Electronic Supplementary Material (ESI) for Journal of Materials Chemistry B.
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Electronic Supplementary Information

Cu^{2+}-embedded carbon nanoparticles as anticancer agents

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Materials

Citric acid, ethylenediamine, NaOH and $CuSO_4.5H_2O$ were purchased from Merck, India. The dialysis membrane (dialysis tubing, benzoylated) was purchased from Sigma-Aldrich, India. All the materials were used as received without further purification. Elix grade water was used in all the experiments. The induction cooker and non-stick frying pan were procured from Philips (model no. HD4908, 220-240V AC, 50/60 Hz) and Prestige, India, respectively.

Experimental Section:

Preparation of Carbon Nanoparticlecs (CNP): CNPs were synthesized by slight modification of a previously reported method from the laboratory.[1] In brief, 50 mL aqueous solution containing a mixture of 630 mg citric acid and 134 µL ethylenediamine (in 3:2 molar ratio) in a non-stick frying pan was heated using induction heater at 100 ^{0}C for 15 min. Then the dark brownish syrup was purified by dialysis method using 1 KDa dialysis membrane. TEM image of the particles synthesized is shown at Fig. S4(f). The longer heating time possibly made the sizes of CNP bigger than those reported earlier.[1]

Preparation of Cu^{2+} embedded Carbon Nanoparticlecs (Cu-CNP): The whole amount of purified aqueous CNP was taken in a round bottom flask and the final volume was made up to 12

mL. Then, solid NaOH (~ 80 mg) was added to it with stirring to ensure the pH of the solution to be in between 9-11. After 5 min of stirring, 100 mg $CuSO_4$ (0.4 mM) was added to it and the mixture was stirred for 2 h at 50 ^{0}C. The color of the reaction mixture turned greenish.

Purification of Cu-CNP: The as-synthesized Cu-CNP was purified by centrifugation. The reaction mixture was centrifuged at 12000 rpm for 10 min using acetone (the ratio of reaction mixture and acetone was 3:1). The dark greenish pellet was isolated but not used for further application as the quantum yield of redispersion of the dark greenish pellet was low (15.9%). On the other hand, the supernatant was separated out and centrifuged at 25000 rpm using acetone (the ratio of reaction mixture and acetone was 11:1) and the final centrifuged Cu-CNP was then dried at 40 ^{0}C for 12 h. The greenish dry pellet was redispersed in water prior to further use as well as for characterizations. The redispersion of Cu-CNP appeared brownish orange in visible light (with a photoluminescence quantum yield of 27.9%, the details of which are mentioned below).

Characterization

The products were characterized using transmission electron microscopy (JEOL 2100 UHR-TEM, operating at 200 KV), UV-vis spectroscopy (Hitachi U 2900 spectrophotometer), photoluminescence spectroscopy (Horiba Fluoromax-4 spectro fluorometer), powder X-ray diffraction (Brucker D8 advanced X-ray diffraction measurement system) with Cu Kα source (λ=1.54 Å), nuclear magnetic resonance spectroscopy (Bruker, 600 MHz), Fourier transform infrared spectroscopy (Perkin Elmer IR spectrometer), electron paramagnetic resonance (JEOL, Model: JESFA200) spectrometry, particle size analysis (Malvern zeta size; Nano ZS 90). Inductive coupled plasma optical emission spectrometer-optical emission spectrometer (ICP-OES) measurement was performed with Thermo-iCAP 6000 Series. Cell cycle was analyzed by FACS (fluorescence-activated cell sorting) Calibur, BD Biosciences.

Quantum yield calculation with respect to quinine sulphate (QS) in 0.1 M H_2SO_4:

We have calculated quantum yield with respect to quinine sulphate using the formula:

$$Q_S = Q_R \times \frac{I_S}{I_R} \times \frac{A_R}{A_S} \times \frac{\eta_S^2}{\eta_R^2}$$

Where,

Q_S = quantum yield of sample; Q_R = quantum yield of reference; I_S = area under PL curve of sample; I_R = area under PL curve of reference; A_R = absorbance of the reference; A_S = absorbance of the sample; η_S = refractive index of sample; η_R = refractive index of reference.

Q.Y. of quinine sulphate = 0.54

Refractive Index of water = 1.33

(The concentration of all samples and the reference quinine sulphate were adjusted so that the optical densities of all samples were 0.100 ± 0.003 at the excitation wavelength (365 nm).

