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Supporting Information

Antioxidant nanozyme: A facile synthesis and evaluation of reactive oxygen species scavenging potential of nanoceria encapsulated albumin nanoparticles

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2. Materials and methods

2.1. Materials and reagents

Bovine serum albumin (BSA) was purchased from HIMEDIA. $(NH_4)_2$ $Ce(NO_3)_6$, CH_3COONa and crosslinking agent glutaraldehyde (50 wt% in H_2O) were procured from Sigma–Aldrich and stored at appropriate storage conditions until used. Acetic acid and hydrogen peroxide (30%) were purchased from Merck. All reagents were of analytical grade and used without further purification. All other chemicals used were molecular biology grade. L-132 cells (Human lung epithelial cells) were received from National Centre for Cell Science, Pune, India. They were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% calf serum and 1% Penicillin-streptomycin in the 37°C incubator with 5% CO_2 and 95% air.

2.2. CNPs synthesis

The nanoceria was synthesised *via* hydrothermal method as reported earlier. [20]. Briefly, $(NH_4)_2$ $Ce(NO_3)_6$ and CH_3COONa were dissolved in deionized water followed by subsequent addition of 10 mL of acetic acid to the solution and stirred for 1h. After mixing, the solution was transferred to a Teflon-lined autoclave for hydrothermal treatment at 200 °C for 12 h. the precipitated particles were separated by centrifugation and thoroughly washed 4–6 times using deionized water and ethanol. Finally nanoparticles were dried in the hot air oven at 50 ^oC and re-suspended in water for further use.

2.3. Preparation of nanoceria encapsulated albumin nanoparticles

The nanoceria encapsulated albumin nanoparticles (BCNPs) were prepared by desolvation method. [21]. In this process ceria nanoparticles were dispersed in water via sonication and

then added dropwise into the 5 mL of 5 mg mL^{-1} BSA solution in water. After one hour of mixing, ethanol was added dropwise into the solution till the turbidity just appeared. The suspension was stirred for additional 10 min before glutaraldehyde was added drop-wise to the suspension. After overnight stirring, the nanoparticles were separated by centrifugation and washed thoroughly with ethanol to remove adsorbed glutaraldehyde and free nanoceria from nanoparticles surface. It is noted that some amount of free CNPs remain attached on the surface of BCNPs even after thorough washing due to the strong electrostatic interaction between the nanoceria and albumin nanoparticles as reported earlier [23], and it is not possible to completely eradicate these adsorbed CNPs.The prepared nanoparticles were lyophilized and redispersed in deionized water for further studies. The control albumin nanoparticles (BNPs) were also prepared by the above procedure without nanoceria addition. The amount of ceria nanoparticles entrapped inside the albumin nanoparticles were determined by using ICP-MS, by digesting a known amount of BCNPs with concentrated nitric acid and then diluted it with deionized water. Later, the concentration was analyzed by using ICP-MS. Moreover, it should be taken into account that the concentration of nanoceria obtained by ICP-MS contains both entrapped and to some extent of the surface adsorbed CNPs, as it is not possible to calculate the exact amount of adsorbed CNPs. So, the entrapment efficiency calculated here too contains some portion of adsorbed CNPs.

2.4. Characterizations

FTIR spectra of BSA (control), nanoceria alone and BCNPs were recorded on Thermo Nicolet FTIR spectrometer in the range 4000–400 cm^{-1} using KBr pellets. The mean size and surface charge for the prepared nanoparticles were determined by dynamic light scattering (DLS, Malvern). Morphology and average grain size of the particles were confirmed using AFM (NTEGRA PNL) operating in semi-contact mode. The images were further processed

using NOVA software. TEM (TECNAI G^2 20 S-TWIN) operating at 200 keV and FE-SEM (Carl Zeiss ULTRA PLUS) equipped with energy dispersive X-ray detector (EDX) operating at an accelerating voltage of 15–20 keV were used to determine the particle size, morphology and compositional analysis of the nanoparticles. XRD analysis was done by using Bruker AXS D8 advance powder X-ray diffractometer (Cu-Kα radiation, λ = 1.5406A˚) in the range of 10–90^o at a scan speed of 0.5^o/min. The concentration of nanoparticles was determined by ICP-MS (Perkin-Elmer ELAN DRC-e). While thermal studies were done by heating 10 mg of respective samples from 30ºC to 700ºC at a constant rate of 10ºC/ min in EXSTAR TG/DTA 6300 under controlled nitrogen atmosphere. Absorbance was measured by using UV-Visible spectrophotometer (Lasany double-beam L1 2800).

