^{-1}	5.0 µL	21.0 µL
7	15.0 µL of albumin coated NCs	1.0 µL	-	5.0 µL	21.0 µL
8	15.0 µL of albumin coated NCs	-	1.0 µL of 500.0 µg mL^{-1}	5.0 µL	21.0 µL

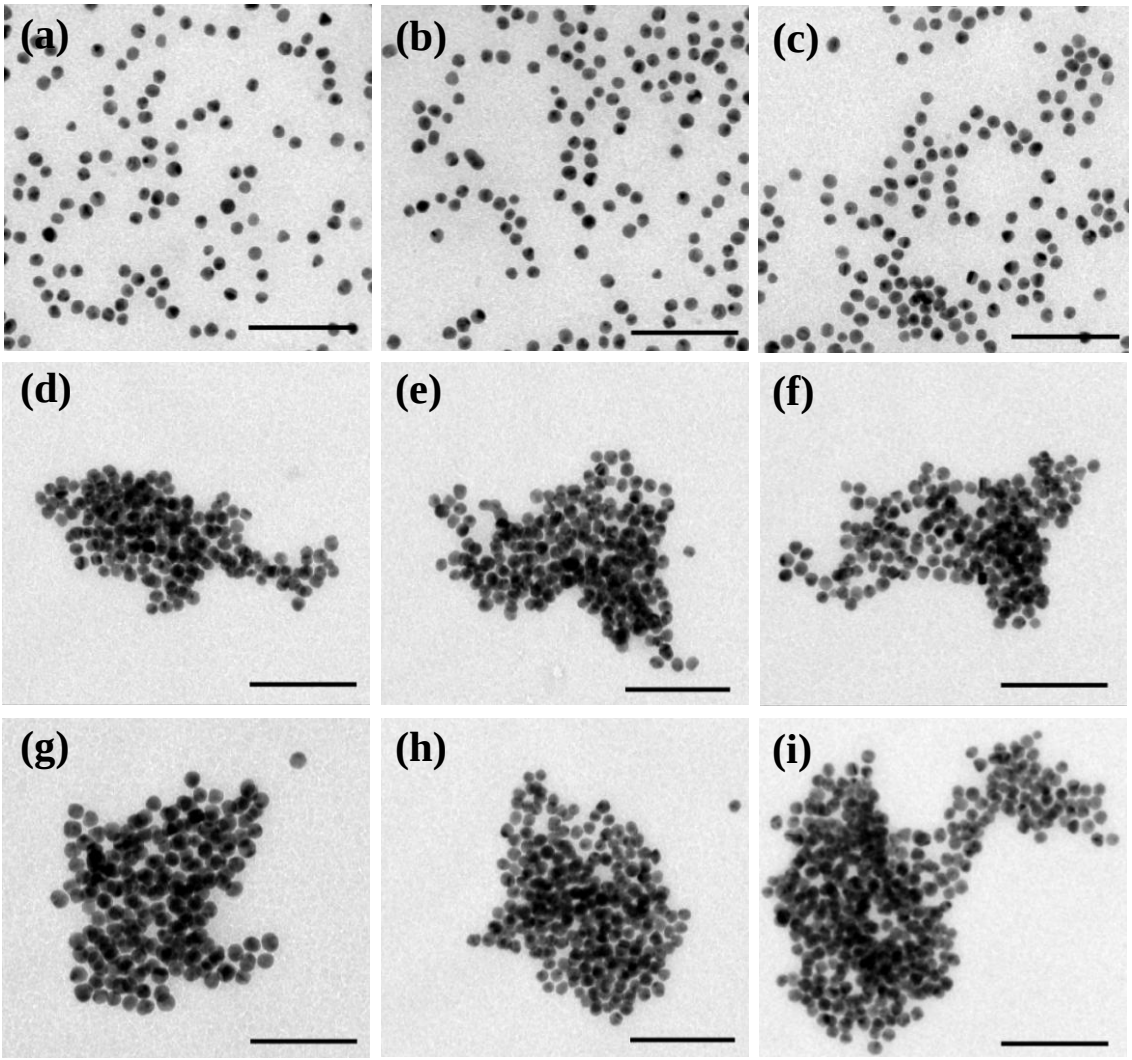

Fig. S1 TEM micrographs of (a-c) Cit-Au NPs; (d-f) Au NP-Lyz agglomerates; and (g-i) CPT loaded Au NP-Lyz agglomerates. Scale bar is 100 nm.