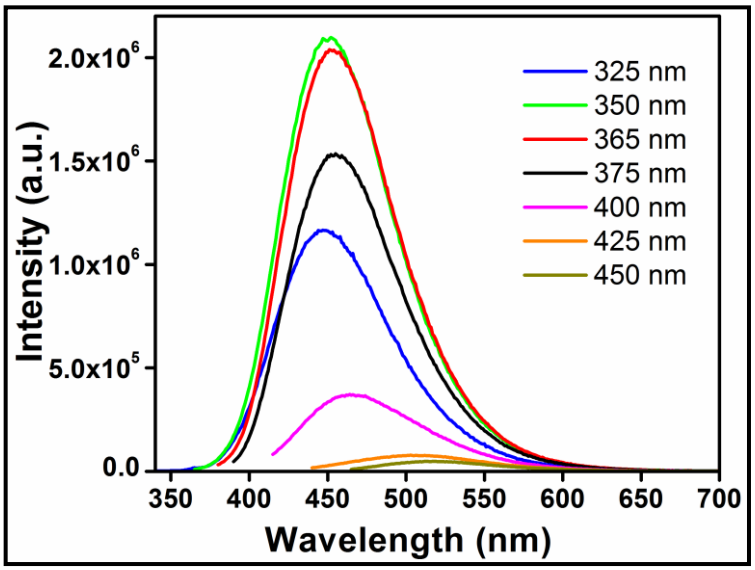

Fig: S1. Excitation wavelength-dependent emission spectra of CNP in water. The wavelengths in the legends indicate excitation wavelengths.

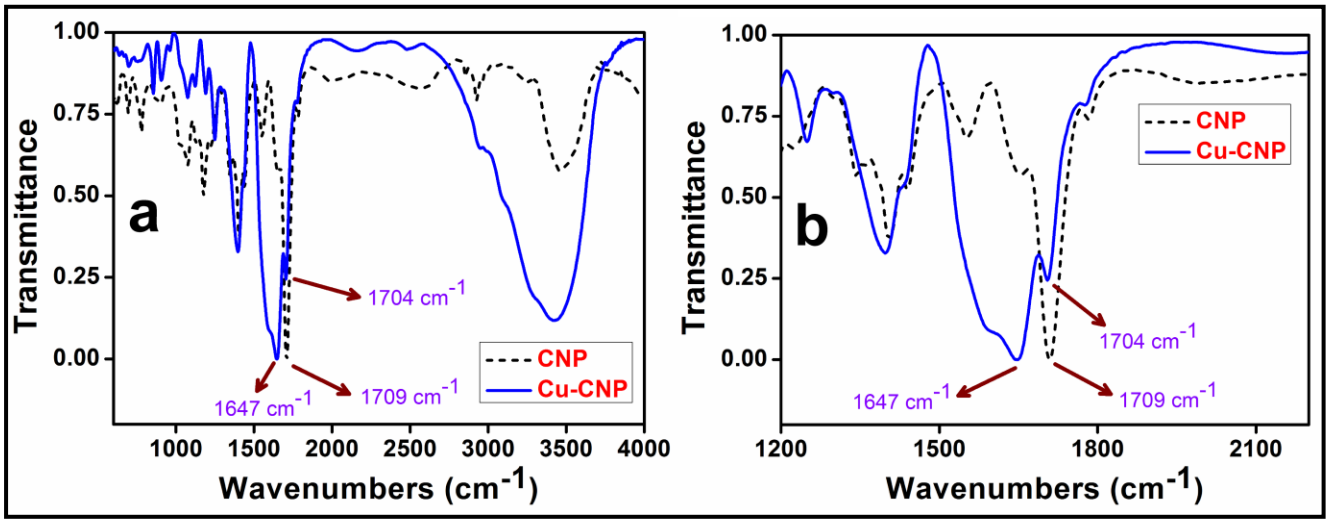

Fig: S2. a) FTIR spectra of CNP and Cu-CNP and b) expanded view of the same for the region 1200 – 2100 cm^{-1}.

