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The interaction of GSSG modified magnetic nanoparticles with SPC-A1 cells *in vitro*

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Magnetic nanoparticles (MNPs) are promising materials for various biomedical applications, including magnetic resonance imaging, stem cell tracking, gene/drug delivery, and cancer treatment. To increase the effectiveness of MNPs, high capture efficiency and controlled uptake of the particles by cells is required. In this paper we report the cytotoxicity and cellular uptake into SPC-A1 cells of oxidized glutathione (GSSG)-modified MNPs (GSSG@Fe_3O_4). Experimental findings indicated that GSSG@Fe_3O_4 were biocompatible, and could be efficiently taken up by SPC-A1 cells (up to 160 pg iron per cell). The internalized GSSG@Fe_3O_4 was retained in the cell cytoplasm for 6 generations. The uptake of GSSG@Fe_3O_4 into SPC-A1 cells was energy-, concentration- and time-dependent. Pinocytosis may be involved in the internalization process of GSSG@Fe_3O_4 into SPC-A1 cells, but this mechanism remains to be elucidated. The controlled and efficient localization of GSSG@Fe_3O_4 into the cytosol and long intracellular retention provides theoretical and experimental insight into the biomedical applications for these molecules.

magnetic nanoparticles, uptake, glutathione, GSSG, SPC-A1

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Magnetic nanoparticles (MNPs) can be used in a wide variety of biomedical applications, ranging from cell labeling, magnetic resonance imaging, and gene/drug delivery for cancer treatment [1–9]. The uptake of MNPs by living cells very much depends on the MNP coating. Surface modification can improve the biocompatibility and enhance cell uptake of MNPs [10–15]. MNPs coated with dextran, or its derivatives, have been frequently used in the context of labeling cells with a magnetic tag [16]. However, cell accumulation of these particles is usually low in non-phagocytic cells, culminating in a low MR detection sensitivity. The application of MNPs is affected by the net amount that are internalized, and those that remain within the cells after initial endocytosis, followed by exocytosis and intracellular degradation [13]. The cellular uptake of MNPs, especially their intracellular retention, is clearly crucial to the above applications. We therefore synthesized GSSG@Fe_3O_4, since glutathione is found in all cells in the body making it a very compatible target for MNP technology. These MNPs were then evaluated for their cytotoxicity, cellular uptake and intracellular retention into SPC-A1 cells.

Most of the promising MNP applications, especially MRI and stem cell tracking, require well-defined and controllable interactions between the MNPs and living cells [1–5,8]. Furthermore, cellular uptake and intracellular pathways are dependent on the MNPs and cell type [9,13,16], so it is crucial to elucidate the uptake kinetics and cellular internalization pathways for GSSG@Fe_3O_4. This study provides theoretical and experimental insight into the biomedical applications of GSSG@Fe_3O_4.

1 Materials and methods

1.1 Materials and equipment

(1) Materials. $FeCl_3 \cdot 6H_2O$ (Analytical Reagent, AR),

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$FeSO_4 \cdot 7H_2O$ (AR), GSSG (AR), nuclear fast red, Prussian blue, glutaraldehyde solution, dimethyl sulfoxide (DMSO), MTT solution and LDH cytotoxicity assay kit were all purchased from Sigma Aldrich (Shanghai, China). Colchicine and cytochalasin B were from Sino-American Biotec (Shanghai, China). SPC-A1 (human lung adenocarcinoma cell line) was purchased from the Cell Bank of the Chinese Academy of Sciences. DMEM culture medium (high glucose) and fetal calf serum were obtained from Hyclone (Shanghai, China). All other reagents were obtained from Shanghai Reagent Company.

(2) Equipment. The equipment used in this study included transmission electron microscopes (TEM, Philips CM-120 for the observation of MNPs and Philips Tecnai 20S-TWIN for the observation of cell samples), Fourier transform infrared spectrometer (FTIR, Bruker EQUINOX 55FTIR), Multilabel Counter (Victor-3, Perkin Elmer, USA) and an inductively coupled plasma optical emission spectrometer (ICP-OES, ICAP-6300, Thermo Fisher, USA).

1.2 Methods

(1) Preparation of GSSG@Fe_3O_4. MNPs were prepared by co-precipitation of ferric and ferrous salts in an alkaline solution [17]. In brief, $FeCl_3 \cdot 6H_2O$ (24.3 g) and $FeSO_4 \cdot 7H_2O$ (16.7 g) were first dissolved in 200 mL deionized water under a blanket of nitrogen gas and heated to 75°C. Then ammonia solution (45 mL, 25% (v/v)) was added dropwise to the mixture with vigorous stirring. After stirring for 30 min, the temperature was raised to 85°C and GSSG (0.2 g) was added to the mixture. After reacting for 1 h, the mixture was cooled down to ambient temperature. The product was retrieved using a magnet, washed 5 times with deionized water and finally dispersed in water.