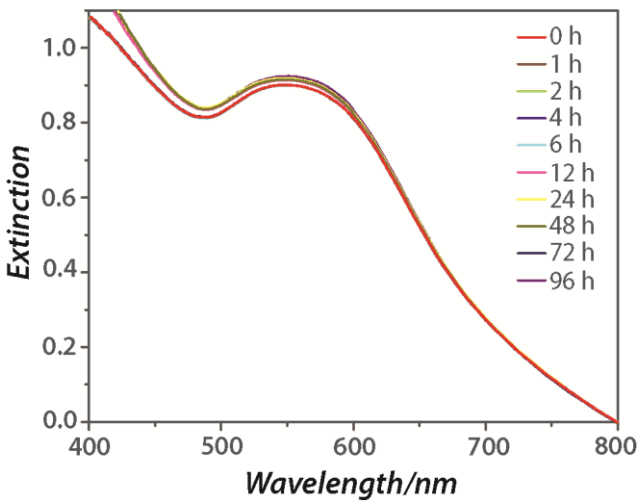

Fig. S2 UV-visible extinction spectra of PLGA coated NCs redispersed in water at different intervals of time as mentioned in the legends.

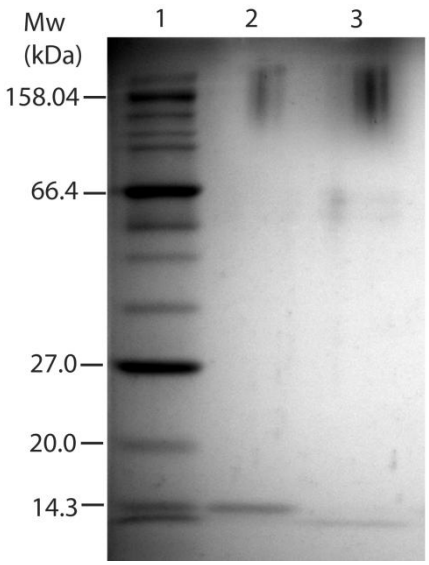

Fig. S3 SDS-PAGE analysis of Au NP-Lyz agglomerates with and without protease treatment. Lane 1: New England Biolabs Protein marker; 2: Au NP-Lyz agglomerates without protease treatment; 3: Au NP-Lyz agglomerates with protease treatment.

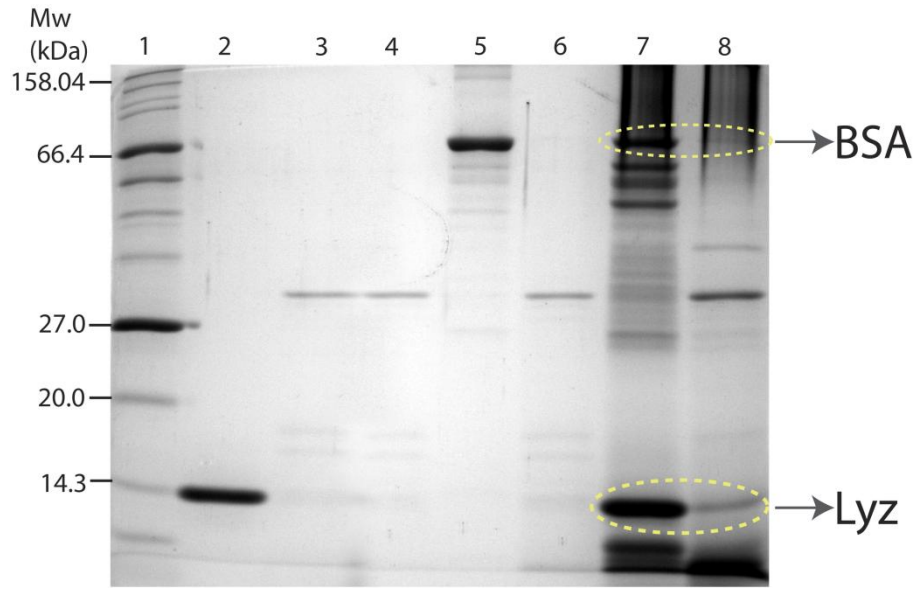

Fig. S4 SDS-PAGE analysis of albumin coated NCs with and without protease treatment. Lane 1: New England Biolabs Protein marker; 2: Lyz without protease treatment; 3: Lyz with protease treatment; 4: Proteinase K only; 5: albumin without protease treatment; 6: albumin with protease treatment; 7: albumin coated NCs without protease treatment; and 8: albumin coated NCs with protease treatment.