2.5. *In vitro* CNPs release studies

In a characteristic release experiment, 5.0 mg of BCNPs were redispersed in 5 mL of 10 mM PBS (pH = 7.4) and incubated at 37 ^{o}C with constant shaking at 150 rpm. After predefined interval the supernatant was withdrawn and replaced immediately with equivalent amount of fresh buffer in order to maintain the sink condition. The supernatant was then digested with nitric acid solution and the concentration was measured by ICP-MS.

2.6. SOD assay

The superoxide anions scavenging activity of released CNPs collected at different time intervals were assessed with a commercial SOD assay kit (Sigma Aldrich). Briefly, a sample solution (20 μL) was mixed with WST-1 working solution (200 μL) in a 96 well plate. With addition of 20 μL enzyme (xanthine oxidase) solution, the reaction was initiated and proceeded at 37 °C for 20 min. Finally, the inhibition activity was measured by taking the

absorbance at 450 nm as compared to the control using a Cytation 3 cell imaging multi mode plate reader (Biotek).

2.7. Cellular Uptake

2.7.1. Quantification of intracellular BCNPs

L-132 cells were initially grown to sub-confluence in cell culture dishes (3.5 cm diameter), and then co-incubated with 100 μg/mL BCNPs in culture medium. After different time intervals, the cells were extensively washed with PBS (pH 7.4) to remove all extracellular nanoparticles and digested with 1 mL nitric acid (16 M) for 24 h. Finally, the digested solution was diluted with deionized water, and the cerium concentration was determined with ICP-MS.

2.7.2. FE-SEM analysis

Cells were seeded over glass cover slips in a 6- well tissue culture plate and then treated with 100 μg/mL BCNPs. After 24 h of incubation, media was removed and the cells were given thorough PBS wash. Thereafter, cells were fixed with 2% glutaraldehyde solution followed by dehydration in graded ethanol solutions. Finally, the cells attached on cover slips were then sputter coated with gold for observation under FE-SEM.

2.7.3. TEM observation

L-132 cells were initially grown to sub-confluence, then co-incubated with 100 μg/mL BCNPs for 24h. After incubation, the cells were thoroughly washed with PBS (pH 7.4) and fixed using 2 % glutaraldehyde solution. Subsequently, the fixed cells were serially dehydrated with graded ethanol solutions. Finally, the cells were mounted on 300-mesh copper grids. The samples were observed using a TEM (TECNAI G^2 20 S-TWIN).

2.8. Cell viability assay

The biocompatibility of CNPs, BNPs and BCNPs were determined by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In a typical experiment, 10,000 cells (L-132 cells) per well were seeded in a 96 well plate. After overnight incubation, cells were treated with different concentrations of CNPs, BNPs and BCNPs for 24 h. After 24 hours, the spent media was removed and cells were given a brief PBS wash. Fresh media (DMEM) (~ 90 µL) containing 10 µL of MTT (stock concentration 5 mg/mL in PBS) was added to each well and incubated at 37 ^{o}C in 5% CO_2 for 3-4 h. Finally, to solubilize the formazan crystals formed by the mitochondrial dehydrogenases enzyme of viable cells, DMSO (dimethyl sulfoxide) was added and incubated at room temperature over gyratory shaker for 15-30 minutes. The optical density of the solution was measured at a wavelength of 570 nm and 690 nm using Cytation 3 cell imaging multi mode plate reader (Biotek). Triplicate samples were analyzed for each experiment.

