Research Square

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Trigger of Apoptosis in Adenocarcinoma Gastric Cell Line (AGS) by a Complex of Thiosemicarbazone and Copper Nanoparticles

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Abstract

Seeking novel anticancer agents with minimal side effects against gastric cancer is vitally important. Copper, as an important trace element, takes roles in different physiologic pathways. Also, there is a higher demand for copper in cancer cells than normal ones. Copper complexes containing a therapeutic ligand could be promising candidates for gastric cancer chemotherapy. In this work, copper oxide nanoparticles were synthesized, functionalized with glutamic acid (CuO@Glu) and conjugated with thiosemicarbazone (Cuo@Glu/TSC NPs). The NPs were characterized and their antiproliferative potential against AGS cancer cells was investigated using MTT, flow cytometry, Hoechst staining, and caspase 3 activation assays. The FT-IR results showed the proper binding of TSC to CuO@Glu NPs and crystallinity of the prepared NPs was confirmed by the XRD pattern. The EDX analysis confirmed the presence of Cu, N, C, O, and S elements and lack of impurities. The Hydrodynamic size and zeta potential of the Cuo@Glu/TSC NPs were 710 nm and 27.5 mv, respectively. The NPs had spherical shape and were in a size range of 20-39 nm in diameter. This work revealed that CuO@Glu/TSC NPs efficiently inhibited the proliferation of AGS cells with significantly lower IC50 value (203 µg/mL) than normal HEK293 cells (IC50=435µg/mL). Flow cytometry and Hoechst staining obviously revealed apoptosis induction among CuO@Glu/TSC treated cells, and caspase-3 activity significantly increased by 1.4 folds. This study introduced CuO@Glu/TSC as an efficient anticancer against gastric cancer cells with lower toxicity toward normal cells which could be employed for cancer treatment after further characterization.

Introduction

Gastric cancer is a prevalent and deadly cancer, with about one million annual new cases, in the world [1]. The disease is highly prevalent around the world especially in eastern and central Asian countries. Gastric cancer causes 783.000 deaths annually, which accounts for 8.3 % of cancer-associated death [1]. Traditional cancer therapy including chemotherapy, radiotherapy, and surgery are expensive, not always efficient and have sometimes considerable side effects [2]. Therefore, finding novel and inexpensive anticancer agents with minimal side effects is crucially important to treat gastric cancer.

Upon introduction of cisplatin, as a novel and efficient anticancer agent, many efforts have been focused on finding metal-containing drugs [3]. Among metal elements, copper complexes have gained more attention due to their fewer side effects and toxicity. Copper is considered an important trace element in the body which is important for the proper activity of cellular proteins and enzymes [4]. Owing to the higher proliferation rate and physiological activity, there is a higher demand for copper in cancer cells in comparison with normal ones [5]. Therefore, due to the increased copper uptake by cancer cells, the preparation of antiproliferative copper complexes might provide selective toxicity against cancer cells.

Several copper complexes with anticancer activity have been reported [3, 6-7]. Among them, the complexes containing therapeutic ligands such as thiosemicarbazones (TSC) have been introduced as novel and efficient anticancer agents [3]. Thiosemicarbazide is able to interact with a variety of metals and to enhance their chemical activity. The anticancer potential metal NPs in conjugation with

thiosemicarbazide was reported in the literature. Owing to the versatile coordination to metal ions and also, broad pharmaceutical potentials, including antibacterial, antiviral, and anti-tumor activities, TSC derivatives have been explored to prepare metal-TSC complexes, to be used as pharmaceutical agents [3, 6-7]. Several Copper/TSC complexes have been evaluated against a variety of cancer cells including colon adenocarcinoma HT-29, breast cancer cells, lymphoblastic leukemia, and cervical carcinoma [3, 6].

Many attempts have been performed to improve the hydrophobicity, anticancer potential, and stability of Copper/TSC complexes and also to minimize their side effects. The stable Copper/TSC complexes are able to reach the desired targets more efficiently and avoid nonselective interactions of the drug with different parts of cells [3]. Modification of TSCs framework to reduce the toxicity of Copper/TSC complexes toward normal cells was the goal of many studies [3, 7-8]. Therefore, in this work we aimed to synthesize copper oxide NPs, functionalize them with glutamic acid (CuO@Glu), and conjugate with TSC (CuO@Glu/TSC NPs). Then, the anticancer effect of CuO@Glu/TSC NPs on AGS cells was evaluated.

Materials And Methods

Preparation of CuO and CuO@Glu/TSC NPs

Preparation of CuO NPs was performed by co-precipitation method, as described elsewhere [9]. Briefly, 300 mg of $CuCl_2.2H_2O$ was dissolved in 300 mL of distilled water; the pH was adjusted to 11 and the mixture was stirred at 80 °C for two hours. Next, the CuO NPs were centrifuged, washed with distilled water, and dried at 70 °C. A similar protocole was used for the synthesis of Cuo@Glu, but 150 mg of L-glutamic acid was also added to the reaction mixture.

To synthesize CuO@Glu/TSC, 300 mg of CuO@Glu and 200 mg of thiosemicarbazone were dissolved in 300 ml of ethanol and stirred at 40 °C for one hour. Then, the mixture was centrifuged; and the resulting NPs were harvested, washed with distilled water, and dried at 70 °C.

Characterization of CuO@Glu/TSC NPs

X-ray diffraction (XRD) pattern of the CuO@Glu/TSC NPs was determined by a Philips X'Pert MPD diffractometer (Co-Ka X-radiation, k = 1.79 Å), and dynamic light scattering (DLS), and Zeta potential analyses were used to characterize the stability and agglomeration state of NPs using a Zeta-sizer instrument (Ver. 6.32, Malvern Instruments Ltd). Also, the morphology, distribution, and size of the prepared CuO@Glu/TSC NPs were determined by scanning electron microscopy (SEM) (KYKY-EM3200) and transmission electron microscopy (TEM) (EM10C). Moreover, to analyze the composition of NPs was energy dispersive X-Ray (EDX) was employed using an X-ray diffraction spectrometer (Delta series KEVEX). Also, a Nicolet IR 100 spectrophotometer was employed to analyze the Fourier-transform infrared (FT–IR) spectra of the NPs.

Cell culture

The adenocarcinoma gastric (AGS) and the human embryonic kidney 293 (HEK293) cells were received from the Pasteur Institute of Iran. The cell culture was performed in 25 cm^2 cell culture flasks using the Dulbecco's modified Eagle (DMEM) medium supplemented with penicillin/streptomycin (100 U/mL) and 100% fetal bovine serum (FBS) (Gibco, Netherland). To prepare cell monolayers, the incubation was performed at 37 °C with 5 % CO_2.

Evaluation of the viability of cells treated with CuO@Glu/TSC NPs

The antiproliferative potential of CuO@Glu/TSC NPs against the AGS (cancer cell line) and HEK293 cells (as normal human cells) was investigated using the 2-(4,5-dimethythiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT) assay. Cisplatin was also used as a control drug against AGS cells. The assay was performed in triplicates.

