Syntheses of Amorphous and Crystalline Cupric Sulfide Nanoparticles and Study on the Specific Activities on Different Cells

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Materials and methods

Preparation of CuS ANPs, NCs and BCs

The preparation of CuS ANPs and NCs were carried out through aqueous chemistry method. In brief, cuprous chloride (CuCl, 0.1 g) was added into double distilled water (30 mL) and stirred violently in the dark for 24 h at 25 ℃ to transform into copper oxychloride. Then the thioacetamide (TAA, 20 mL, Tianjin Chemical Reagent Factory, Tianjin, China) aqueous solution (10 mg/mL) was slowly added into the reaction system and mixed completely. The black solid-state products after reaction for 3 days or 5 days were centrifuged for one hour at 20, 000 g and washed three times with double distilled water and ethanol, respectively. Then the precipitates were dried under vacuum condition at room temperature for 24 h. The products for 3 days are CuS ANPs, while the products for 5 days are CuS NCs. To understand the importance of TAA during the preparation of nanoparticles, the control experiment was performed. CuS BCs were prepared by using the synthetic method of ANPs and NCs in sodium sulfide aqueous solution without adding TAA. All the experiments were performed in triplicate.

Characterization of CuS ANPs, NCs, and BCs

Powder X-ray diffraction (XRD) patterns were recorded on a Bruker D&Advance X-ray powder diffractometer with graphite monochromatized Cu Kα (λ=0.15406nm). A scanning rate of 0.05 deg/s was applied to record the pattern in the 2θ range of 10-70º. To prepare the transmission electron microscope samples (TEM, FEI-Philips Tecnai G220, Philips, Holland), a 5 μl droplet of dilute alcohol solution was dripped onto a holey carbon-coated formvar support. High-resolution

transmission electron microscopy (HRTEM) equipped with selected-area electron diffraction (SAED) was used to confirm the samples size and determine the crystallinity.

Cell culture

HL-60 human acute promyelocytic leukemia cells (ATCC No. CCL-240) and Hep G2 human hepatocellular carcinoma cells (ATCC No. HB-8065) were cultured in RPMI 1640 medium supplemented with heat-inactivated FBS (10%), Penicillin (100 units/mL), Streptomycin (100 μg/mL), amphotericin B (fungizone, 0.25 μg/mL) and sodium bicarbonate (2 mg/mL) in a humidified incubator at fully humidified atmosphere at 37°C, 5% CO_2 and 95% room air. V79-4 Chinese hamster lung cells (ATCC No. CCL-93) were cultured in DMEM medium supplemented with 10% heat-inactivated FBS, Penicillin (100 units/mL), Streptomycin (100 μg/mL), amphotericin B (fungizone, 0.25 μg/mL) and sodium bicarbonate (3.7 mg/mL) in a humidified incubator at fully humidified atmosphere at 37°C , 5% CO_2 and 95% room air.

Treatment of HL-60, Hep G2 and V79-4 Cells with CuS ANPs, NCs, and BCs

For HL-60 cells, fifty microliters of HL-60 cells with initial cell density of 5×10^4 cells/mL were seeded separately in the wells of sterile 96-well flat bottom culture microplates and incubated with fifty microliters of CuS ANPs, NCs, or BCs at final concentrations of 1.5625, 3.125, 6.25, 12.5 25, 50 and 100 μg/mL each for 48 hours in a humidified incubator at fully humidified atmosphere at 37°C, 5% CO_2 and 95% room air. For Hep G2 and V79-4 cells, one hundred microliters of culture media containing cells, Hep G2 or V79-4, with initial cell density of 2.5×10^4 cells/mL were seeded separately in the wells of sterile 96-well flat bottom culture microplates and acclimated for 24 hours. It was then mixed with one hundred microliters of CuS ANPs, NCs, or BCs at concentrations of 1.5625, 3.125, 6.25, 12.5 25, 50 and 100 μg/mL each for 48 hours of incubation in a humidified incubator at fully humidified atmosphere at 37°C, 5% CO_2 and 95% room air. The treatment of the

cells with culture medium rather than CuS was perpared as the control. The cytotoxic effects of CuS ANPs, NCs, and BCs on HL-60, Hep G2 and V79-4 cells were determined by MTT colorimetric assay.