Cellular ROS detection assay

L-132 cells were seeded into six-well plates and grown to sub-confluence. Subsequently, the cells were incubated with 100 µg/mL of BCNPs for different time period whereas for free nanoceria, cells were incubated with different concentration of CNPs for 24 h. After incubation, the cells were given thrice PBS wash followed by exposure to 800 µM H_2O_2 in culture medium for 8 h at 37 °C. The 2',7'- dichlorfluorescein diacetate (DCFH-DA, Sigma Aldrich) dye was used to measure intracellular ROS production. Inside the cell, endogenous esterases deacetylate the DCFH-DA into non-fluorescent 2',7'-dichlorfluorescein (DCFH), which further converted into a green fluorescent dichlorofluorescein (DCF) compound in respond to ROS production that was analyzed by flow cytometry. Stock solution of DCFH-DA was prepared in dimethyl sulfoxide (DMSO, Sigma Aldrich). After the treatment the

cells were given thorough PBS wash, followed by incubation with 20 μM DCFH-DA at 37 °C for 30 min. At the end of incubation, the cells were washed thoroughly, and then harvested. The fluorescent intensity was analyzed with a Flowsight flow cytometer (Amnis), and the data were acquired from 10,000 cells per sample. The relative fluorescence intensity was measured as the ratio of mean intensity of the experimental cells and mean intensity of control cells. The data were analyzed by IDEAS version 6.0 software. The intracellular antioxidant activity of BCNPs was also studied by EVOS cell imaging system (life technologies, USA). L-132 cells were treated following the same procedures as mentioned above. After incubation with DCFH-DA and subsequent washing, the cells were visualized. The intracellular ROS level in each sample was reflected by the fluorescent intensity of the images.

2.8. Semi-quantitative RT-PCR analysis

For gene expression analysis, L-132 cells were grown in 3.5 cm plates. After overnight incubation, cells were treated with required concentration of BCNPs (100 μg/mL) for 48 h followed by H_2O_2 (500 μM for 24 h) treatment. The total RNA was extracted by using Tri reagent (Sigma-Aldrich). cDNA synthesis was done at 42^{o}C for 50 min by using Superscript II Reverse Transcriptase (Invitrogen, USA). Expression of antioxidant and apoptotic genes were analysed by RT-PCR analysis using housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal control. Semi quantitative PCR was done by taking 1 μL of cDNA (5 times diluted stock of the prepared RT product) along with gene-specific forward and reverse primers in Veriti 96 well thermal cycler (Applied Biosystems). The initial denaturation was done at 94 ^{o}C for 3 min, followed by PCR cycles with parameters mentioned below (i) denaturation at 94 ^{o}C for 30 s (ii) annealing at 60 ^{o}C for 30 s (iii) extension at 72 ^{o}C for 1 min and (iv) final extension at 72 ^{o}C for 10 min. The PCR amplified

products were loaded and run on a 1.2% agarose gel and visualized under UV light. Finally, the band intensity was computed by using Image lab 4.0 software.