At first, the cell monolayers with a cell population of 1×10^4 cell/well were prepared in microtiter plates. The cells were exposed to CuO@Glu/TSC NPs at different concentrations in a range of 0-500 µg/mL for 24 h. As a positive control, similar concentrations of cisplatin were also used against the AGS cells. After that, the MTT solution (0.5 mg/mL) was added to each well and the plate was incubated for 4 hours. After the incubation period, the medium was removed and 200 µL of dimethyl sulfoxide was added to the wells, and the plate was shaken for 30 min. Finally, the optical absorption of the wells was determined at 590 nm using a microplate reader (Bio-Rad, Hercules, CA, USA). The following formula was used to determine the 50% inhibition concentration (IC50) values of CuO@Glu/TSC NPs and cisplatin against the studied cells [10]:

$$Inhibition\ (\%) = \frac{Abs\ of\ control - Abs\ of\ Test}{Abs\ of\ control} \times 100 \qquad (1)$$

Flow cytometry assay

Flow cytometry analysis was performed to determine the apoptosis/necrosis rate of the AGS cells treated with CuO@Glu/TSC NPs using a flow cytometry kit (Roche, Germany). The AGS cells were treated with the NPs at IC_{50} concentration and incubated for 24 h. Then, the cells were harvested and washed with phosphate-buffered saline (PBS). Then, 10 µL of Annexin-V FLUOS and 5 µL of propidium iodide (PI) were added to the cells and the population of apoptotic/necrotic cells was determined by a flow cytometry device (Partec, Germany).

Hoechst 33258 staining

The nuclear damages caused by CuO@Glu/TSC NPs in treated AGS cells were evaluated by the Hoechst staining method [11]. At first, the AGS cells (4×10^5 cells/well) were prepared and treated with CuO@Glu/TSC NPs at IC_{50} concentration. The AGS cells without NPs exposure were regarded as control cells. After 24 h incubation, the cells were stained with Hoechst 33258 dye and visualized using a fluorescent microscope (Incell Analyser 2000, USA).

Caspase-3 activity

The activity of *caspase-3* among the AGS cells, treated with CuO@Glu/TSC NPs and also control cells was evaluated using a colorimetric assay kit (Sigma-Aldrich, CASP3C). In brief, the AGS cells with a population of 5×10^6 cells/well were prepared in 6-well plates and treated with CuO@Glu/TSC NPs at IC_{50} concentration for 24 h. Then, the cells were harvested, lysed using a cell lysis buffer, and centrifuged. Finally, DEVD-pNA was added to the cell supernatant and the *caspase-3* activity was determined by measuring the absorbance of p-nitroaniline (μM) released from the substrate at 405 nm [12].

Statistical analysis

The cellular toxicity assays were conducted in triplicates and the results were presented as mean ±SD. Statistical analyses were performed using SPSS. 16.0 software (SPSS Inc., Chicago, IL). The cellular toxicity of the compounds was expressed as mean ±SD, and the statistical significance of data was analyzed by one-way ANOVA. The p-value less than 0.05 was considered as statistically significant.

Results And Discussion

Characterization of CuO@Glu/TSC NPs

The X-ray diffraction (XRD) pattern for the CuO@Glu/TSC nanoparticles was displayed in Figure 1. The XRD pattern for CuO@Glu/TSC showed the typical reflection peaks at 2θ values of 32.3°, 38.6°, 48.7°, 58.1.0° and 68.0° corresponding to (110), (111), (202), (202) and (220) planes of CuO nanoparticles. The reflections could be indexed to face-centered cubic phase of CuO, in accordance with the JCPDS card no. 80-1268 [13]. The sharpness and the intensity of the peaks imply the high crystallinity of the CuO@Glu/TSC NPs.

The FT-IR spectra of CuO@Glu and CuO@Glu/TSC NPs in the range of 400 cm^{-1} to 4000 cm^{-1} were presented in Figure 2. There are two sharp absorption peaks at 427 cm^{-1} and 535 cm^{-1} that are corresponding to the stretching vibrations of metal–O bonds supporting the presence of the monoclinic

phase of copper oxide NPs [14]. The band at 1607 cm^{-1} corresponds to the C=O vibration of −COOH groups for glutamic acid on the surface of CuO NPs.

The band at 3392 cm−1 corresponding to the O-H stretching vibrations show the vibrational modes of water molecules adsorbed on the surface of the NPs. Also, the peak at around 1955 cm−1 in FT–IR spectrum of CuO@Glu/TSC is associated with the O-H bending. The comparison of FT—IR spectra of synthesized CuO@Glu and CuO@Glu/TSC confirms the successful conjugation of thiosemicarbazide compound on the surface of CuO@Glu NPs. The strong band observed at 1339 cm−1 in the spectrum of CuO@Glu/TSC can be devoted to the v (C—N) stretching of thiosemicarbazone in the synthesized nanoparticles. The comparison of FT—IR spectra of the CuO@Glu NPs and synthesized CuO@Glu/TSC confirms the successful conjugation of this organic compound on the surface of CuO@Glu NPs. For instance, the band around 800 cm−1 in the IR spectrum is corresponding to v(C=S) stretching [11, 14].

The morphology and size of the CuO@Glu/TSC NPs were evaluated by scanning and transmission electron microscopy. The particles were approximately spherical with the particle size in a range of 20-39 nm. Figures 3 and 4 display the SEM and TEM images of the CuO@Glu/TSC NPs.

Energy dispersive X-Ray (EDX) was employed to analyze the Elemental composition of CuO@Glu/TSC NPs. The EDX analysis showed strong signals for the presence of Cu element, and also signals for the C, O, N, and S atoms. The presence of no other EDX signal clearly indicates the purity of the synthesized NPs (Figure 5, Table 1). Moreover, the hydrodynamic size and zeta potential of CuO@Glu/TSC NPs were measured 710 nm and 27.5 mv, respectively. The proper zeta potential of the CuO@Glu/TSC NPs could provide sufficient repulsive force between particles to avoid agglomeration of the NPs and provides satisfactory colloidal stability [15] (Figure 6).

Table 1

EDS analysis of CuO@Glu/TSC NPs

Elements	K	Kr	Weight (%)	Atomic (%)	ZAF
C	0.0707	0.0357	17.9	31.72	0.1996
N	0.0720	0.0364	17.2	26.14	0.2114
O	0.0828	0.0418	17.1	22.78	0.2442
S	0.1689	0.0853	10.2	6.78	0.8357
Cu	0.6055	0.3058	37.6	12.58	0.8140
Total	1.0000	0.5050	100.00	100.00	

Cytotoxic effect of CuO@Glu/TSC NPs

The anti-proliferative potential of CuO@Glu/TSC NPs against AGS and HEK293 cell lines was evaluated. A considerable anti-proliferative effect of CuO@Glu/TSC NPs against AGS cells was observed with IC50 value of 203µg/mL. Also, the prepared NPs showed a significantly less anti-proliferative effect on the HEK293, as normal human cells, with an IC50 value of 435µg/mL. However, the anticancer potential of the NPs against AGS cells was significantly less than cisplatin, which exhibited the IC50 value of 65µg/mL. The relative viability of the cells exposed to different concentrations of CuO@Glu/TSC NPs and cisplatin was presented in Figure 7.