MTT colorimetric assay

For HL-60, Hep G2 and V79-4 cells, after treated with CuS ANPs, NCs, or BCs, freshly prepared MTT (3-(4,5-dimethylthiazol-2-yl)-2,5,diphenyl tetrazolium bromide, 20 μL, 5 mg/mL, Sigma-Aldrich, Co., MO, USA) in filtered PBS was added to each well of the control and the CuS-treated cells and incubated at 37°C, under 5% CO_2 for 5 hours. After the incubation, the medium was carefully removed by centrifuged for 15 min with 300 g. Then, 150 microliters of DMSO were added to each well to dissolve the dark blue crystals completely. The absorbance of the solution in each well at the wavelength 570 nm was measured by a microplate reader. The extent of cell proliferation was reflected by the average value of absorbance while the cytotoxic effects of CuS ANPs, NCs, and BCs were calculated.

Flow cytometric analysis of apoptosis

HL-60 cells with the cell density of 1×10^6 cells/mL and CuS ANPs or NCs were incubated together in culture medium for 48 hours in a humidified incubator at fully humidified atmosphere at 37°C, 5% CO_2 and 95% room air. Hep G2 cells with the cell density of 5×10^5 cells/mL were seeded in culture medium and acclimated for 24 hours. The cells were then incubated with CuS ANPs or NCs for 48 hours in a humidified incubator at fully humidified atmosphere at 37°C, 5% CO_2 and 95% room air. Cells without addition of CuS ANPs or NCs were prepared as the control. After treatment, Hep G2 cells were trypsinized with trypsin-EDTA solution (0.25%) firstly. All HL-60 cells and Hep G2 cells were collected and pelleted by centrifugation at 400g and 10°C for 5 minutes. The cell pellets were washed with cold PBS, fixed with ethanol (70%, 3 mL) precooled to

-20°C, and incubated at -20°C for at least 1 hr. The fixed cells were centrifuged and washed with cold PBS. The cells were stained with propidium iodide (PI, 10 μg/mL in PBS, Sigma-Aldrich, Co., MO, USA) at 37°C in the dark for 30 minutes. The stained cells were filtered through 41 μm Nylon net filters and analyzed by a COULTER Epics XL flow cytometry system (Beckman Coulter, Inc., CA, USA) in sextuplicate and the percentages of sub G1, G1, G2, and S phases were recorded.

Measurement of apoptosis using fluorescent dyes and fluorescence microscopy

HL-60 cells or pre-seeded Hep G2 cells were incubated together with CuS ANPs or NCs in culture medium in a 96-well round bottom microplate for 48 hours (sextuplicate) in a humidified incubator at fully humidified atmosphere at 37°C , 5% CO_2 and 95% room air. The cancer cells without adding CuS ANPs or NCs were prepared as the control. After the treatment, cell mixtures were pelleted by centrifugation (200g, 10 min) and the supernatant were carefully removed except for approx 25–30 μL. Then the cell pellet was resuspended and Hoechst 33342 (20 μg/mL in PBS) + PI (10 μg/mL in PBS) staining solution (2 μL) were added. Ten microlitres of this mixture were placed on clean microscope slides and were covered with a coverslips. The slides were examined with different objectives (40× to 60×) using the epiilumination and a filter combination at 340-380 nm.

Transmission electron microscopy of cells

HL-60 and Hep G2 cells were seeded on culture flasks and treated with CuS ANPs or NCs for 48 hours. After treatment, cells were collected, fixed with glutaraldehyde (2.5%) and washed three times with PBS. Subsequently, post-fixation with osmium tetroxide (1%) was performed followed by washed with PBS, dehydration with ascending series of alcohol before embedding in Spurr. Ultrathin sections with 70 nm were cut by Leica Reichert ultracut and doubly stained with uranyl acetate and lead citrate. Images were acquired using transmission electron microscope.

Data processing and statistical analysis

All the data in this study were analyzed by one-way ANOVA together with Fisher LSD test using OriginPro 8 SR1 V8.0773 (OriginLab Corporation, MA, USA). IC_{50} values were obtained by fitting dose-response curves with the Hill equation.

