# Syntheses of amorphous and crystalline cupric sulfide nanoparticles and study on the specific activities on different cells†

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*Received 27th January 2010, Accepted 10th March 2010*
*First published as an Advance Article on the web 7th April 2010*
**DOI: 10.1039/c001714k**

**Copper sulfide amorphous nanoparticles and nanocrystals were prepared successfully by a special process. These CuS nanoparticles could specifically and significantly induce the apoptosis and inhibit the proliferation of human cancer cells rather than normal cells. Moreover, the biological activities of these nanoparticles are related to their polymorphs.**

Recently, with the development of nanoscience and nanotechnology, the potential application of inorganic nanomaterials in life sciences has become increasingly extensive, such as the fluorescent biological labeling of cells,[1–3] drug and gene delivery,[4,5] biological detection of pathogens and proteins,[6] and probing of DNA structure.[7] This leads to considerable concerns about the biological effects of the nanoparticles, especially on cells and proteins.[8–12] Some results expand the understanding of the effects of nanoparticles on cells. For example, after treatment with inorganic nanoparticles, such as CdSe, ceria, titania, and zirconia, the viabilities of normal cells and cancer cells decreased significantly.[8,9] Our previous study demonstrated that silver sulfide nanocrystals could inhibit the viability of tumor cells.[13] However, most of these results showed that the inhibition effects of the inorganic nanoparticles on cells were unspecific. Hitherto, the specific effects of inorganic nanomaterials on cells, especially the relationship between the biological activities and the polymorphs, have scarcely been reported. In addition, from biomineralization studies, amorphous nanoparticles can be formed through biological mineralization, which are stabilized by organisms and play crucial and specific roles. However, amorphous nanoparticles are thermodynamically metastable *in vitro* and tend to transform into the more stable crystalline form. Therefore, the successful *in vitro* preparation of amorphous nanoparticles and the understanding of their properties are difficult and limited. Reports about properties and biological effects of the amorphous nanoparticles are few. In our current study, copper sulfide (CuS) amorphous nanoparticles (ANPs) and nanocrystals (NCs) were prepared successfully by a special process. For comparison, bulk crystals (BCs) were also prepared. Biological assays indicated that CuS ANPs and NCs could significantly inhibit the proliferation of cancer cells rather than normal cells. Moreover, CuS ANPs and NCs could significantly induce the apoptosis of cancer cells. However the BCs did not show the anti-proliferation and apoptosis-inducing activities. Furthermore, the anti-proliferation and apoptosis-inducing activities of ANPs were significantly stronger than those of NCs.

The preparations of CuS ANPs and NCs were carried out through a special process. In brief, cuprous chloride (CuCl) was added into double distilled water and stirred violently in the dark to transform into copper oxychloride. Then thioacetamide (TAA) aqueous solution was slowly added into the reaction system. The black solid-state products after reaction for 3 days or 5 days were collected and dried under vacuum. The products for 3 days are CuS ANPs, while the products for 5 days are CuS NCs. For comparison, CuS BCs were also prepared under the same conditions in sodium sulfide aqueous solution without adding TAA. Also, to determine the importance of the slow release of the cupric ions from the freshly prepared copper oxychloride, cupric salts such as $CuCl_2$ and $CuSO_4$ were used as the copper sources to perform the experiments. In addition, the anti-proliferation activities and apoptosis-inducing activities of the samples on Hep G2 human hepatocellular carcinoma cells, HL-60 human leukemia cells, and V79-4 hamster normal cells were determined through MTT assay, flow cytometric analysis and fluorescent staining analysis.

Fig. 1 shows the XRD spectra and SAED images of CuS ANPs and NCs, respectively. The absence of any diffraction peaks in the XRD spectrum shown in Fig. 1a indicates the samples of 3 days are amorphous. The existence of diffraction rings rather than spots in the SAED image further reveals the amorphous state. Diffraction peaks of the XRD patterns and the typical diffraction spots in the SAED image shown in Fig. 1b reveal that the samples of 5 days are crystals. The XRD patterns of the BCs are similar to NCs (See ESI†), indicating the BCs are crystals. These results show that the slow release of sulfur ions from the hydrolysis of TAA is important to the formation of nanoparticles. In addition, when cupric salts such as $CuCl_2$ and $CuSO_4$ rather than CuCl were used as the copper source, the products were neither ANPs nor NCs, but bulk crystals, showing that the slow release of Cu(II) from the freshly prepared copper oxychloride is also important to the formation of nanoparticles. These results reveal that amorphous nanoparticles and nanocrystals can be successfully prepared through the adjustment of the experimental conditions. Moreover, according to the XRD monitoring result, the ANPs can remain in the amorphous state for at least six months in the ambient environment,