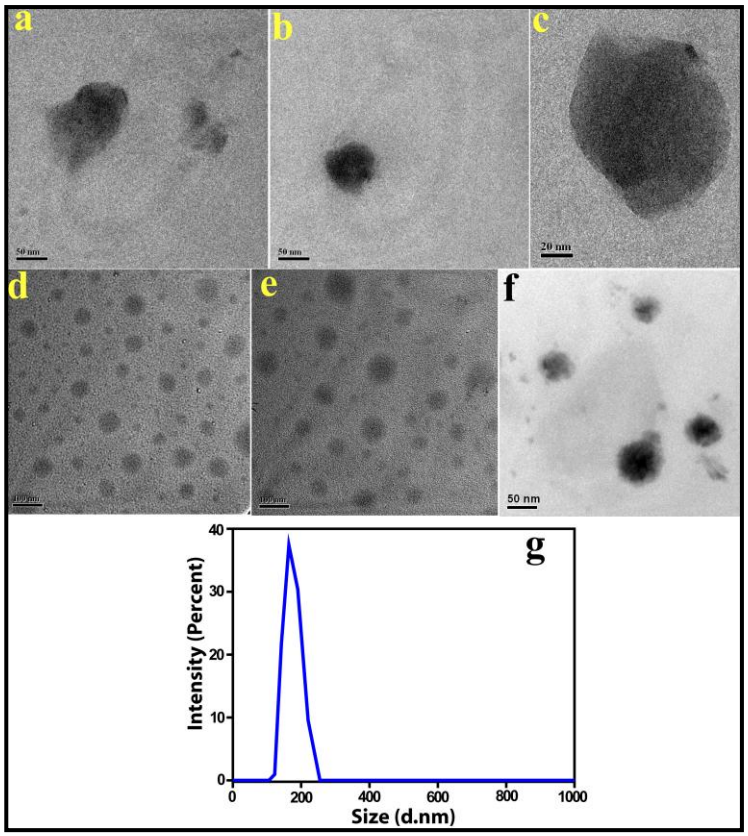

Fig. S3. (a, b & c) Representative additional TEM images of the Cu-CNP. (d & e) TEM images of Cu-CNP at lower magnification in a scale of 100 nm. (f) TEM image of CNP. (g) Dynamic light scattering based particle size analysis of Cu-CNP.

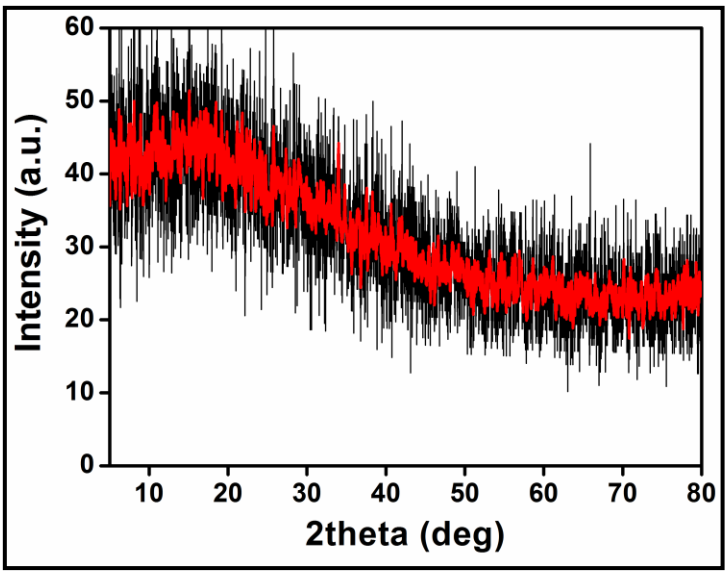

Fig: S4. Powder XRD pattern of Cu-CNP.

Loading of Cu^{2+} on the surface of CNP:

Metal concentration on the surface of CNP was determined by atomic absorption spectroscopy (AAS). To carry out this experiment, first we recorded the absorbance of standard samples of copper (2 ppm to 12 ppm). Then the absorbance of stock solution of Cu-CNP was recorded. The sample Cu-CNP was digested with HNO_3 before recording absorbance.

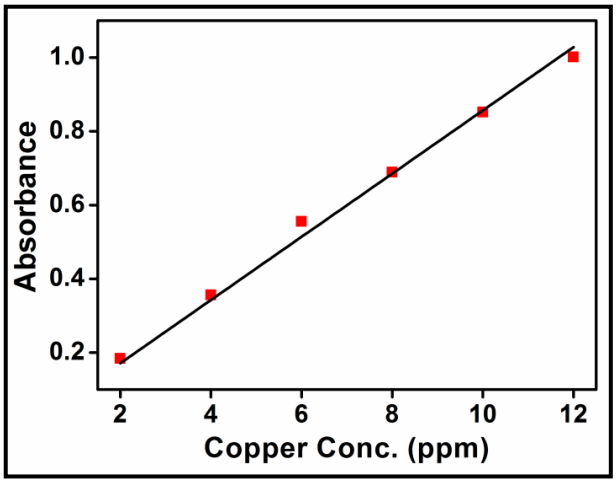

Fig. S5. Calibration Curve representing measurement of concentrations of copper (standard), using atomic absorption spectroscopy.

To digest the sample, desired amount of sample was taken in a vial and 0.2 mL HNO_3 was added to it, followed by sonication for 2 min. It was kept for 2 h and then required amount of water was added to make the final volume 10 mL, such that the conc. of HNO_3 was 2%.