As a trace element with specific functions inside the eukaryotic cells, this element has lower toxicity to normal human cells [3]. Also, due to the higher physiological state of cancer cells, they show a higher demand for this element, which could provide selective toxicity of copper-containing drugs against cancer cells [3, 6]. Combination of copper with a variety of therapeutic ligands, including imidazoles and phosphines, as well as TSC derivatives showed a promising anticancer potential [3, 16].

Several anticancer mechanisms have been proposed to be mediated by copper. The anticancer potential of copper has been mainly associated with the oxidative stress caused by the production of reactive oxygen species (ROS) and also, Glutathione (GSH) oxidation [3, 6]. The oxidative imbalance damages cellular molecules, including nucleic acids, lipids, and proteins, which triggers cell apoptosis [6, 17-18].

According to the literature, copper complexes have shown proteasome inhibitory potential in different cell lines and copper element had a crucial role in this issue. The proteasome, a large and multi-catalytic protease, is responsible for the degradation of damaged proteins and also regulating the concentrations of cell cycle regulatory proteins, including cyclins and p53 (144 and 145 from 4). Due to the higher load of copper inside cancer cells than normal cells, a higher proteasome inhibitory potential and thus, apoptosis induction by copper complexes was reported, which efficiently inhibited the proliferation of cancer cells [19-20].

Similar to other metal complexes, copper complexes can undergo redox reaction and ligand substitution which enable them to interact with a variety of biological substrates. Thus, conjugation of therapeutic ligands with copper could provide efficient cytotoxicity against cancer cells by the action of both partners [3, 6].

TSCs are considered as efficient cytotoxic agents, which supress cancer cells proliferation by inhibition of cellular ribonucleotide phosphate reductase, the enzyme necessary for DNA replication [6]. Also, it was reported that the cytotoxicity of copper complexes containing coordinating ligands, including TSCs could be associated to the binding of the ligands to cell DNA which inhibits genome replication and transcription, and finally results in cell apoptosis [6].

Based on the results and above considerations we found that the conjugation of TSC to copper oxide NPs could inhibit the proliferation of cancer cells and results in higher toxicity to AGS cells compared with normal human cells. In addition, the functionalization of CuO@Glu/TSC NPs using glutamic acid seems to improve their stability and bioavailability. In agreement with our finding, several studies aimed to

increase the hydrophilicity of Cu/TSC complexes by modification of TSCs framework to improve their stability and reported promising anticancer characteristics [8, 21].

Moreover, investigating the antitumor effect of copper- 2-keto-3- ethoxybutyraldehyde-bis(thiosemicarbazone) (KTS) revealed that the KTS ligand could stabilize the oxidation state of $^{+2}$copper. Following uptake of the complex by cancer cells, the complex is dissociated by interaction with cellular thiols. This dissociation generates Cu(I), which may act as heavy metal poison [6, 22]. The similar synergistic potential could be also hypothesized to other CuO-TSC complexes.

Apoptosis induction of CuO@Glu/TSC NPs in AGS cells

Figure 8 displays the apoptosis of CuO@Glu/TSC treated and control AGS cells. The results were presented in the diagrams with four regions where Q1 region displays cell necrosis, Q2 and Q3 represent late and early apoptosis, and Q4 for live cells. Our results showed that early apoptosis occurred among the majority of CuO@Glu/TSC treated cells (69 %) and 11.5 % of cells experienced late apoptosis, which was significantly higher than control cells. In addition, 96.7 % and 19.3 % of control and NPs treated cells remained alive. Also, only 0.27 % of cell necrosis was found among CuO@Glu/TSC treated cells. The apoptosis induction by copper NPs conjugated to the TSC derivatives against Burkitt's lymphoma cells has been reported previously, which is in agreement with our finding [22]. As described above, the apoptotic effect of CuO@Glu/TSC NPs could be associated with the DNA damages caused by oxidative stress, inhibition of DNA replication, and cellular proteasome inhibition [3, 6].

Hoechst 33258 staining

Hoechst staining was performed on CuO@Glu/TSC treated and control cells to evaluate the presence of compact chromatin of apoptotic nuclei. The apoptosis morphological characteristics, including condensed chromatin and chromatin fragmentation, and apoptotic bodies were found among the AGS cells treated with CuO@Glu/TSC NPs. In contrast, the control cells did not show considerable changes (Figure 9). As stated above, the increased ROS generation by CuO@Glu/TSC could results in DNA strand breakage and apoptosis induction [6]. The apoptotic morphological characteristics of CuO@Glu/TSC treated cells comply well with the results from the MTT and flow cytometry assays.

Caspase 3 activity

The activity of caspase-3 among CuO@Glu/TSC treated AGS cells and control cells was investigated. The results showed that treating AGS cells with the NPs resulted in the increased activation of caspase-3. In other words, among NPs treated cells the caspase-3 activity significantly increased by 1.4 folds in comparison with the control cells (Figure 10). Caspases play a critical role in triggering of apoptosis [23]. In this study a significantly increased activity of caspase-3 was noticed, confirming the apoptosis

induction in AGS cells following exposure to CuO@Glu/TSC NPs. In agreement with our work, previous studies showed increased transcription and activation of caspase-3 in a variety of cancer cells following treatment with copper NPs or copper complexes [4, 6, 11, 23]. Generation of ROS and subsequently damages to cellular DNA is considered a major signaling for triggering apoptosis pathway [23]. In our opinion, after exposure of AGS cells to CuO@Glu/TSC NPs, generation of ROS by copper and also inhibition of cell proteasome, which regulates the concentration of p53 proteins, are major causes of apoptosis induction following upregulation of caspase proteins. In agreement with this, previous works reported that the activation of p53 among cancer cells treated with copper NPs indirectly promotes caspase-3 activity [24-25].

Conclusion

In this study, CuO@Glu/TSC NPs were synthesized and their anti-proliferative effect on AGS gastric cancer cells was investigated. Our results showed that the prepared NPs efficiently inhibited the cancer cell proliferation with significantly less IC50 than normal HKE293 cells. Also, the NPs induced early apoptosis, chromatin fragmentation, and increased activity of caspase-3 in AGS cells, which describe the cytotoxic mechanism of CuO@Glu/TSC NPs. This work showed a promising anti-proliferative effect of CuO@Glu/TSC against AGS cells, to be used for gastric cancer chemotherapy after further characterizations.

Declarations

Ethics approval

Not applicable.