(2) Characterization of GSSG@Fe_3O_4 nanoparticles. The morphology and size of the particles were observed and analyzed by TEM. To do this, the particle suspension was directly deposited onto a carbon-coated copper grid and air-dried at room temperature. The particle size and size distribution were calculated using an image analysis software of Image J by measuring the diameter of at least 300 particles. The obtained particles were dissolved in deionized water and analyzed by FTIR.

(3) Cytotoxic evaluation of GSSG@Fe_3O_4. SPC-A1 cells were cultured in DMEM medium, supplemented with 10% fetal calf serum at 37°C in a 95∶5 air/CO_2 water-saturated atmosphere. Cell viability was investigated with MTT and LDH assays. For MTT assay, cells were seeded in 96-well plates (1×10^5 cells/well) and incubated with culture medium containing GSSG@Fe_3O_4 at iron concentrations of 0.6, 1.0 or 1.5 mg/mL for different periods of time (6, 12, 24 or 48 h). After incubation, 20 μL of MTT solution (5 mg/mL in PBS) was added to each well and incubated for 4 h. Then, the MTT containing medium was removed and 150 μL DMSO was added to dissolve the formazan crystals produced from the reduction of MTT by viable cells. The optical density was measured at 570 nm using a Multilabel Counter. The cell viability was calculated using the following equation: cell viability=$OD_{experiment}/OD_{control}\times100\%$. For the LDH assay, after co-culturing with GSSG@Fe_3O_4 (0.6, 1.0 or 1.5 mg Fe/mL) for 24, 48 or 72 h, the supernatant was analyzed with a LDH cytotoxicity assay kit according to the manufacturer's protocol. SPC-A1 cells incubated with nanoparticle-free medium served as controls (100% cell viability).

(4) Intracellular retention of GSSG@Fe_3O_4. The intracellular retention of GSSG@Fe_3O_4 was evaluated by TEM observation. SPC-A1 cells were washed twice after a 24 h incubation period in culture medium containing 1.0 mg/mL GSSG@Fe_3O_4, and cultured in fresh medium up to the 6th subpassage. Culture medium was changed every third day. After incubation, the culture medium was removed. The adhered cells of the 2nd, 4th or 6th generations were washed three times with PBS and harvested by trypsinization. The cells were centrifuged and redispersed into 4% (w/v) glutaraldehyde and processed for analysis by TEM using previously published methods [18].

(5) Uptake kinetics of GSSG@Fe_3O_4 by SPC-A1 cells. To evaluate the effects of temperature on cellular uptake, SPC-A1 cells were incubated with culture medium containing 0.2 mg/mL GSSG@Fe_3O_4 at 4°C for 24 h, or at 37°C for 1 h. After incubation, the culture medium was removed. The adhered cells were washed three times with PBS, harvested by trypsinization, fixed and processed for TEM analysis.

To evaluate the effects of time and dose on cellular uptake, intracellular iron uptake was quantitatively analyzed using ICP-OES and qualitatively analyzed by Prussian blue iron stain assays. For quantitative analysis, SPC-A1 cells were incubated with a culture medium containing GSSG@-Fe_3O_4 at the concentrations of 0.1, 0.4, 0.7 or 1.0 mg Fe/mL at 37°C for different periods of time (3, 6, 12, 24, 48 or 72 h). After incubation, the culture medium was removed. The adhered cells were washed, harvested, and digested with aqua regia, and then diluted to 10 mL. The iron content in cells was determined by ICP-OES. The iron content was expressed in picogram (pg) of Fe per cell.

For qualitative analysis, SPC-A1 cells were incubated with GSSG@Fe_3O_4 at the same iron concentration (0.4 mg/mL) for 6 h, 24 h or co-cultured for the same time period (24 h) at an iron concentration of 0.1 or 1.0 mg/mL. After incubation, adhered cells were washed and observed by Prussian blue staining. For Prussian blue staining, the collected cells were fixed with methanol for 5 min, then acetone for 1 min, under −20°C and then air dried for 15 min. Slides were soaked in PBS for 5 min, and then incubated with 10% Prussian blue [$K_4Fe(CN)_6 \cdot 3H_2O$] for 5 min. Subsequently, slides were further incubated with a mixture of 10% Prussian blue and 20% HCl (1:1) for 30 min, water for 10 min and finally nuclear fast red (Sigma Aldrich) for 10 min. Slides were then washed with water, 70%, 80%, 100% ethanol (twice)

and subsequently xylene. The slides were coverslipped with Vitro Clud coverslipping medium in preparation for microscopy.