Results

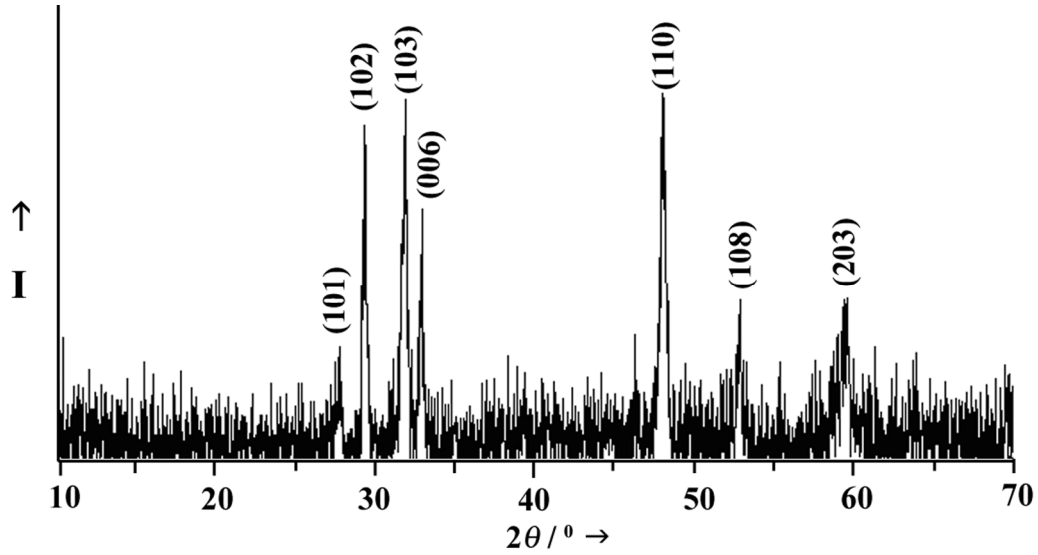

Fig. 1 X-ray diffraction patterns and SAED images of CuS bulk crystals (BCs) prepared in control system.

Fig. 1 shows the XRD pattern of BCs. The XRD patterns show characteristic diffraction peaks, which is consistent with that of the 06-0464 CuS. This shows that the BCs are crystals.

To determine the nanoparticle size distribution and the mean particle diameter quantitatively, the particle size of CuS ANPs and NCs were fitted with lognormal distribution function for P(R) whose expression is shown in equation 3, where R^0 denotes the mean particle radius of the distribution and σ is the lognormal dispersion or width in size. From the results shown in Fig. 2, CuS ANPs and NCs both exhibit the narrow particle size distribution, with the average particle diameters are 50 nm and 60 nm，respectively. This indicates that ANPs is smaller than NCs.

$$P(R) = \exp\left[-\frac{1}{2}\frac{\left(\ln\frac{R}{R^0}\right)^2}{\sigma^2}\right]\frac{\exp\left[-\frac{1}{2}\sigma^2\right]}{R\sigma\sqrt{2\pi}} \qquad (3)$$

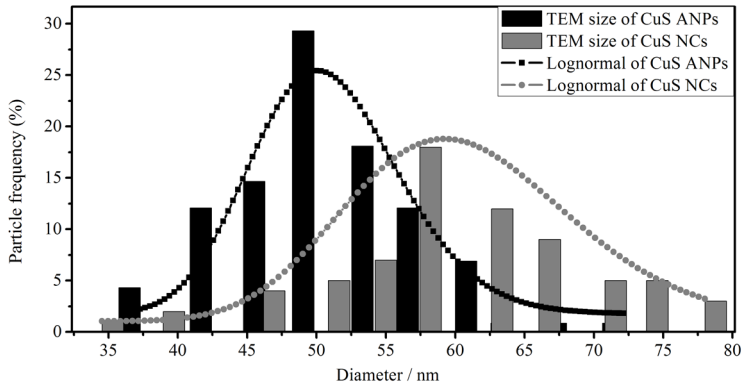

Fig. 2 Particle size distribution of (a) CuS ANPs and (b) NCs fitted by lognormal functions.