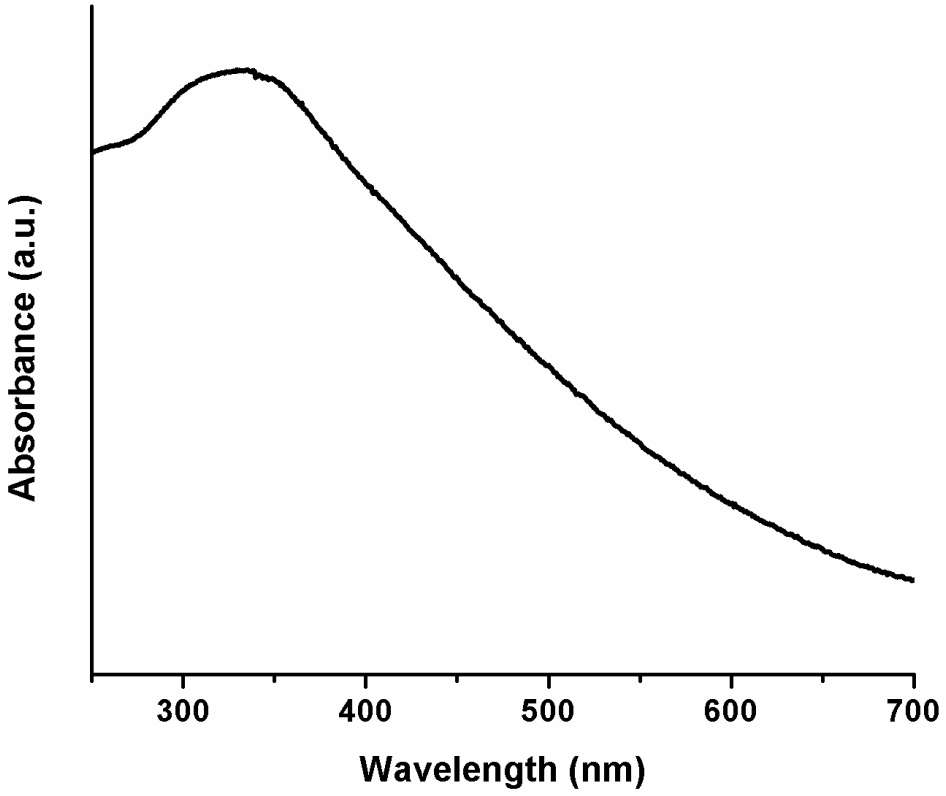

Fig. S1 UV–visible absorption spectra of prepared CNPs.