Cell Based Experiments:

Cell Culture: HeLa cells (human cervical carcinoma) were acquired from National Centre for Cell Sciences (NCCS) Pune. For growing HeLa cells Dulbecco's Modified Eagle's Medium was used which was purchased from Sigma Aldrich along with L-glutamine (4 mM), penicillin (50 units/mL), streptomycin (50 mg/mL), which were mixed with the medium. Into that, 10% (v/v) fetal bovine serum was added, which was procured from PAA Laboratories, Austria. The cells were grown in CO_2 incubator with 5% CO_2 at 37 °C.

Cell viability assay: To quantify the viable cells following the 48 h treatment with Cu-CNP, the cell viability assay was carried out. In this regard, approximately 1×10^4 cells/well were seeded in 96-well micro plate and was kept for overnight incubation, in the presence of 5% CO_2 at 37 °C. The grown cells were then treated with various concentrations of Cu-CNP with copper concentration of (0.75 ppm – 2.5 ppm), along with various CNP concentrations separately (20-80 ppm), under identical conditions for 48 h. After the treatment, the number of viable cells was estimated by performing MTT assay. For MTT reaction, 7 µL of [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was added and incubated for 2 h in the above mentioned condition wherein respiratory mitochondria reduced MTT into color formazan. After the incubation, the medium was discarded and the absorbance of formazan was measured at 550 nm by adding 70 µL of DMSO, which provides the number of viable cells. All the experiments were carried out in three sets with 100% number of viable cells for each experiment.

FACS Analysis for Detecting Reactive Oxygen Species Generation:

Generation of reactive oxygen species was analysed by fluorescence based assay in FACS with the help of 2, 7-dichlorofluoresceindiacetate (DCFH-DA) dye. For this, the overnight grown cells (2×10^5 in 6 well plates) were treated with IC_{50} value dose of Cu-CNP and CNP for 3 h,

prior to the incubation with 10 µL of 1 mM DCFH-DA at 37 ^{0}C for 10 min. DCFH-DA a non-fluorescent dye after its diffusion inside the cell is deacetylated by cellular esterase and is oxidized by ROS into 2, 7-dichlorofluroscein (DCF), which has high fluorescence with absorbance at 480 nm and emission at 530 nm. To determine the fluorescence, the treated cells were trypsinized and redispersed in fresh 1 mL DMEM medium and analyzed in a flow cytometer (FACS Calibur, BD Biosciences). The fluorescence data were recorded in cell Quest Program with fixed 15000 cells for each sample and was collected in Fl-1 channel (530/30), which corresponds to green fluorescence.

FESEM Analysis for Untreated and Treated Cells:

For FESEM analysis cells were grown in 35 mm culture plate for 12 h followed by treatment with IC_{50} dose of Cu-CNP for 24 h in case of treated sample. After trypsinization, the cells were fixed with 4% formaldehyde and kept for 10 min incubation at 37 ^{0}C. The cells were collected after centrifuging at 670 rcf for 6 min and immediately redispersed in chilled 70% ethanol. Then 20 µL of the each sample (control and treated) sample was drop casted on a clean glass side with aluminum foil over it.

Cell Cycle Analysis by Propidium Iodide

Cell cycle analysis in FACS was carried out by using propidium iodide (PI) dye. For this, 2×10^5 cells were seeded in 6 well culture plates for 12 h, in the same condition as mentioned above. The Cu-CNP at IC_{50} dose as well as CNP (separately) was added and kept for 48 h. After trypsinization, the cells were fixed with chilled 70% ethanol and kept for 1 h incubation at -20 ^{0}C. The fixed cells were re-dispersed in 1 mL PBS and were incubated in water bath at 37 ^{0}C with 100 µg/mL of RNase A for 45 min, followed by addition of 10 µL of PI under dark condition. After 30 min of incubation the PI fluorescence data for 15000 cells were analyzed in FACS Calibur by using cell quest program. PI binds with DNA and gives red emission, which is collected at FL-2 channel (band pass filter 585/42nm).

Caspase 3-Assay

To further study apoptosis caspase 3-assay was conducted. The 6 well plates were seeded with 2 × 10^5 cells and were kept for 12 h as mentioned earlier. The cells were treated in a same manner as mentioned in other experiments and were incubated for 24 h. The cells were trypsinized and were fixed with 0.1% (v/v) formaldehyde and kept in dark for 10 min. To permeabilise the harvested cells, 0.5% Tween20 was added along with 1 mL of PBS. This was followed by two repetitive washing with PBS buffer and finally 8 µL of Caspase 3 (PE conjugated antibody) was added with 500 µL PBS buffer under dark condition. After 30 min, the data were collected in FL-2 channel (band pass filter 585/42nm) with the help of cell quest program in FACS.