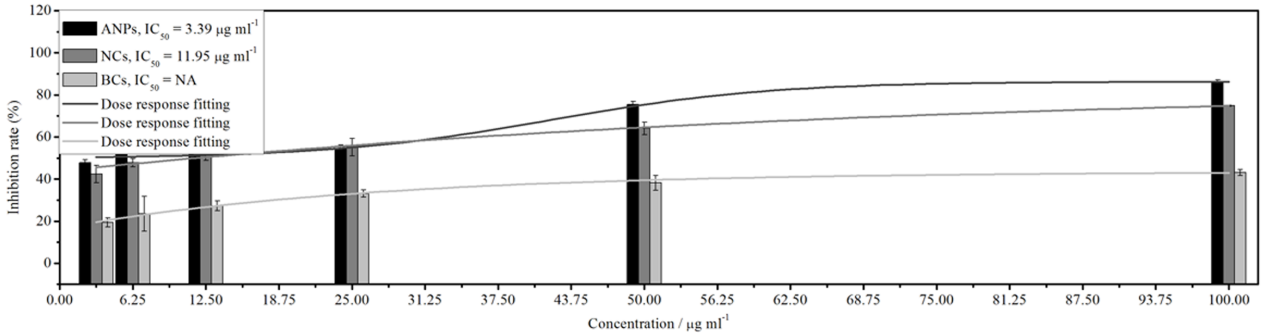

Fig.3 Anti-proliferation effects of CuS ANPs, NCs, and BCs on Hep G2 cells. Each bar represents the mean ± S.D.. NA: not available.

In this work, the anti-proliferation effects of CuS ANPs, NCs and BCs on HL-60 cancer cells, Hep G2 cancer cells and V79-4 normal cells were determined through MTT colorimetric assay. In this assay, the lower the IC_{50} value, the stronger the inhibition effect. The results of the samples on Hep G2 cancer cells are shown in Fig. 3. From Fig. 3, the IC_{50} values of CuS ANPs and NCs on Hep G2 cells are 3.39 μg/mL and 11.95 μg/mL, indicating that CuS ANPs and NCs can significantly inhibit the proliferation of Hep G2 cancer cells. In addition, the IC_{50} of CuS BCs on

Hep G2 cancer cells is not available, showing that CuS BCs just inhibit the proliferation of Hep G2 cancer cells slightly.

In addition, the cell densities were also monitored through light microscope to further determine the anti-proliferation effects of ANPs and NCs. Fig. 4a, b, and c present the cell densities of Hep G2 cells of control system, treated with ANPs, and NCs, respectively. From the Fig.s, compared with the control, after treated with ANPs and NCs, the cell densities decrease significantly. This further indicates the anti-proliferation effects of ANPs and NCs. These results reveal that CuS ANPs and NCs can specifically and significantly inhibit the proliferation of cancer cells rather than normal cells.

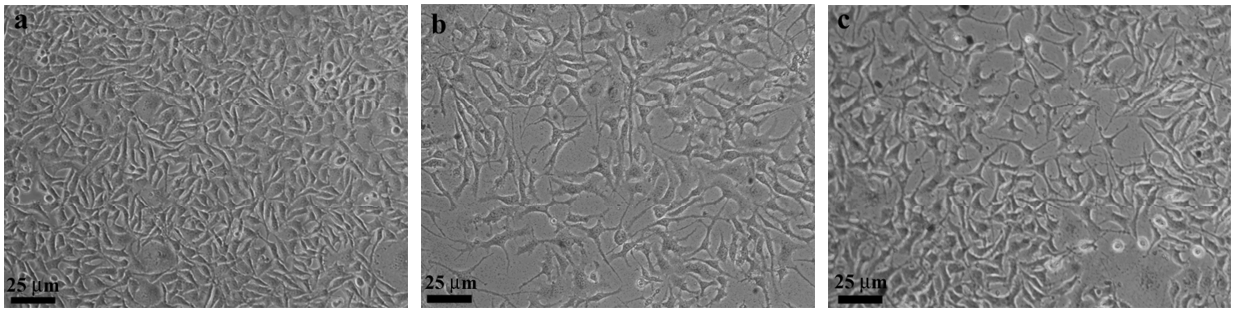

Fig. 4 Light microscopy images of Hep G2 cells of (a) control, (b) treated with 25 μg/mL of CuS ANPs for 48 hours, (c) treated with 25 μg/mL of CuS NCs for 48 hours

Fig. 5a and b show the transmission electron microscopy (TEM) images of HL-60 cells and Hep G2 cells treated with 25 μg/mL of CuS ANPs. Fig. 5a shows the presence of CuS ANPs in the cytosol, vesicles and around the vesicular membrane of HL-60 cells. Fig. 5b indicates the presence of CuS ANPs in the cytosol and mitochondria of Hep G2 cells. The locations of CuS NCs are the same as those of the ANPs. These TEM results indicate that CuS ANPs can enter into the cancer cells through the membrane and locate to different organelles of the cancer cells.