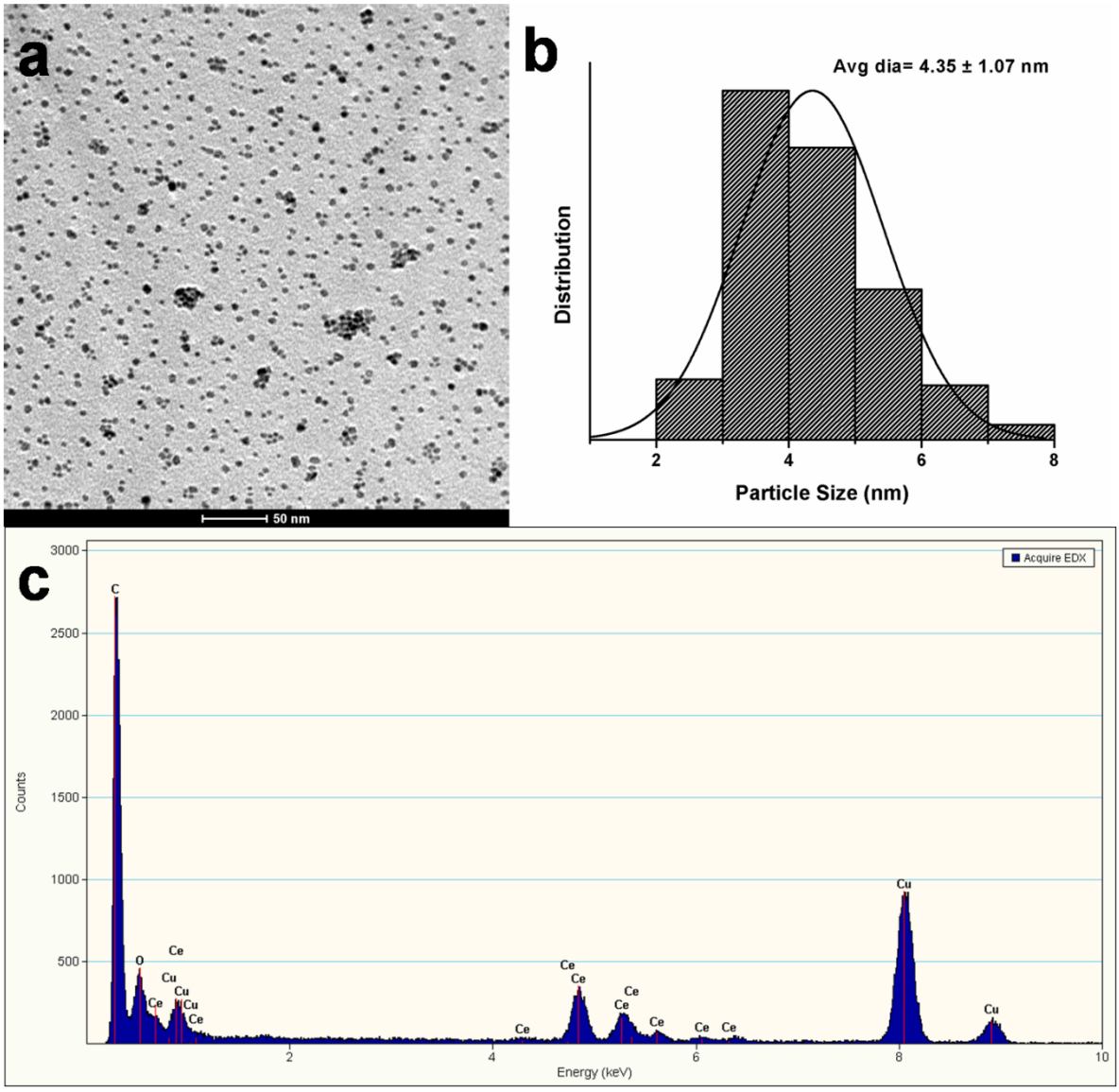

Fig. S2 (a) TEM image of prepared CNPs. (scale bar: 50 nm) (b) Particle size distribution histogram of prepared CNPs. (c) Energy dispersive spectra (EDS) of CNPs.

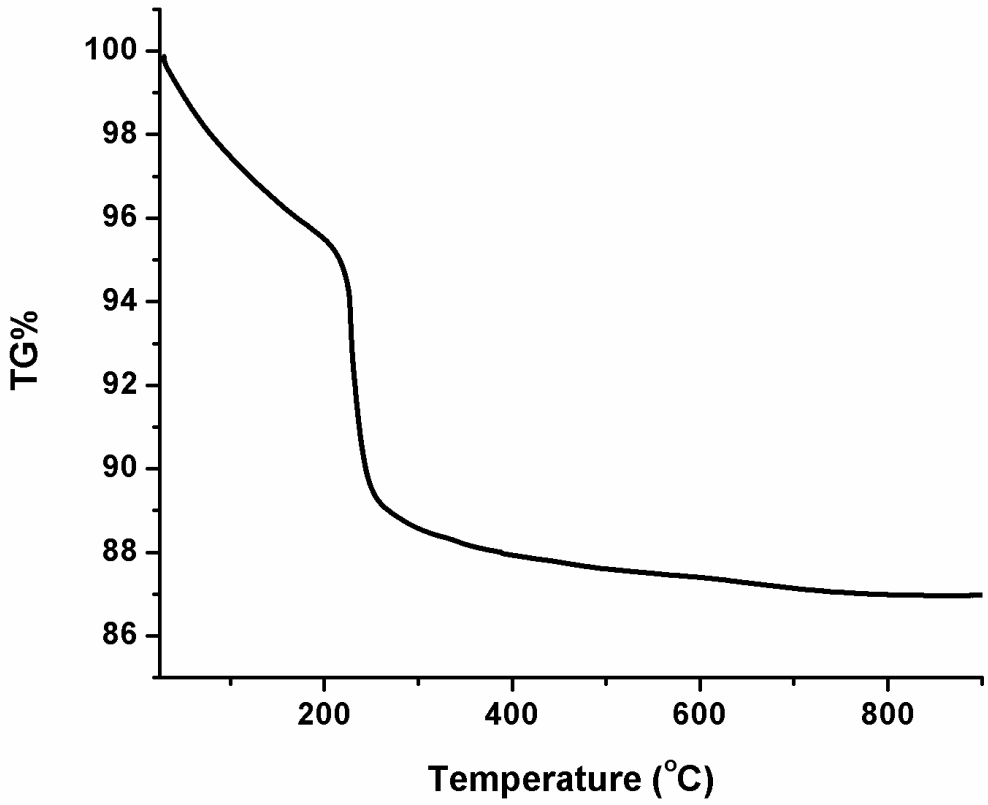

Fig. S3 TG spectra of CNPs.