(6) Internalization pathway of GSSG@Fe_3O_4 in SPC-A1 cells. To investigate the possible pathway of cellular internalization of GSSG@Fe_3O_4, SPC-A1 cells were pretreated with 50 μg/mL colchicine (an inhibitor of pinocytosis) or 50 μg/mL cytochalasin B (an inhibitor of phagocytosis) [19,20] for 4 h at 37°C. After incubation, the pretreatment solution was removed and the cells were washed with PBS. Cells were then co-cultured with 0.2 mg/mL GSSG@Fe_3O_4 for 24 h. The intracellular iron was measured by ICP-OES and the cellular uptake of GSSG@Fe_3O_4 was observed by TEM. Statistical analyses were performed using SPSS 13.0 software. The results were presented as the mean±SD and compared using Student's *t*-test. $P<0.05$ was considered significant. To further investigate the uptake pathway of GSSG@-Fe_3O_4, TEM assays were performed. SPC-A1 cells were incubated in medium containing 1.0 mg/mL GSSG@Fe_3O_4 for 1, 3 or 6 h. After incubation, the cells were washed, collected, fixed and processed for analysis by TEM.

2 Results and discussion

2.1 Characterization of GSSG@Fe_3O_4 nanoparticles

GSSG@Fe_3O_4 were well dispersed and the visualized diameters were roughly 7–15 nm (Figure 1). FTIR spectra of GSSG@Fe_3O_4 and uncoated MNPs are shown in Figure 1. The FTIR spectra of GSSG@Fe_3O_4 exhibited characteristic absorption bands of an amido bond at 1640 cm^{-1} due to the $V_{C=O}$ stretching vibration, which meant when GSSG was binding to iron ions found on the MNPs. The amido bonds were not involved in the reaction and the carboxyl groups of GSSG were probably chelated on the surface of MNPs. The δ_{NH} bending vibration at 1550 cm^{-1} and V_{CN} stretching vibration at 1405 cm^{-1} were observed in GSSG@Fe_3O_4, but not observed in naked MNPs, indicating that MNPs were successfully coated with GSSG.

2.2 Cytotoxic evaluation of GSSG@Fe_3O_4

As shown in Figure 2(a), after incubation with GSSG@-Fe_3O_4, cell viability was similar to the control cells cultured with GSSG@Fe_3O_4-free medium. Even when cells were co-cultured with medium containing GSSG@Fe_3O_4 at 1.5 mg/mL for 48 h, cell viability was still more than 90% (Figure 2(a)). LDH cytotoxicity assays were performed after co-culture with different concentrations of GSSG@Fe_3O_4 for different periods of time. LDH release from SPC-A1 cells was not obviously different compared with the control (Figure 2(b)). These results demonstrated that GSSG@Fe_3O_4 did not cause severe cytotoxicity under the current conditions.

Unmodified MNPs have been shown to cause cytotoxicity above a certain concentration [10]. This cytotoxicity has been shown to increase in a concentration dependent manner [21]. Although the MNPs were coated with biocompatible molecules, such as dextran, to reduce cytotoxicity [22], the reported concentrations of MNPs in culture medium shown to cause cytotoxicity were seldom more than 1 mg/mL.

2.3 Intracellular retention of GSSG@Fe_3O_4

After incubation with culture medium containing 1.0 mg/mL GSSG@Fe_3O_4 for 24 h, SPC-A1 cells were cultured in culture medium free of GSSG@Fe_3O_4 for up to the six generations. As shown in Figure 3(a), many nanoparticles in the cytoplasm of the 2nd generation SPC-A1 cells could be observed using TEM. Intracellular nanoparticles reduced in