Author contributions

Conceptualization: MB and AS; Methodology: AS; Formal analysis and investigation: MB, SHJ, SRM, MJ and MH; Writing - Original Draft Preparation: AS; Editing: AS; Resources: MB, SHJ, SRM, MJ, TGH and MH; Supervision: AS.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Figures

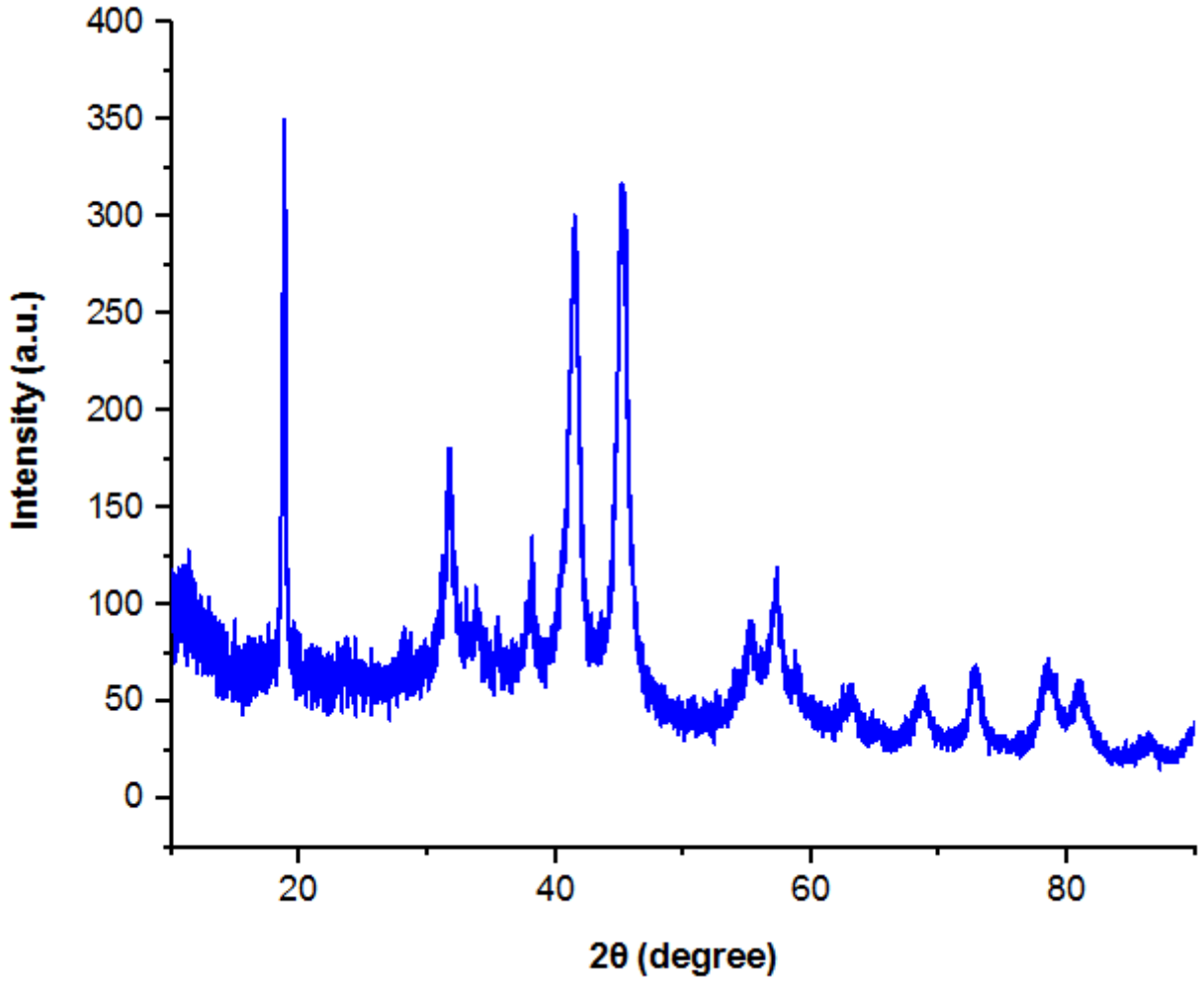

Figure 1

XRD pattern of the synthesized CuO@Glu/TSC NPs

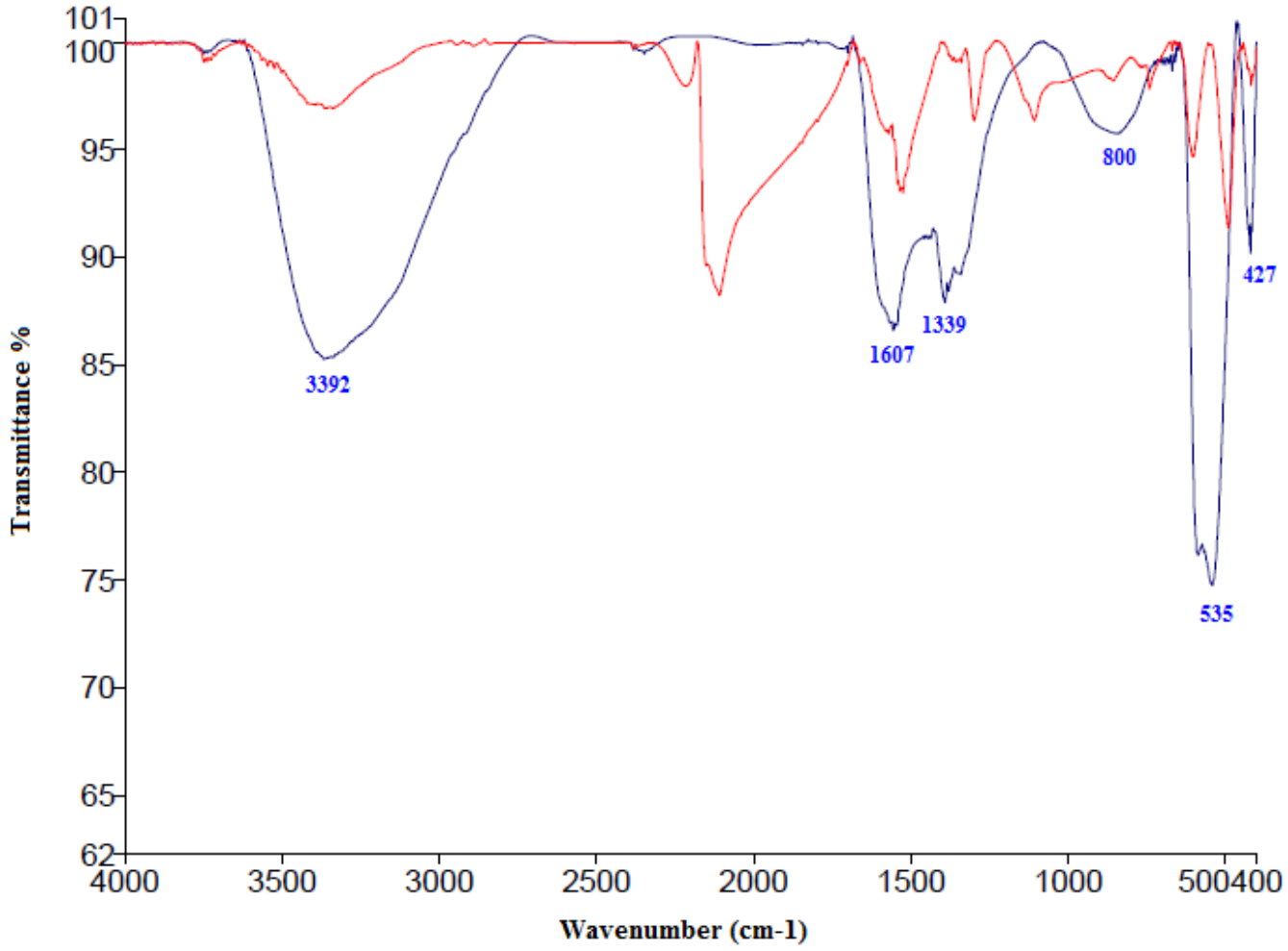

Figure 2

FT-IR spectra of CuO@Glu NPs (red graph), CuO@Glu/TSC NPs (blue graph)