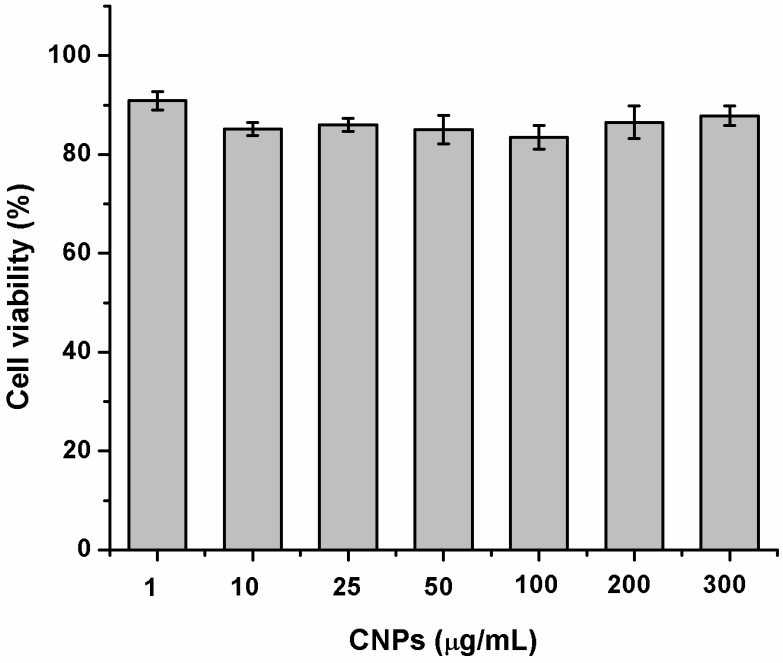

Fig. S4 Biocompatibility and cell viability assay (MTT assay) of CNPs on L-132 cells.

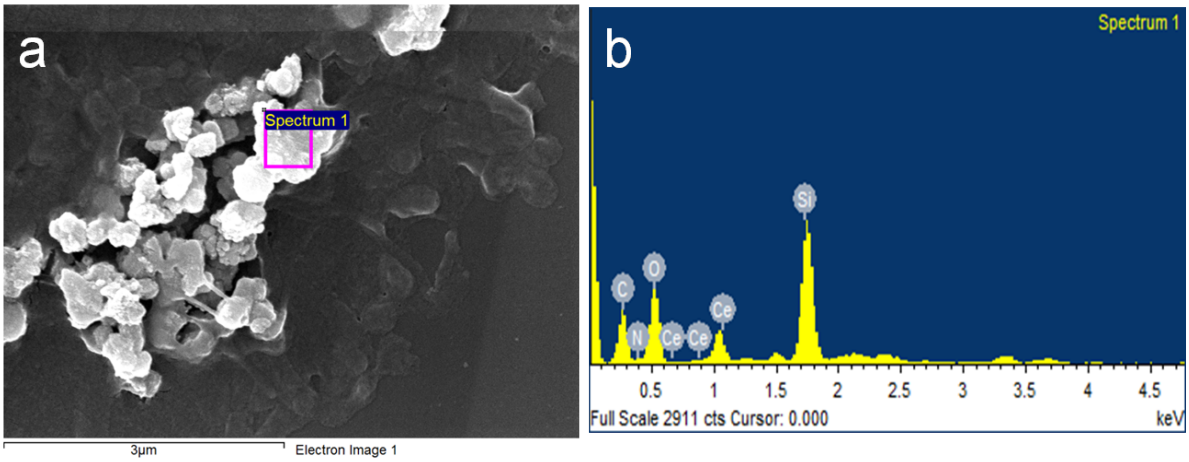

Fig. S5 (a) Magnified FE-SEM image of internalizing BCNPs and corresponding (b) Energy dispersive spectra (EDS) of selected proportion of cell.