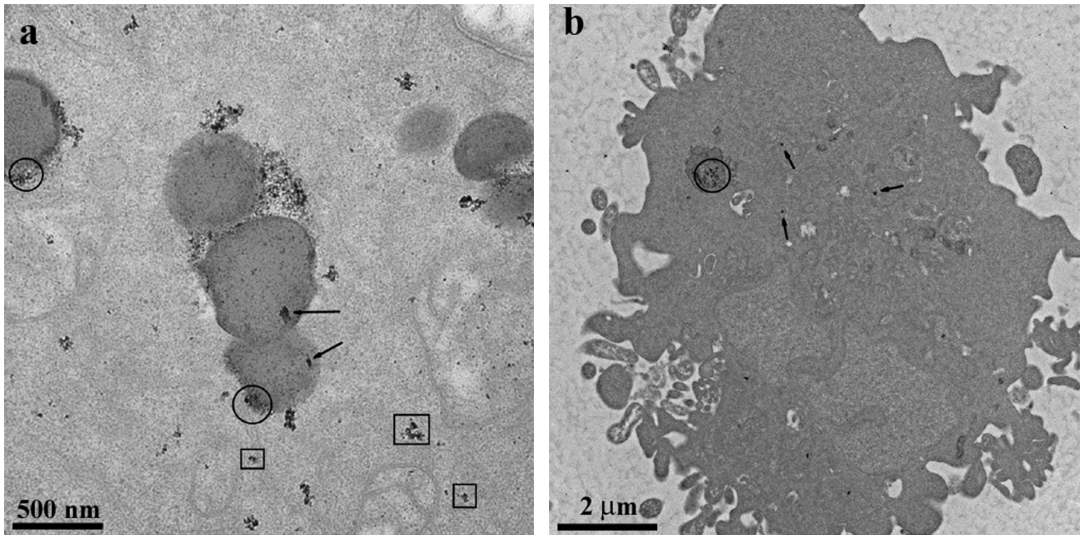

Fig. 5 Transmission electron microscopy images of (a) HL-60 cells treated with 25 μg/mL of CuS ANPs for 48 hours showing the presence of CuS nanoparticle clusters in cytosol (rectangles), vesicles (arrows) and the vesicular membrane (circle) and (b) Hep G2 cells treated with 25 μg/mL of CuS ANPs for 48 hours showing the presence of CuS nanoparticle clusters in cytosol (arrows) and mitochondria (circle).

Fig. 6 a, b, and c present DNA content frequency histograms of HL-60 cells of the control, treated with CuS ANPs, and NCs, respectively. From the Fig.6, compared with the control, after treated with ANPs and NC, the percentages of Sub G1 phases of HL-60 cells increase significantly，revealing that both of them can induce the apoptosis of HL-60 cells significantly. Fig. 6d, f, and f present DNA content frequency histograms of Hep G2 cells of the control, treated with CuS ANPs, and NCs, respectively. From the Fig.s, compared with the control, after treated with ANPs and NC, the percentages of Sub G1 phases of Hep G2 cells increase significantly, showing that both of them can induce the apoptosis of Hep G2 cells significantly. In addition, apoptosis-inducing effects of ANPs and NCs on Hep G2 cancer cells were compared through statistical analysis. The results shown in Fig. 6g reveal that the apoptosis-inducing effects of CuS ANPs are significantly stronger than those of NCs, which is consistent with the anti-proliferation effects.