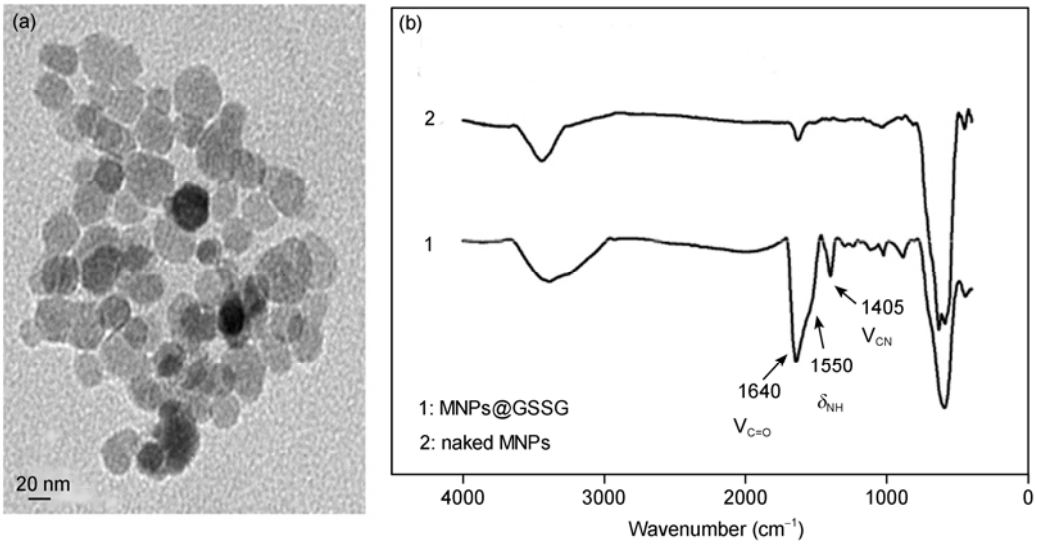

Figure 1 (a) TEM image of GSSG@Fe_3O_4 (scale bar=20 nm) and (b) IR spectra of GSSG@Fe_3O_4 (1) and uncoated MNPs (2).

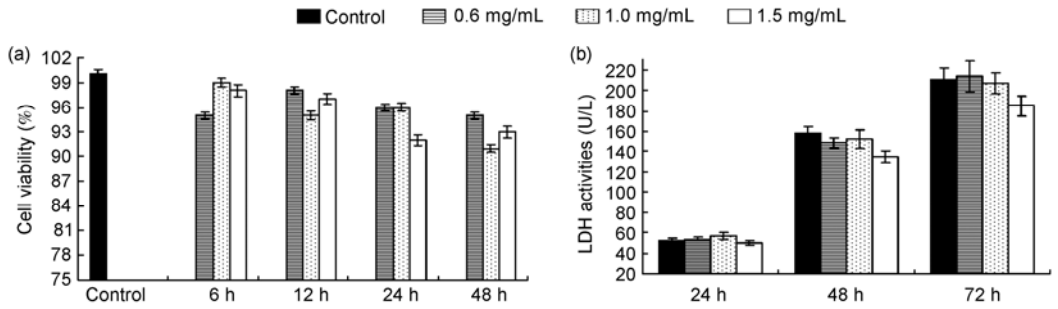

Figure 2 Cytotoxic evaluation of GSSG@Fe_3O_4. SPC-A1 cells were incubated with culture medium containing GSSG@Fe_3O_4 at various iron concentrations for different periods of time. Subsequently, cell survival and LDH activities were determined by MTT (a) and LDH assays (b) (mean±SD, n=3). SPC-A1 cells incubated with GSSG@Fe_3O_4-free medium served as controls (blank, 100% cell viability).

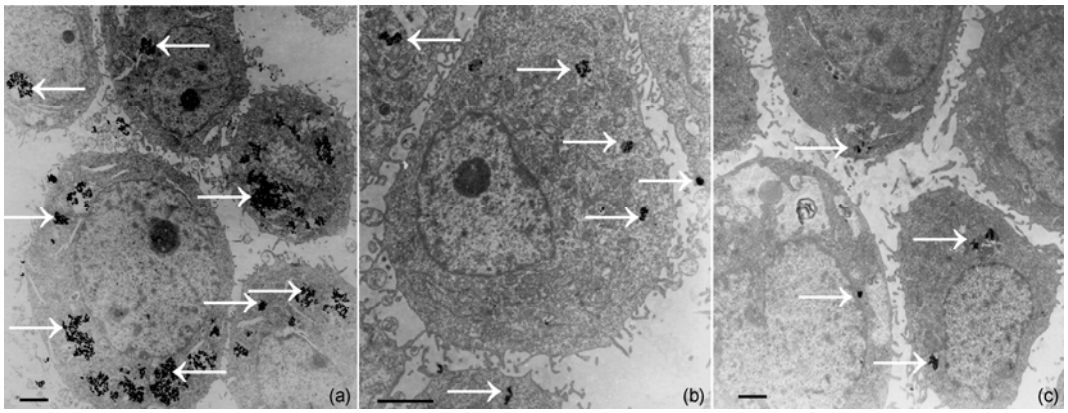

Figure 3 TEM images of sub-cultured cells after co-culture with GSSG@Fe_3O_4. The 2nd (a), the 4th (b) and the 6th (c) generation of SPC-A1 cells after co-culture with GSSG@Fe_3O_4. Arrows show GSSG@Fe_3O_4 located in the cytoplasm. Scale bar=1 μm.

number from generation to generation, but remained visible until the 6th subpassage (Figure 3(c)). After the 6th generation nanoparticles were hardly observed in the cytoplasm as GSSG@Fe_3O_4 may be distributed to subpassaged cells via mitosis and eliminated via exocytosis.