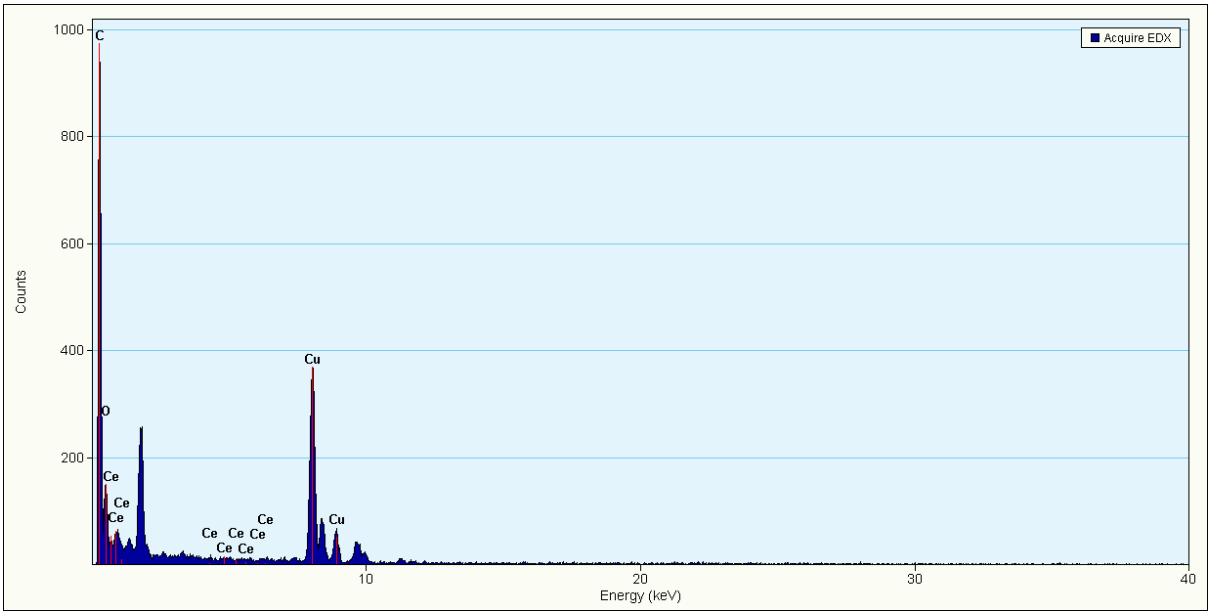

Fig. S6 EDS analysis of internalized BCNPs on the TEM.

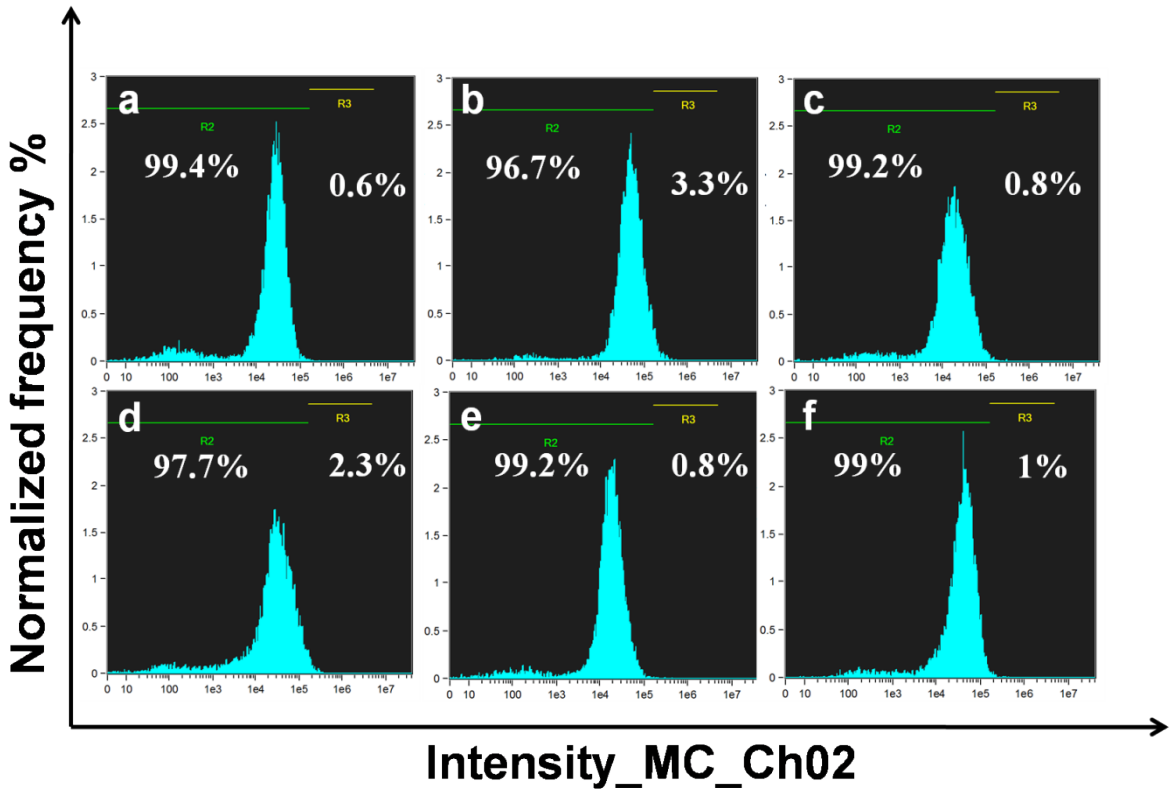

Fig. S7 Flow cytometric analyses of ROS production in L-132 cells treated with BCNPs for different time intervals. (a) Control (b) 12 h (c) 24 h (d) 48 h (e) 72 h (f) 96 h.