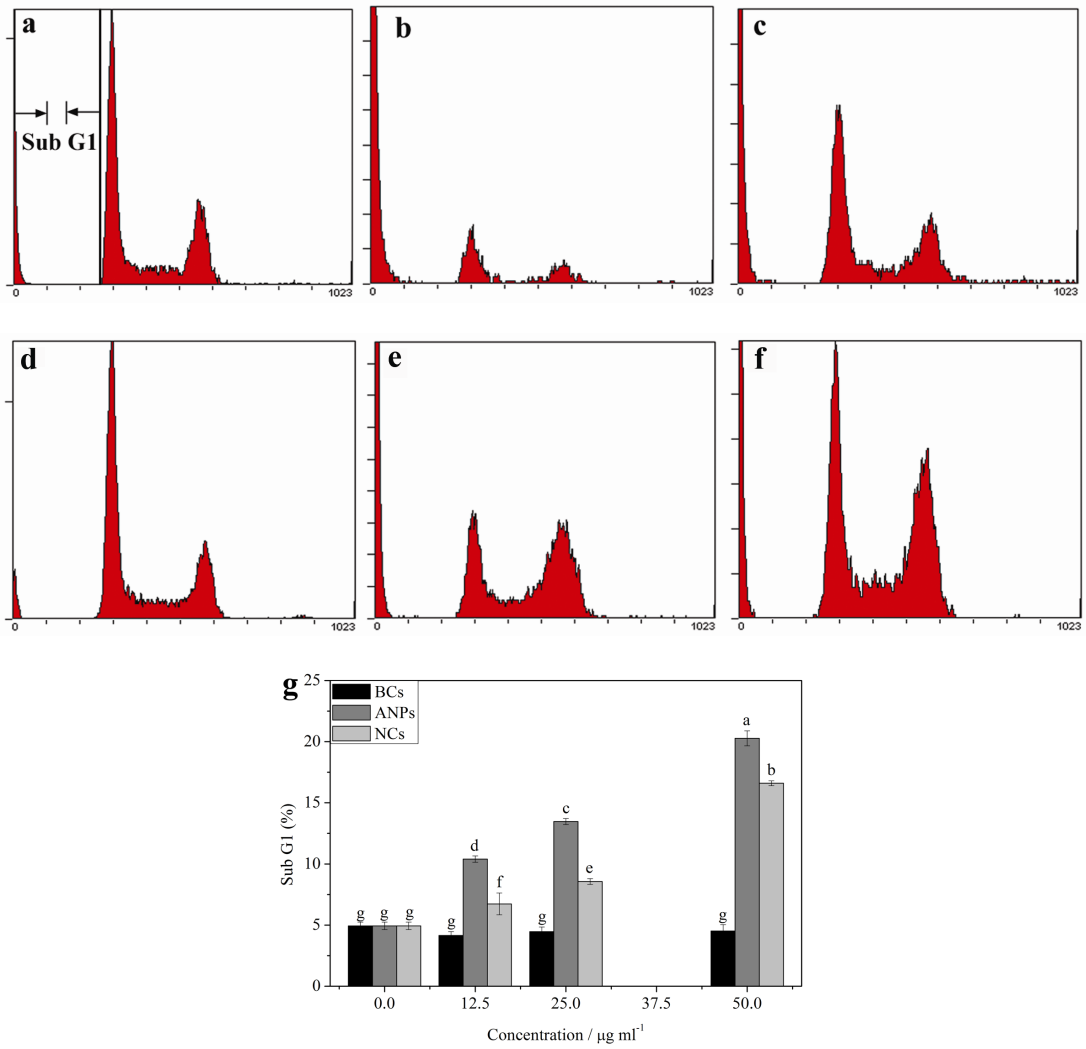

Fig. 6 DNA fluorescence flow cytometric profiles of PI-stained HL-60 cells (a) control, (b) 25 μg/mL CuS ANPs, (c) 25 μg/mL CuS NCs, and DNA fluorescence flow cytometric profiles of PI-stained Hep G2 cells (d) control, (e) 25 μg/mL CuS ANPs, (f) 25 μg/mL CuS NCs, and (g) effects of CuS ANPs and NCs on the apoptosis of Hep G2 cells. Each bar represents the mean ± S.D.. Asterisk means significant difference (T-test, $p \leq 0.05$), n = 6.

Based on the morphological features of the apoptotic cells, cells with blue chromatin and organized structure are live cells with normal nuclei (LN), cells with blue chromatin that is highly condensed or fragmented are the live cells with apoptotic nuclei (LA), cells with pink chromatin and organized structure are dead cells with normal nuclei (DN), and cells with pink chromatin that

is highly condensed or fragmented are the dead cells with apoptotic nuclei (DA). Fig. 7 a, b, and c show the fluorescent microscopy images of HL-60 cells of the control, treated with CuS ANPs, and NCs, respectively. From the figures, compared with the control, after treated with ANPs and NCs, the chromatin of HL-60 highly condensed, even changed into the featureless, bright spherical beads. Fig. 7 d, e, and f show the fluorescent microscopy images of Hep G2 cells of the control, treated with CuS ANPs, and NCs, respectively. From the figures, similar to HL-60 cells, after treated with ANPs and NCs, the chromatin of Hep G2 also highly condensed, even changed into the featureless, bright spherical beads. This result further confirms that CuS ANPs and NCs can induce the apoptosis of HL-60 and Hep G2 cancer cells significantly.