Inductive coupled plasma optical emission spectrometer (ICP-OES) Analysis for Untreated and Treated Cells:

Metal concentration inside the cells was determined by an ICP-OES. To carry out these experiments, 6 well plates with 1×10^5 cells in 1.8 mL medium for each was incubated. The cells were than treated with IC_{50} dose of Cu-CNP for 4 h. After treatment and washing, the cells were treated with 3% sub-boiled HNO_3 for 2 h at room temperature. Afterwards the solutions were collected in 15 mL tubes and prepared for ICP measurement. For the untreated cells the same procedure was repeated (in the absence of any nanoparticles).

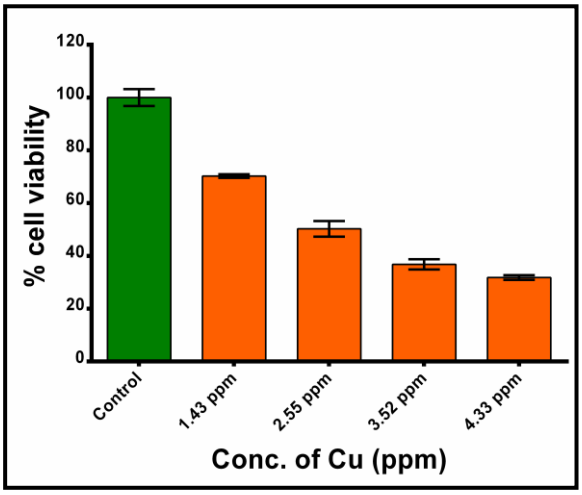

Fig. S6. Cytotoxicity analysis of Cu-CNP (following 48 h treatment) by MTT based cell viability assay.

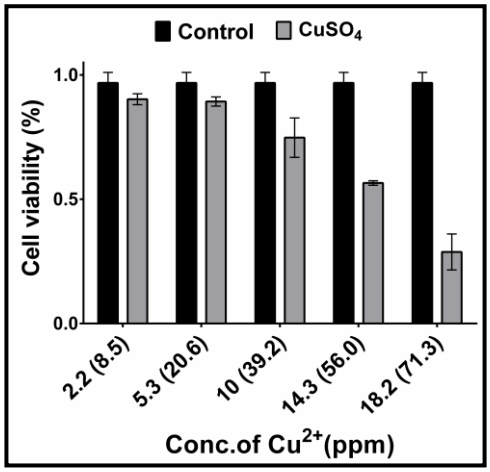

Fig. S7. Cytotoxicity analysis of the $CuSO_4$ (24 h post-treatment) by MTT based cell viability assay. The conc. of $CuSO_4$. $5H_2O$ is represented by the number inside the parenthesis.

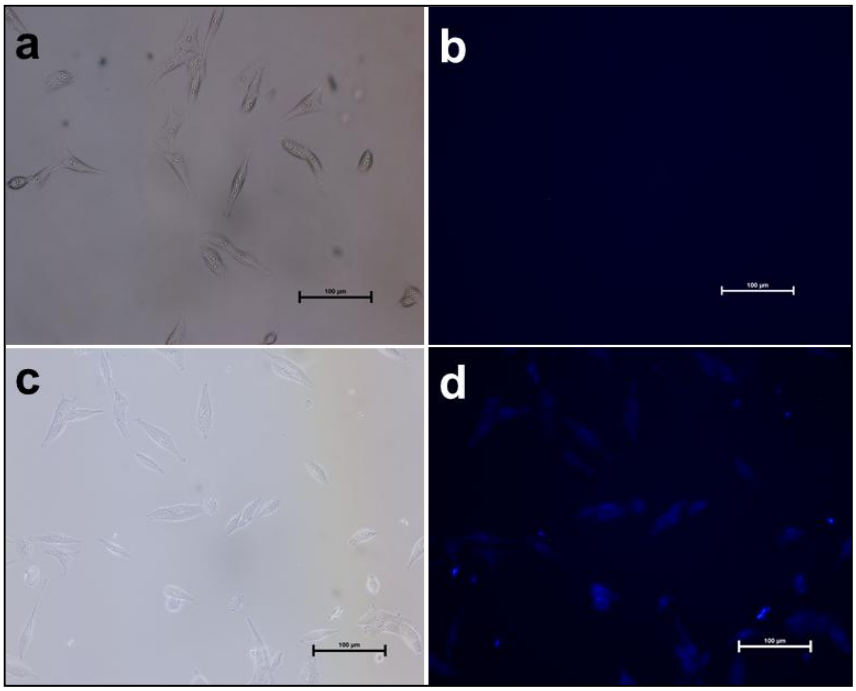

Fig. S8. Epifluorescence microscopic images of HeLa cells treated with Cu-CNP composite. (a) Bright field image of control HeLa cells. (b) Fluorescent image of control HeLa cells, when excited by UV light. (c) Bright field image of HeLa cells treated with Cu-CNP. (d) Fluorescent image of HeLa cell treated with Cu-CNP, when excited by UV light. The images were recorded following treatments for 4 h. Scale bar: 100 μm.