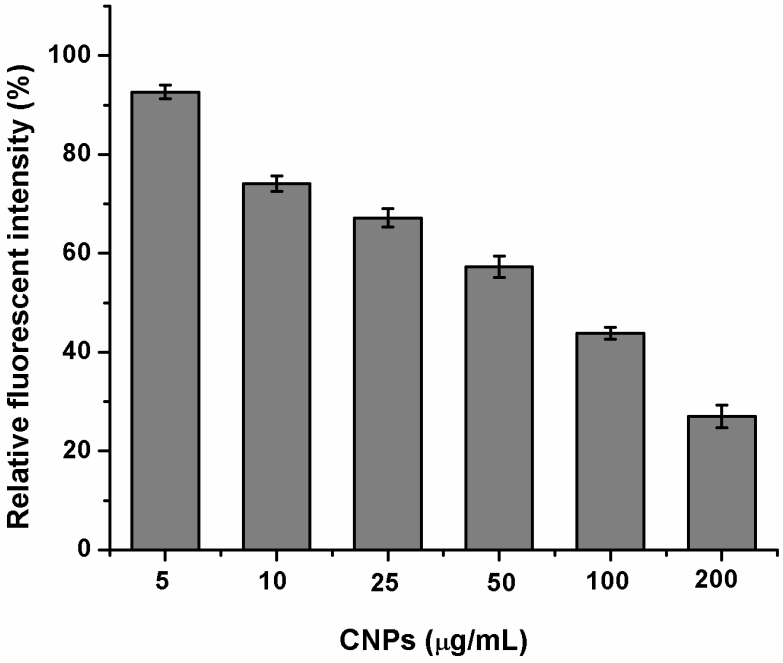

Fig. S8 (a) Scavenging of ROS in L-132 cells preincubated with different concentration of CNPs.

Functional groups	-OH	-OH	C-H	C=O	C=O	N-O	Ce-O	Ce-O
Wave No. (cm^{-1})	3431.12	1630.32	2925.38	1455.76	1538.41	1338.93	873.88	439.51
Vibration	-OH stretch	-OH bend	C-H stretch	C=O stretch	C=O stretch	N-O stretch	Ce-O stretch	Ce-O stretch

Table S1. Characteristic major absorption bands in the IR spectra of the CNPs.

Genes	Primers
GAPDH	Forward: 5’ CCACCCATGGCAAATTCCATGGCA 3’ Reverse : 5’ TCTAGACGGCAGGTCAGGTCCACC 3’
MnSOD	Forward: 5’ TCCACCACCGTTAGGGCTGAGG 3’ Reverse : 5’ CACCAGCAGGCAGCTGGCTCC3’
Caspase-3	Forward : 5’ TTCAGAGGGGATCGTTGTAGAAGTC 3’ Reverse : 5’ CAAGCTTGTCGGCATACTGTTTCAG 3’
Cu/Zn SOD	Forward : 5’ CAATAGACACATCGGCCACAC 3’ Reverse : 5’ AAGGCCGTGTGCGTGCTGAA 3’
GPx	Forward : 5’ CCACCAGGAACTTCTCAAA 3’ Reverse : 5’ TGGCTTCTTGGACAATTGCG3’
Catalase	Forward : 5’ AAGACCAGTTTACCAACTGGG 3’ Reverse : 5’ CAGATGGACATGCGCACATG3’
Bcl-xl	Forward : 5’ATGGCAGCAGTAAAGCAAGC 3’ Reverse : 5’CGGAAGAGTTCATTCACTACCTGT 3’

Table S2. List of antioxidant and apoptotic genes primers used in semi-quantitative RT-PCR analysis.