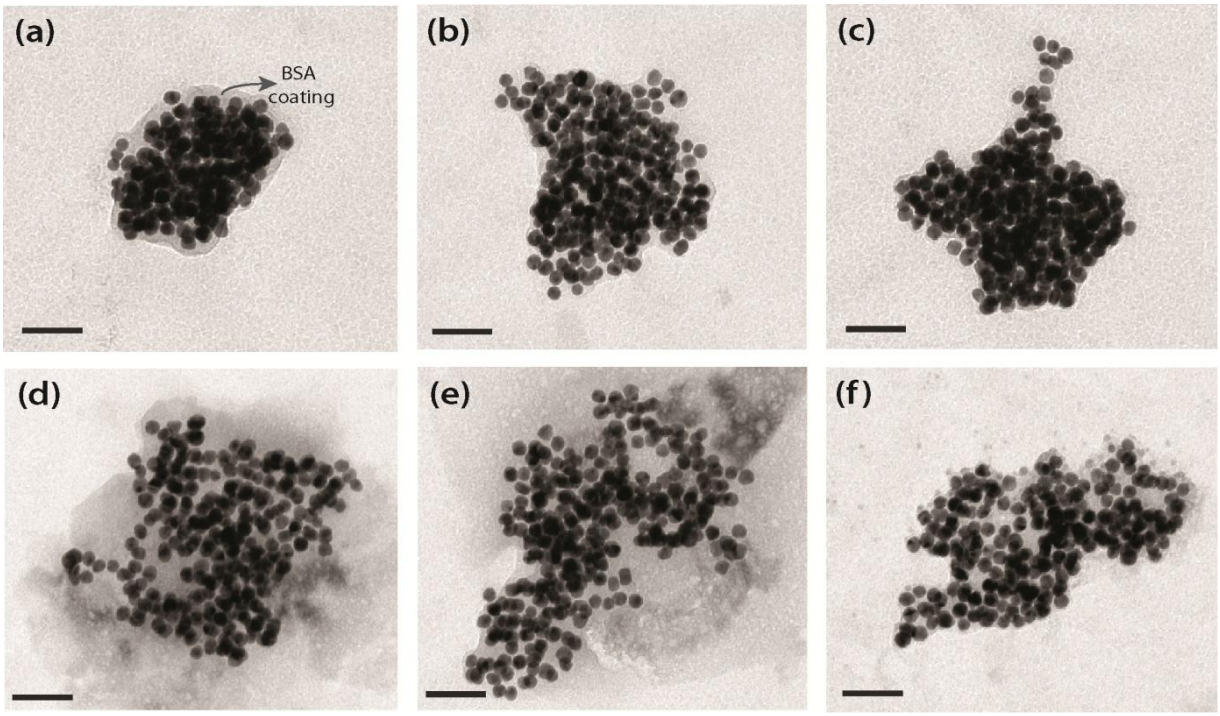

Fig. S5 TEM micrographs of albumin coated NCs (a-c) without and (d-f) with protease treatment. Scale bar is 50 nm.