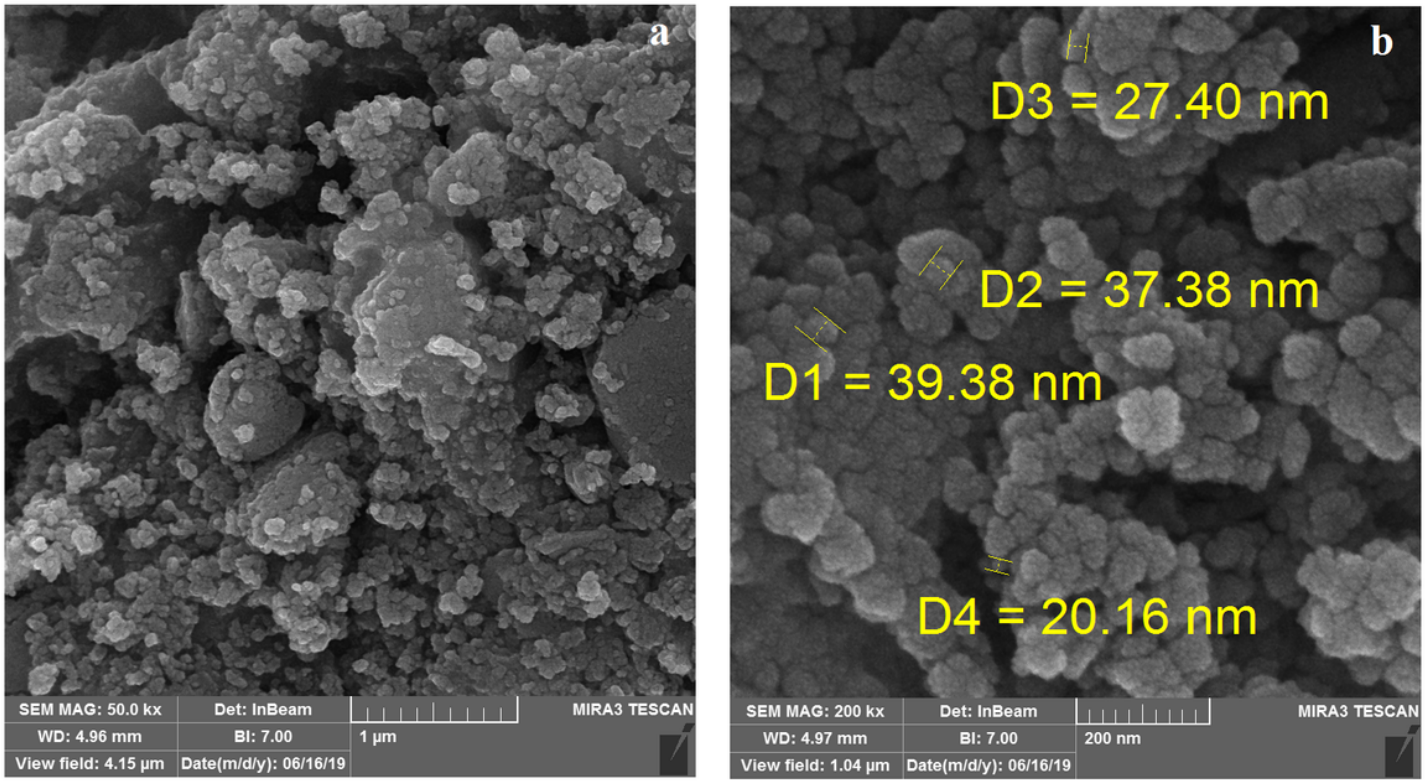

Figure 3

Scanning electron micrograph of the CuO@Glu/TSC NPs

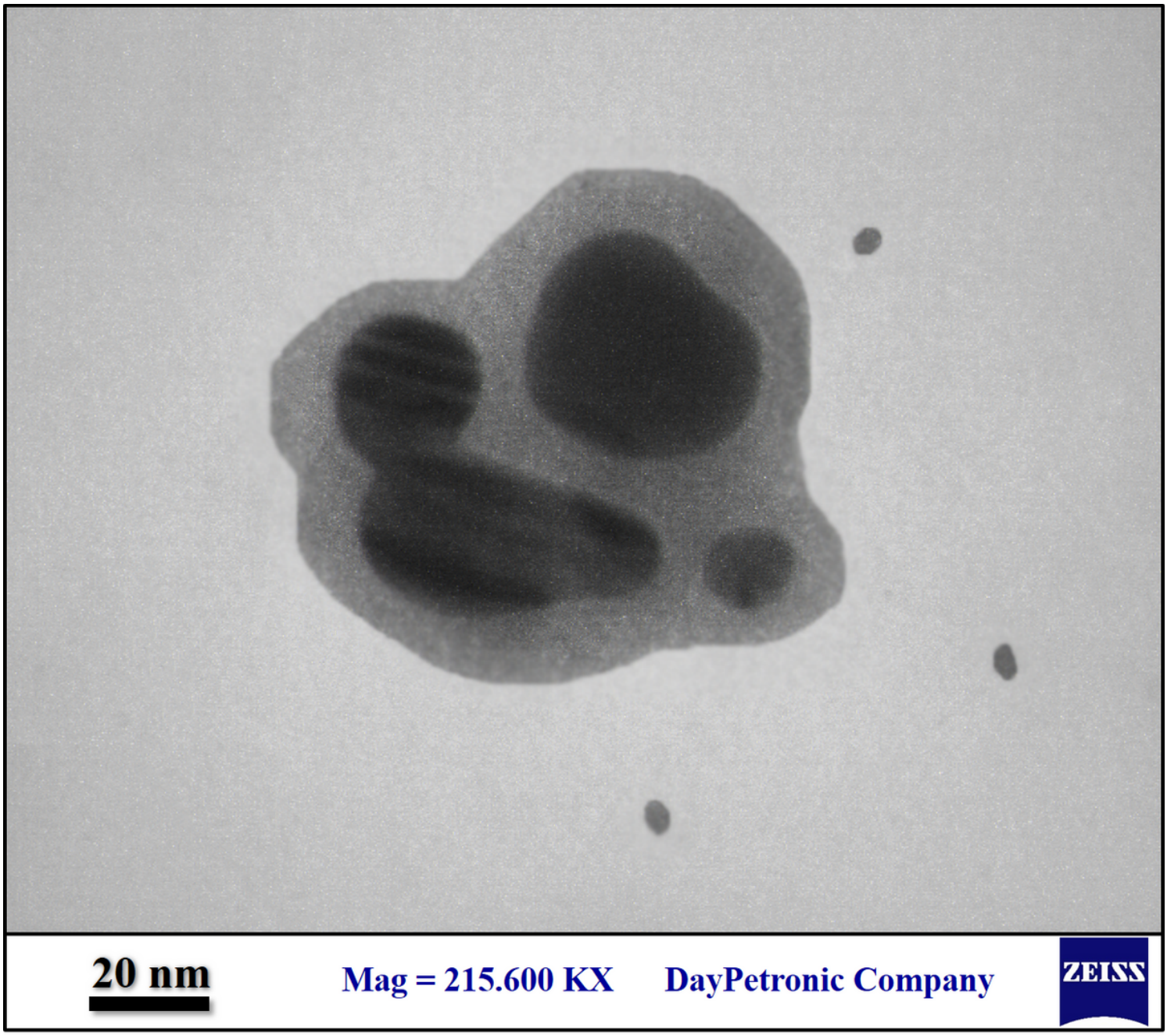

Figure 4

Transmission electron micrograph of the CuO@Glu/TSC NPs