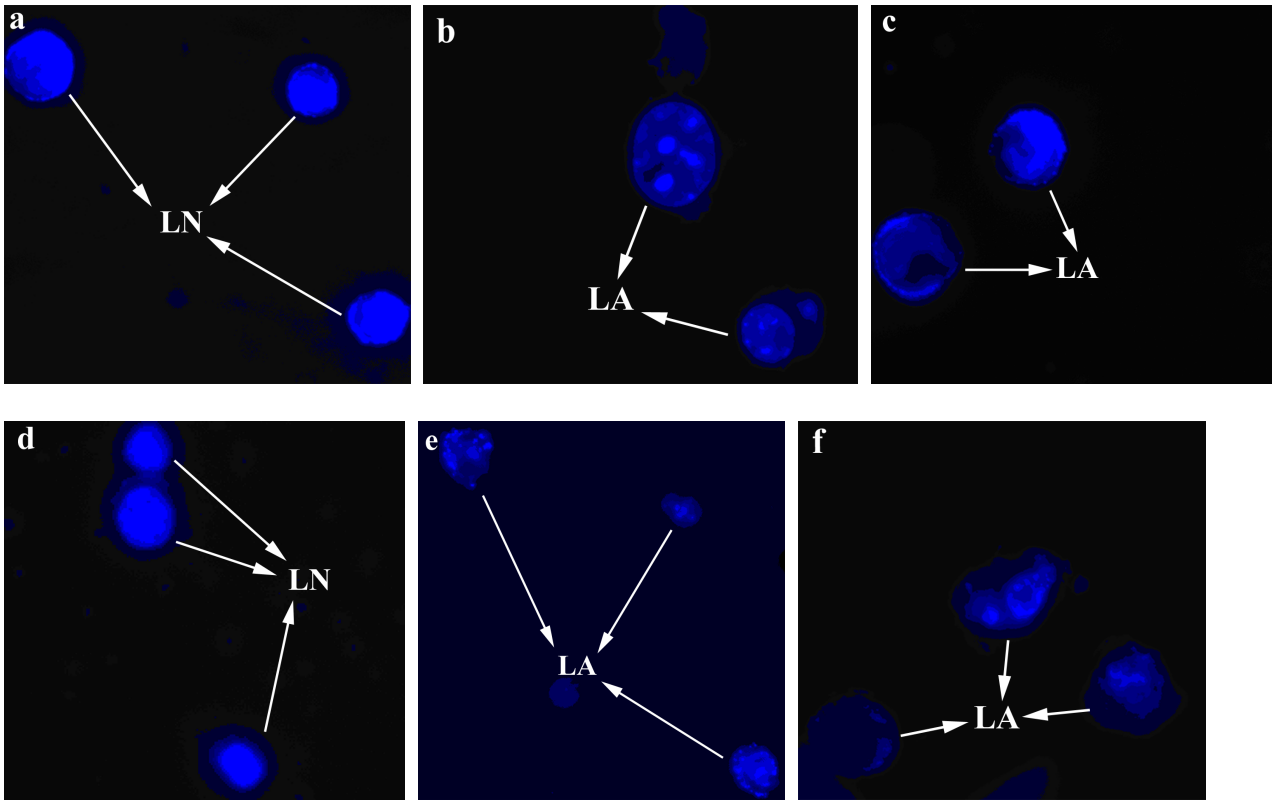

Fig. 7 Fluorescent microscopy images of HL-60 cells (a) control, (b) treated by 25 μg/mL CuS ANPs for 48 hours, (c) treated by 25 μg/mL CuS NCs for 48 hours, and fluorescent microscopy images of Hep G2 cells (d) control, (e) treated by 25 μg/mL CuS ANPs for 48 hours, (f) treated by 25 μg/mL CuS NCs for 48 hours.