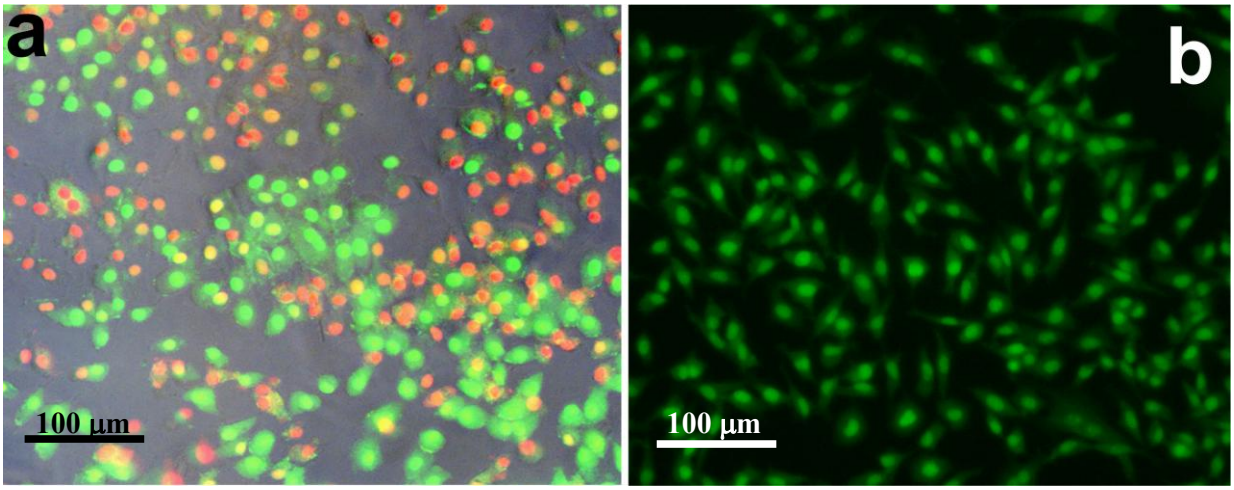

Fig. S9. Optical microscopic images recorded following dual-staining (acridine orange / ethidium bromide) of HeLa Cells treated with a) Cu-CNP and b) CNP.

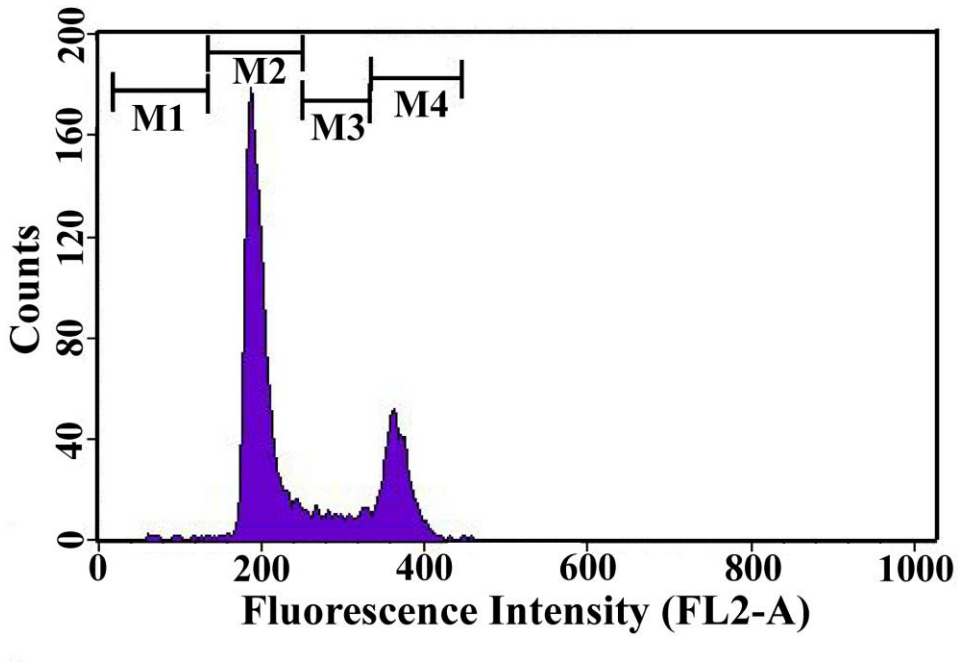

Fig. S10. Cell cycle analysis by FACS of PI-stained HeLa cells (control).