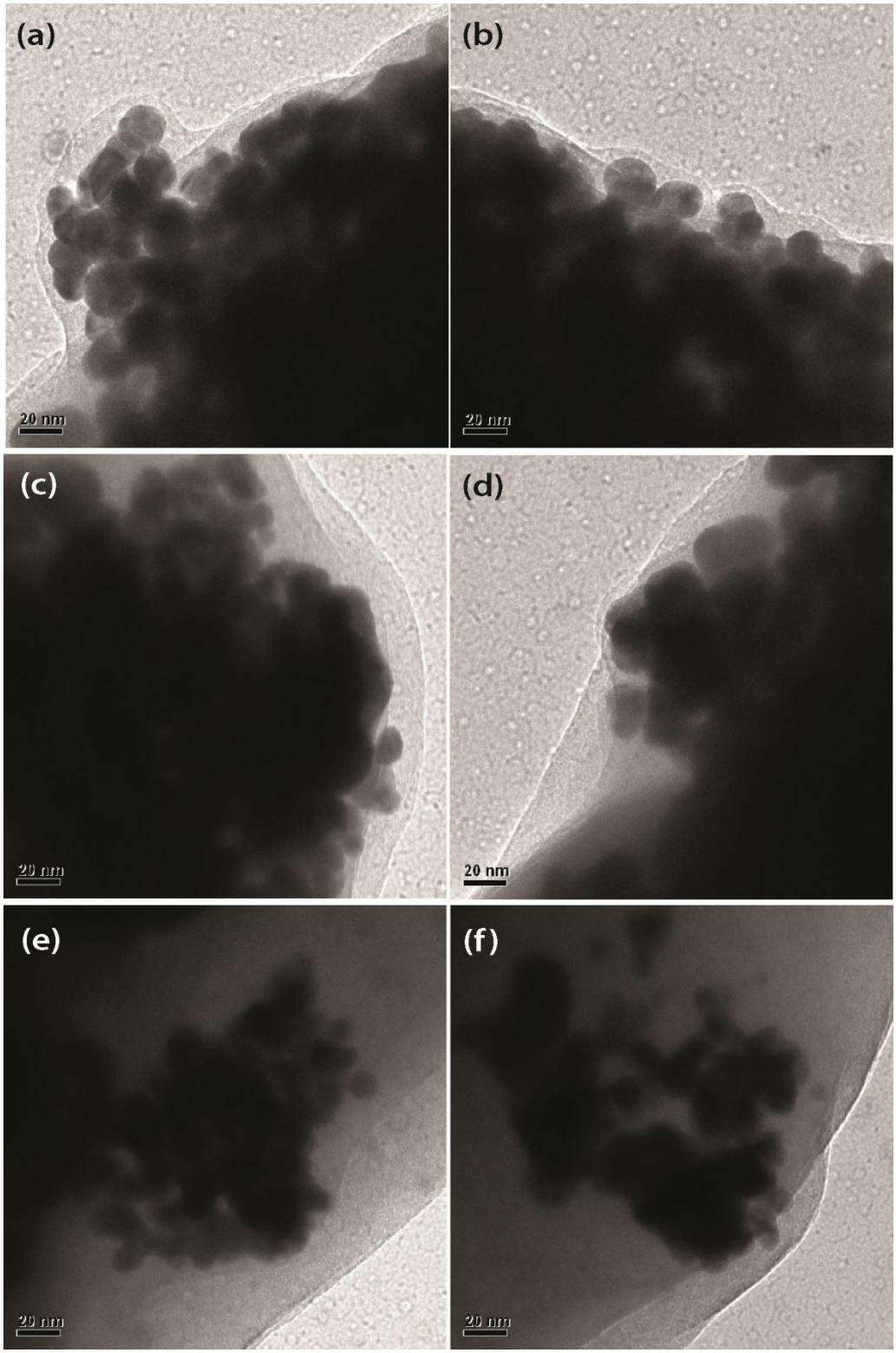

Fig. S6 TEM micrographs of the various regions of the HeLa cell having the PLGA coated NCs internalized.

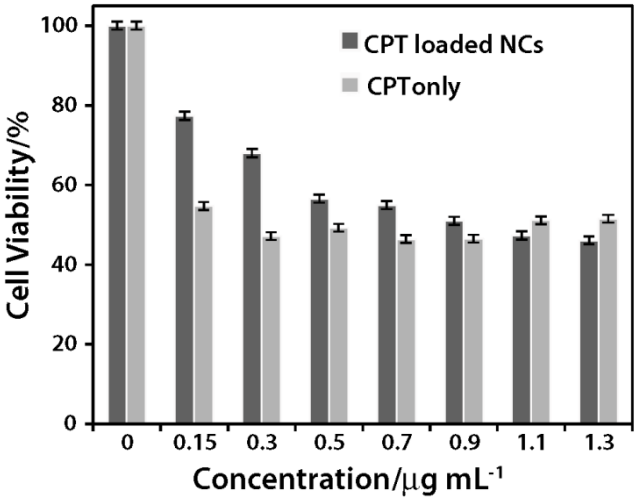

Fig. S7 Viability of HeLa cells after 24 h treatment with CPT loaded NCs or CPT only at varying CPT concentrations.

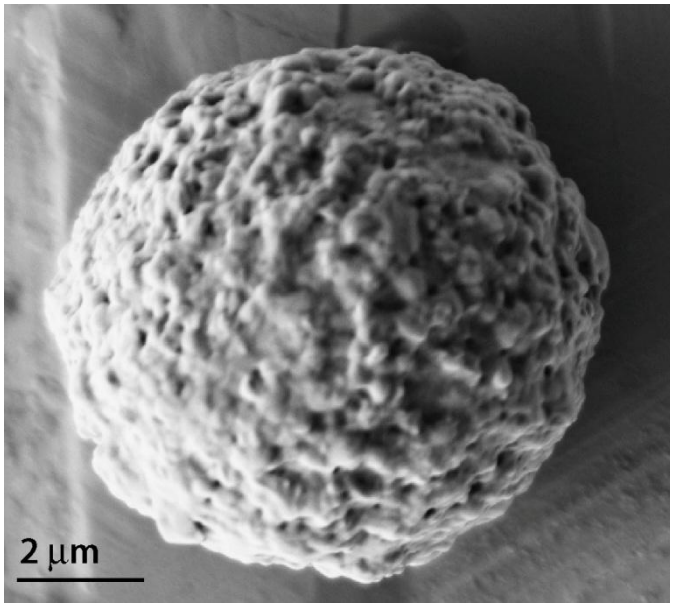

Fig. S8 FESEM image of a control HeLa cell.