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† Electronic supplementary information (ESI) available: Experimental details. See DOI: 10.1039/c001714k

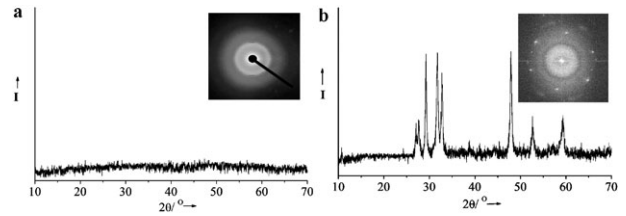

**Fig. 1** X-ray diffraction patterns and SAED images of CuS particles with different size and crystal forms. (a) ANPs prepared in TAA solution for 3 days, inset, SAED; (b) NCs prepared in TAA solution for 5 days, inset, SAED.

showing that the ANPs are comparatively stable in the ambient environment.

Fig. 2 presents the TEM images of CuS BCs, ANPs, and NCs, respectively. From Fig. 2a, the CuS BCs prepared from sodium sulfide exhibit irregular bulk morphology and inhomogeneous size distribution, ranging from 500 nm to several microns. However, the CuS ANPs prepared from TAA have a flake-like structure and are obviously well dispersed (Fig. 2b). The CuS NCs show similar morphology and dispersity to the CuS ANPs (Fig. 2c). Based on size distribution analysis through a lognormal distribution function, CuS ANPs and NCs both exhibit a narrow particle size distribution, and the average particle diameters are 50 nm and 60 nm, respectively, indicating that the ANPs are smaller than the NCs (See ESI†).

Moreover, the anti-proliferation activities of CuS ANPs, NCs, and BCs on three different cells were determined through MTT assay. From the results, the $IC_{50}$ values of CuS ANPs on HL-60 cells and Hep G2 cells are 18.53 μg $mL^{-1}$ and 3.39 μg $mL^{-1}$, respectively (Fig. 3a and ESI†). The $IC_{50}$ values of CuS NCs on HL-60 cells and Hep G2 cells are 29.26 μg $mL^{-1}$ and 11.95 μg $mL^{-1}$, respectively (Fig. 3a and and ESI†). This indicates that CuS ANPs and NCs can significantly inhibit the proliferation of the cancer cells. Based on the $IC_{50}$ values, the anti-proliferation activities of CuS ANPs are significantly stronger than those of CuS NCs. However, the $IC_{50}$ of BCs is not available, showing that the BCs just slightly inhibit the proliferation of these two cancer cells. In addition, the $IC_{50}$ values of CuS ANPs, NCs, and BCs on V79-4 normal cells are not available (Fig. 3b), revealing that these samples just inhibit the proliferation of V79-4 normal cells slightly. The cell densities monitored through a light microscope further confirm the anti-proliferation activities of ANPs and NCs on two human cancer cells (See ESI†). Thermodynamically, the energy of amorphous nanoparticles is higher than that of nanocrystals and metastable,[14,15] therefore the amorphous nanoparticles should be more active and

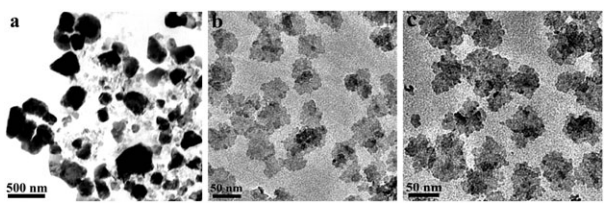

**Fig. 2** TEM images of (a) CuS bulk crystals, (b) amorphous nanoparticles, and (c) nanocrystals.

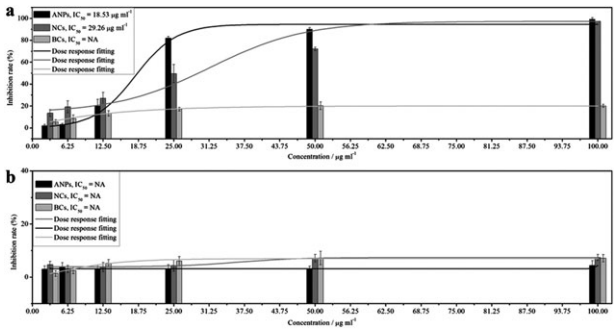

**Fig. 3** Anti-proliferation effects of CuS ANPs, NCs, and BCs on (a) HL-60 cells, (b) V79-4 cells. Each bar represents the mean ± S.D. NA: not available.

comparatively soluble than nanocrystals and might have much more active sites on the particle surface. This might be the reason for the stronger effects of CuS ANPs.