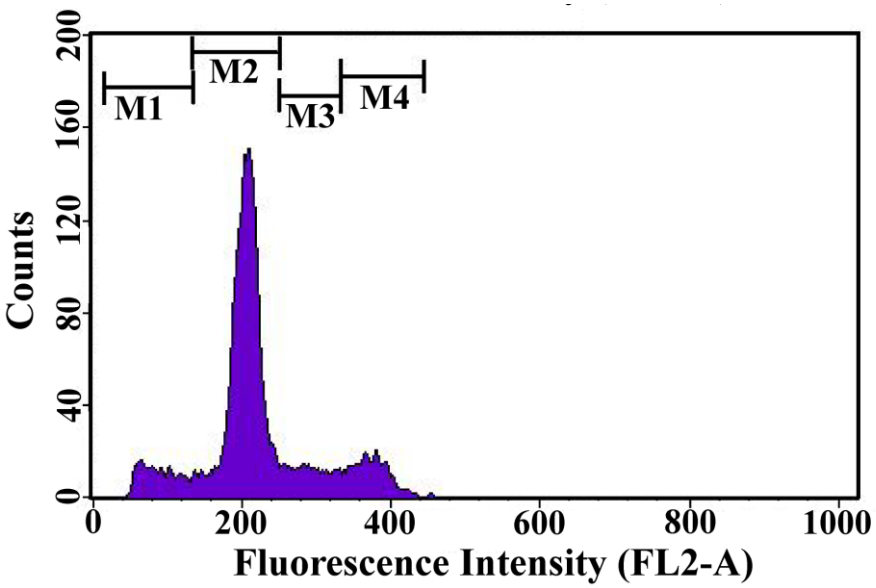

Fig. S11. Cell cycle analysis by FACS of PI-stained HeLa cells treated with Cu-CNP.

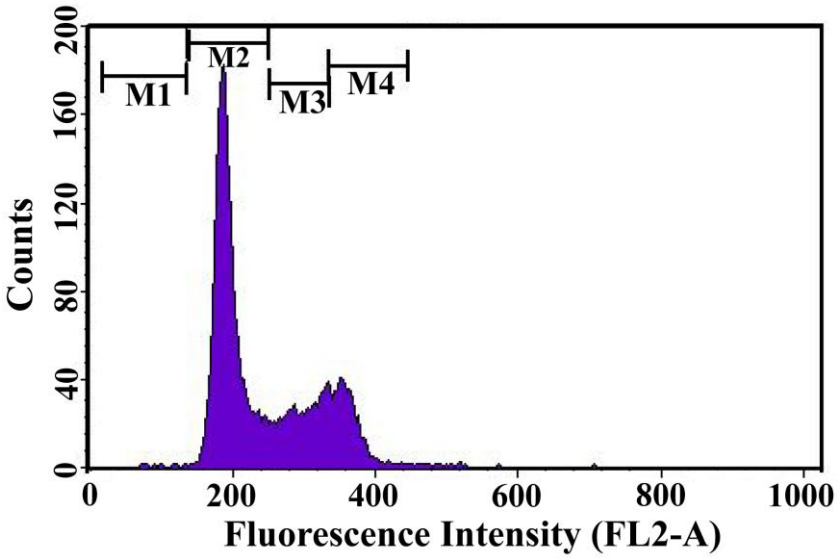

Fig. S12. Cell cycle analysis by FACS of PI-stained HeLa cells treated with CNP.