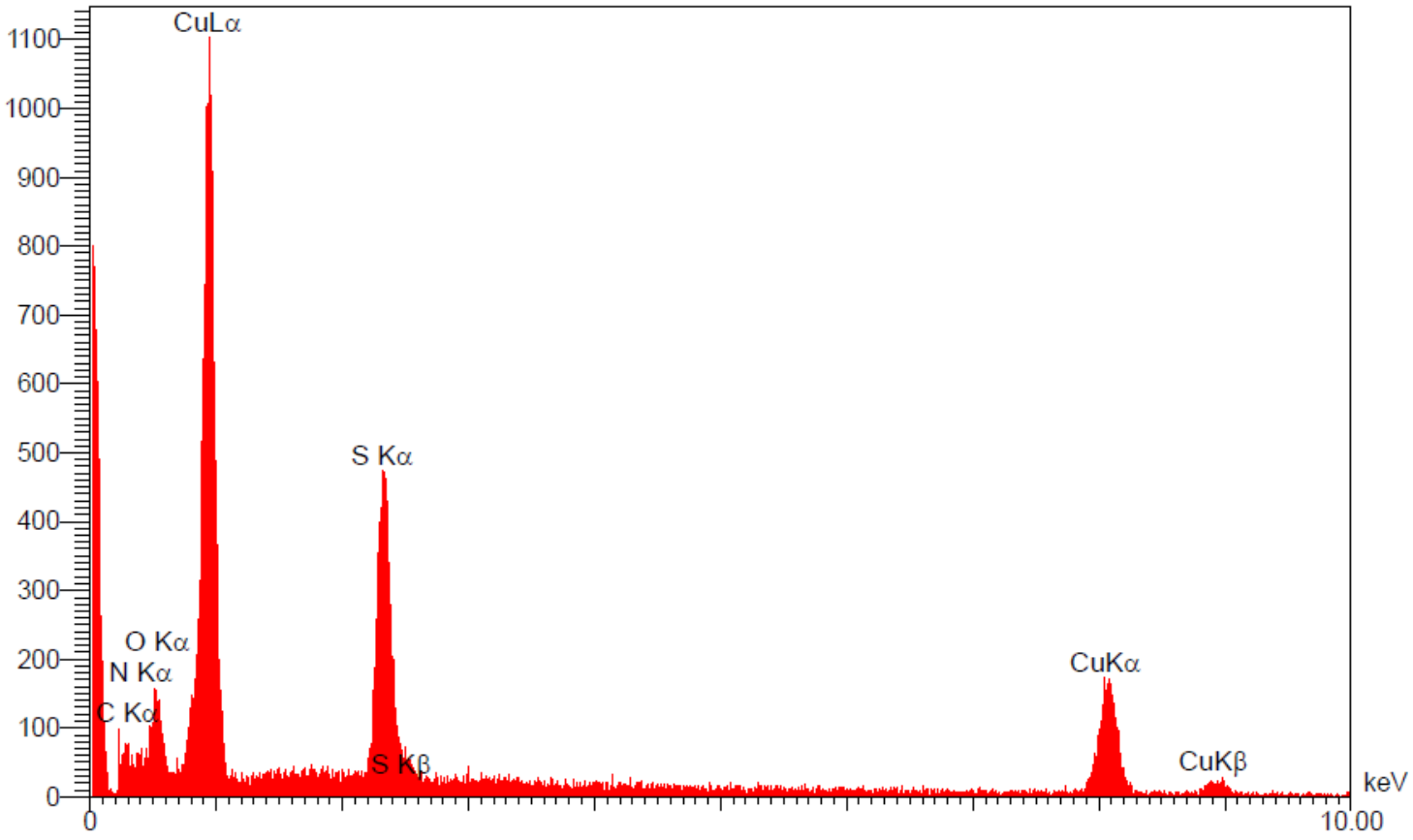

Figure 5

EDX analysis of synthesized CuO@Glu/TSC NPs

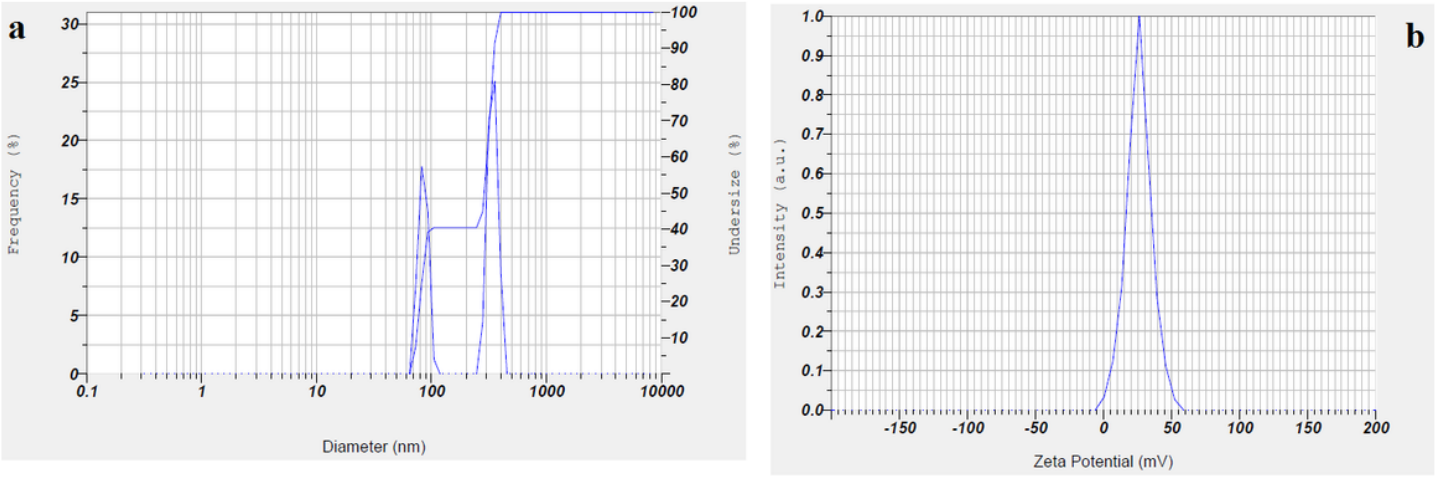

Figure 6

DLS (a) and Zeta potential (b) analyses of CuO@Glu/TSC NPs