Because CuS ANPs and NCs could inhibit the proliferation of HL-60 and Hep G2 cells significantly, their effects on the apoptosis of these two kinds of human cancer cells were determined through flow cytometric analysis and fluorescent staining analysis. From the results, compared with the control without treatment with samples, after treatment with ANPs and NCs, 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. 4a). Similarly, after treatment with ANPs and NCs, the percentages of Sub G1 phases of Hep G2 cells increase significantly (See ESI†), showing that both of them can also induce the apoptosis of Hep G2 cells significantly. Furthermore, apoptosis-inducing activities of ANPs and NCs on the two human cancer cells were compared through statistical analysis. The results reveal that the apoptosis-inducing activities of CuS ANPs are significantly stronger than those of NCs (Fig. 4a and ESI†), which is consistent with the anti-proliferation activities. In addition, the apoptosis-inducing activities of ANPs and NCs were also determined through fluorescent staining analysis. From the results shown in Fig. 4b, after treatment with ANPs, the chromatin of HL-60 became highly condensed, even changed into featureless, bright spherical beads, further revealing that ANPs can induce the apoptosis of HL-60 cancer cells significantly. Similarly, NCs can also induce the apoptosis of HL-60. In addition, ANPs and NCs also show significant apoptosis-inducing activities on Hep G2 (See ESI†). However, the BCs do not show apoptosis-inducing activity on HL-60 or Hep G2 cancer cells.

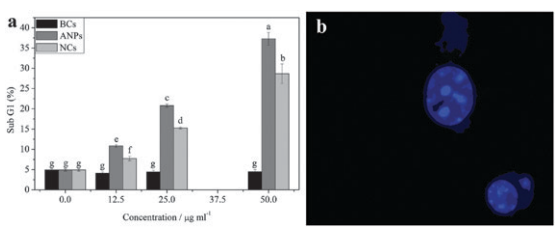

**Fig. 4** Effects of CuS ANPs and NCs on the apoptosis of HL-60 human cancer cells. (a) Flow cytometric analysis, each bar represents the mean ± S.D. Different letter means significant difference (ANOVA, $p \leq 0.05$), $n = 6$. (b) Fluorescent staining analysis.

The distributions of ANPs and NCs in HL-60 and Hep G2 cells were monitored by TEM. From the results, these CuS nanoparticles could enter into HL-60 and Hep G2 cancer cells and be located in the vesicles of HL-60 cells and the mitochondria of Hep G2 cells, respectively (See ESI†).

Based on the TEM results, the mechanisms of the apoptosis-inducing activities of the samples are hypothesized. For HL-60 cells, the CuS ANPs and NCs located in the vesicles might alter the calcium channels on the vesicular membrane. The alteration of the calcium channels further results in a decrease in capacitative calcium entry. This decrease in the capacitative calcium entry might lead to the apoptosis of HL-60 cells. For Hep G2 cells, CuS ANPs in the mitochondria might interact with the apoptotic-related proteins, *e.g.* Bcl-2 families, in the mitochondria. These regulating effects might stimulate a series of apoptosis processes, and lead the cells to apoptosis.

The anti-proliferation activities and apoptosis-inducing activities of CuS ANPs and NCs on two human cancer cells suggest the possible medicinal use of the inorganic nanomaterials in cancer treatment. Therefore, tumor treatment in *in vivo* models by a local injection method should be examined, and also the mechanisms of the activities of the CuS ANPs and NCs on cancer cells need to be studied in detail.

To conclude, this paper provides a special method to prepare bioactive CuS amorphous nanoparticles and nanocrystals. Biological assays reveal that the amorphous nanoparticles and nanocrystals can enter into the cancer cells, locate themselves in the different organelles of the different cancer cells, and exhibit different anti-proliferation and apoptosis-inducing activities. However, the bulk crystals do not show the biological activities. This work was financially supported by the National Science Foundation of China (Grant No. 20771036) and the National Key Basic Research and Development Program of China (Grant No. 2009CB626610, 2005CB724306).

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