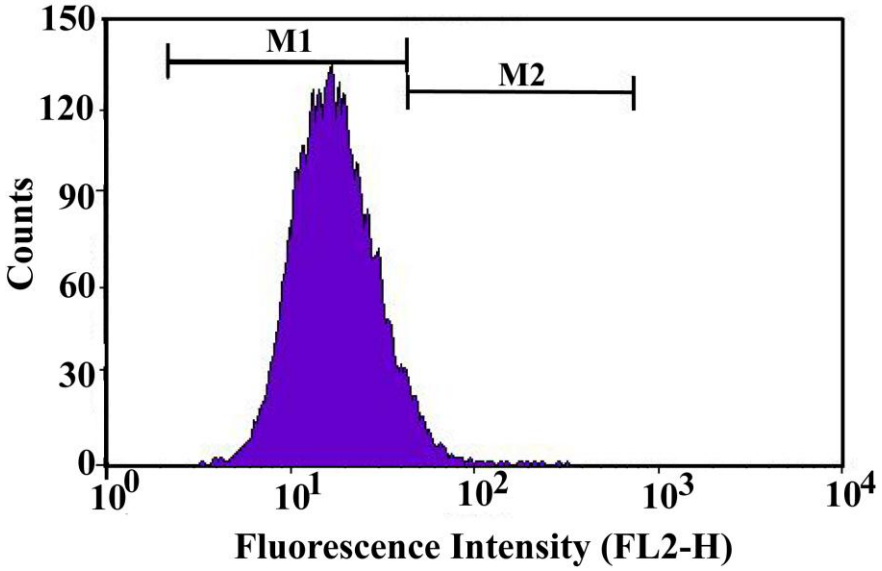

Fig. S13. Caspase 3 assay of control HeLa cells.

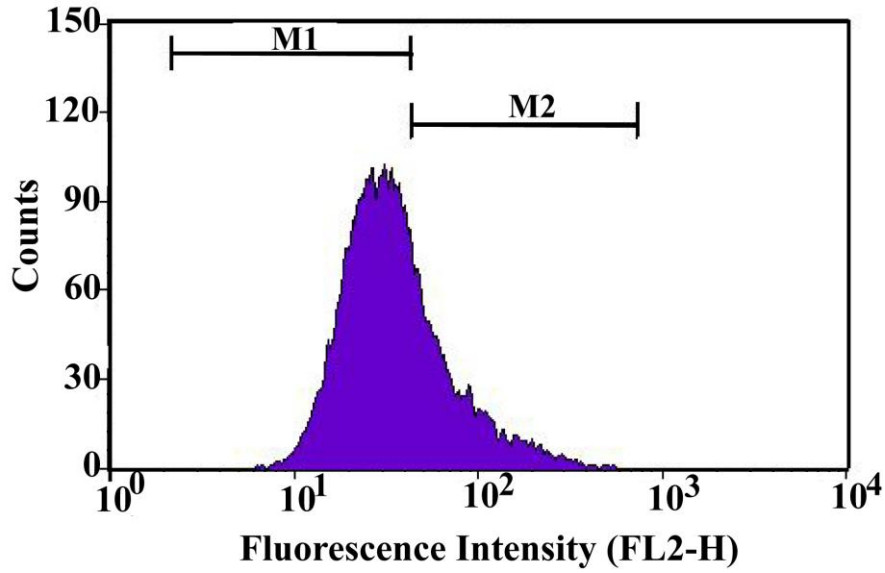

Fig. S14. Caspase 3 assay of Cu-CNP treated HeLa cells.

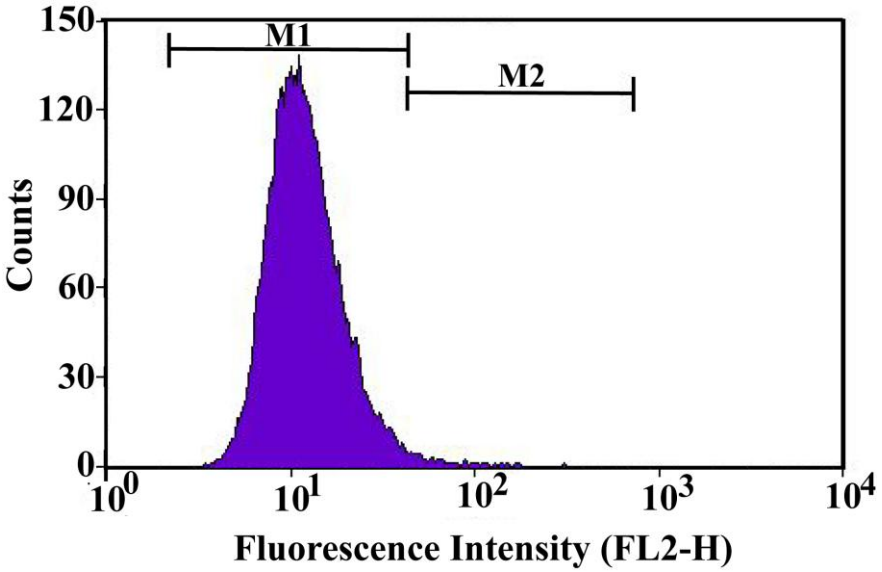

Fig. S15. Caspase 3 assay of CNP treated HeLa cells.

Reference

1. M. P. Sk and A. Chattopadhyay, *RSC Adv*., 2014, **4**, 31994.