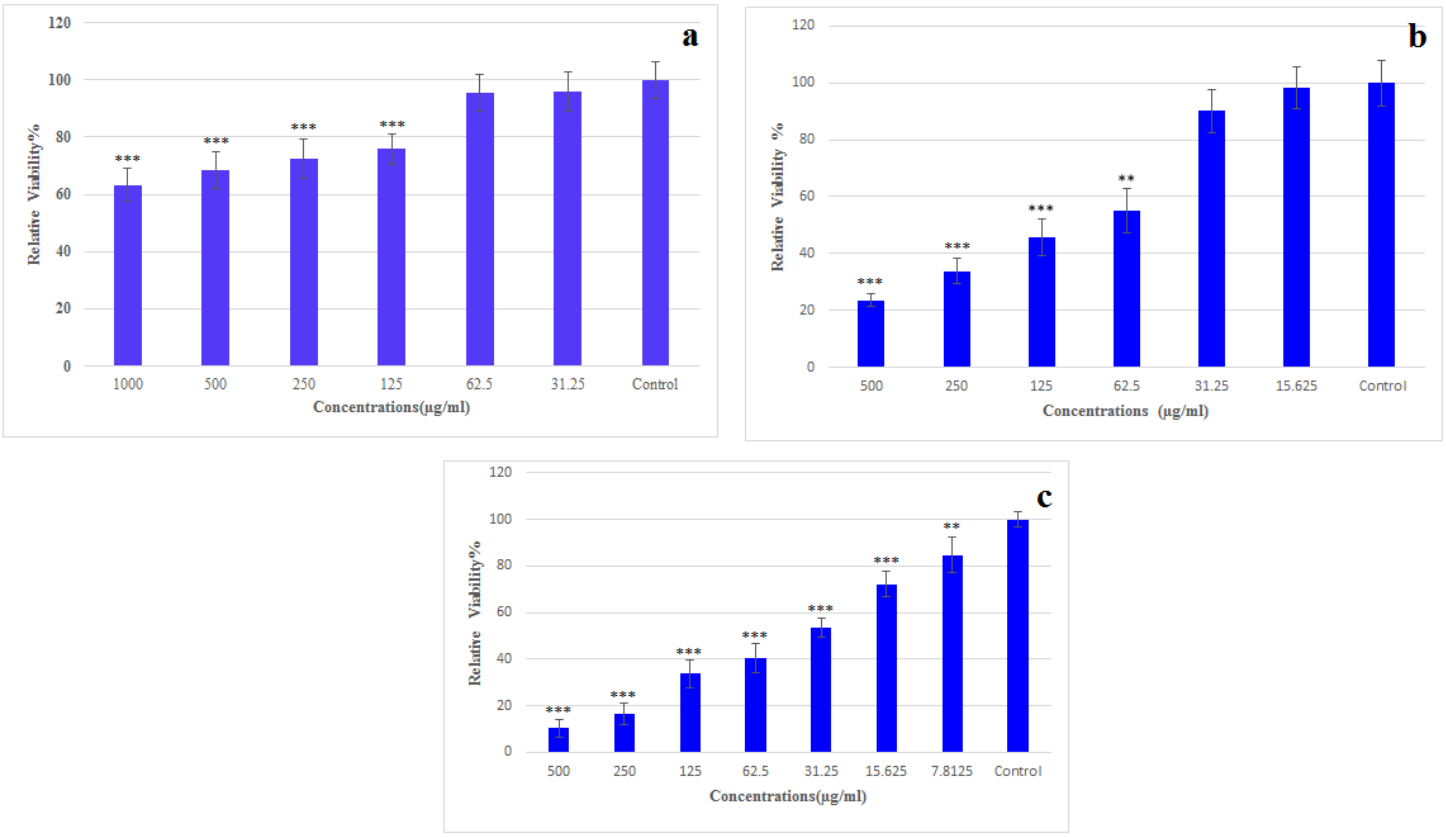

Figure 7

Cytotoxic effect of CuO@Glu/TSC NPs on a) normal fibroblast cells, and b) AGS cell, and c) cytotoxicity of cisplatin for AGS cells

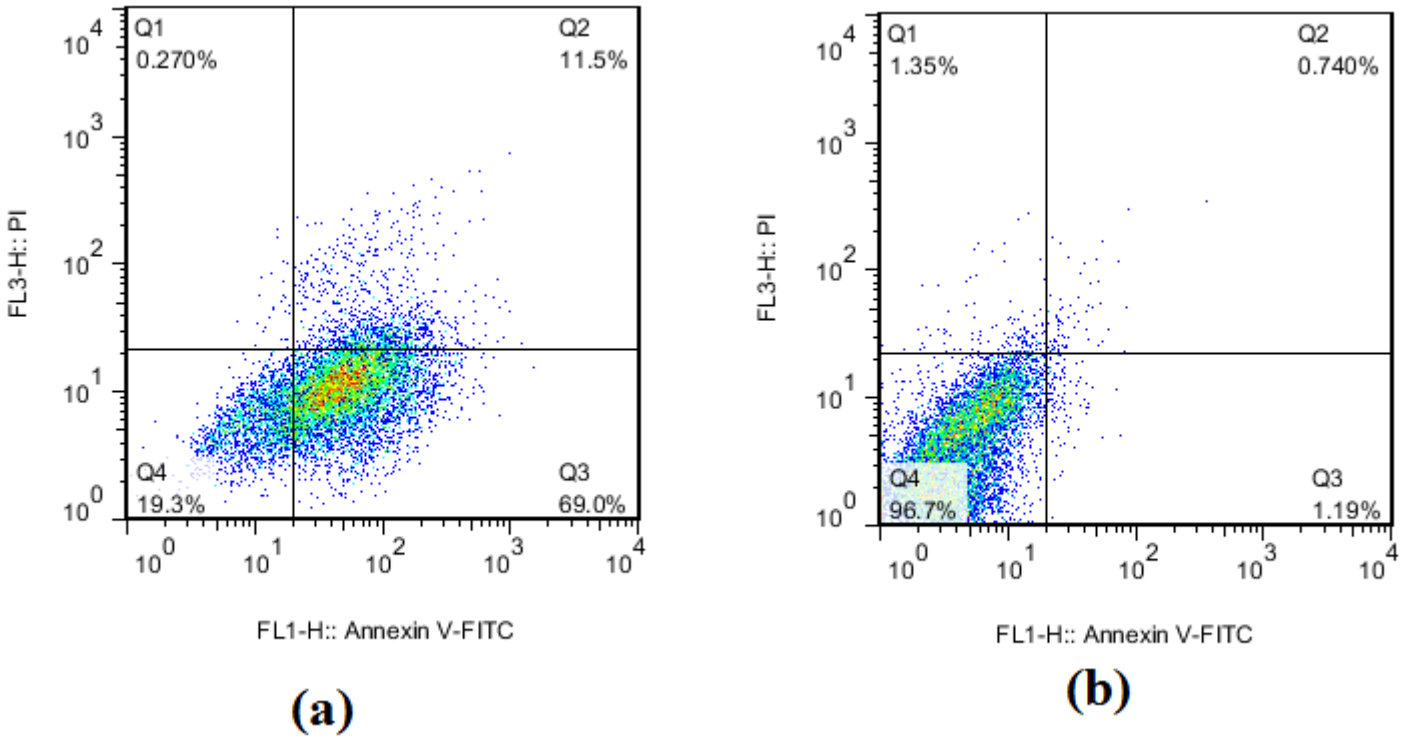

Figure 8

Flow-cytometry analysis of a) CuO@Glu/TSC treated, and (b) control AGS cells

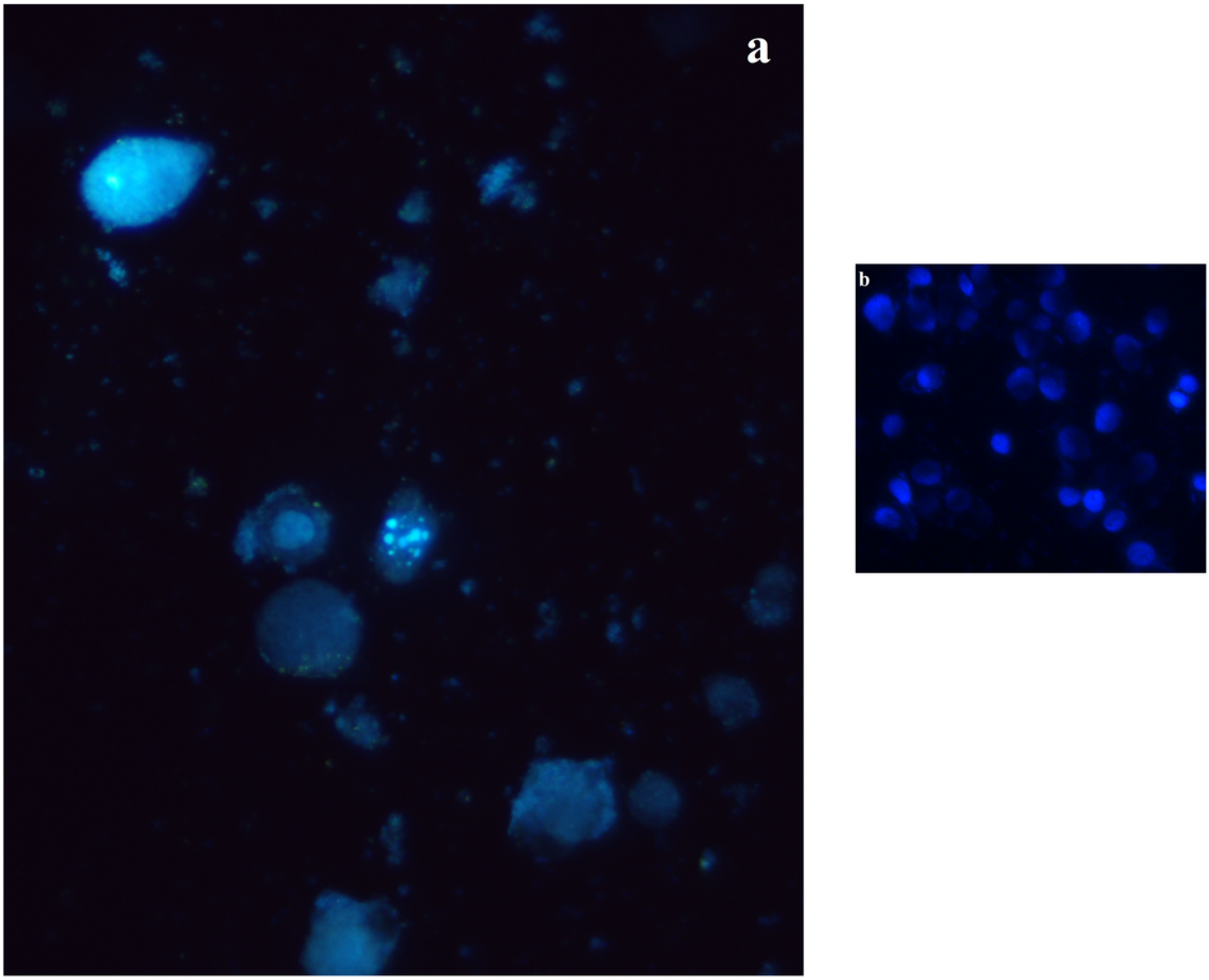

Figure 9

Hoechst staining of a) CuO@Glu/TSC treated, and b) control AGS cells. The apoptosis changes induced by CuO@Glu/TSC could be observed in the NPs treated cells.

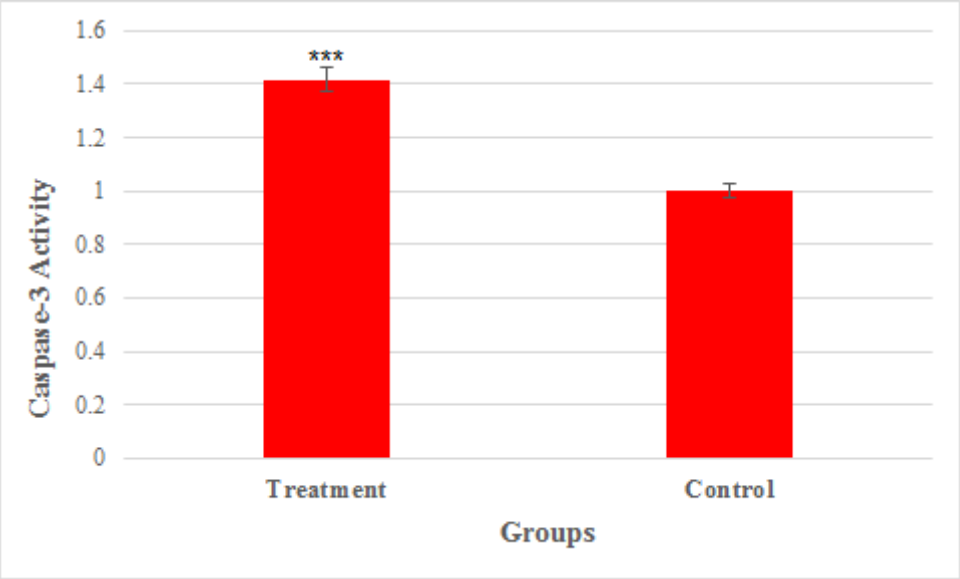

Figure 10

Caspase-3 activity among CuO@Glu/TSC treated and control AGS cells