Cytotoxicity and intracellular retention assays demonstrated that at a certain concentration range (0–1.5 mg/mL), GSSG@Fe_3O_4 had no obvious cytotoxic effects on SPC-A1 cells, and that internalized GSSG@Fe_3O_4 could remain in SPC-A1 cells following several generations. These results imply the potential applied value of GSSG@Fe_3O_4 to cell tracking, MRI and intracellular hyperthermia.

2.4 Uptake kinetics of GSSG@Fe_3O_4 into SPC-A1 cells

GSSG@Fe_3O_4 absorbed to the cell membrane after co-culture with SPC-A1 cells at 4°C for 1 h. After culture for 24 h, there were no nanoparticles observed in the cytoplasm (Figure 4(a)). When SPC-A1 cells were co-cultured with GSSG@Fe_3O_4 at 37°C for 1 h, nanoparticles were observed in the cytoplasm (Figure 4(b)). As endocytosis is inhibited at low temperatures, these findings indicate that the endocytosis of GSSG@Fe_3O_4 was an energy-dependent and active process [23].

Both the results of iron content measurement assays and Prussian blue iron stain assays showed intracellular iron content was increased in a time and concentration dependent manner in SPC-A1 cells (Figure 5), which is in accordance with previous reports [12,18,24]. When co-cultured for 48 h at 1.0 mg/mL of GSSG@Fe_3O_4, the iron content reached a plateau of 160 pg iron per cell (Figure 5A), suggesting the uptake of GSSG@Fe_3O_4 was a saturable process [23].

Cell labeling with MNPs is an increasingly common method for *in vitro* cell separation as well as for *in vivo* imaging. Magnetic cell labeling also raises very promising developments for therapy by allowing magnetic intracellular hyperthermia. All these applications require that cells consistently and efficiently capture MNPs [21,23]. Our results demonstrated that the uptake of GSSG@Fe_3O_4 by SPC-A1 cells was efficient and occurred in a concentration and time-dependent manner, which is crucial for their use in the above-mentioned biomedical applications.

2.5 Internalization pathway of GSSG@Fe_3O_4 in SPC-A1 cells

Historically, endocytic machinery has been classified into

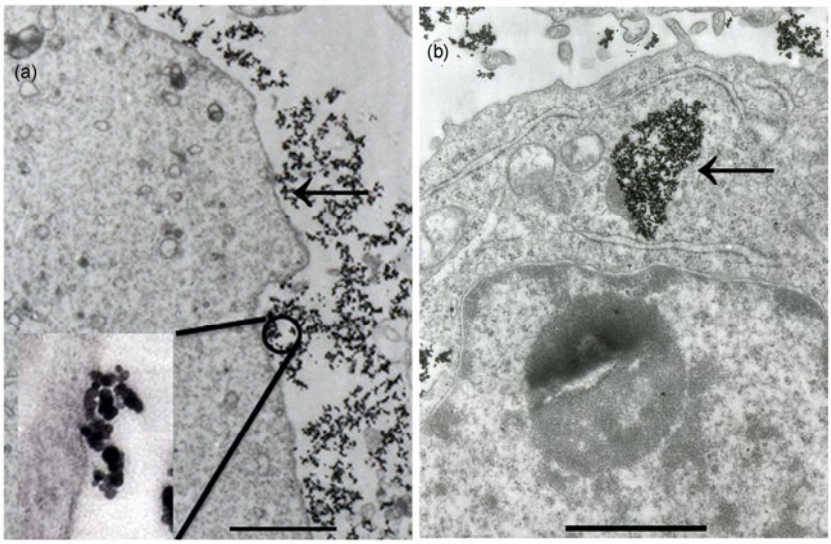

Figure 4 Temperature-dependent uptake of GSSG@Fe_3O_4 by SPC-A1 cells. (a) Co-cultured SPC-A1 cells with 0.2 mg/mL GSSG@Fe_3O_4 at 4°C for 24 h; (b) co-cultured SPC-A1 cells with 0.2 mg/mL GSSG@Fe_3O_4 at 37°C for 1 h. Scale bar=1 μm.

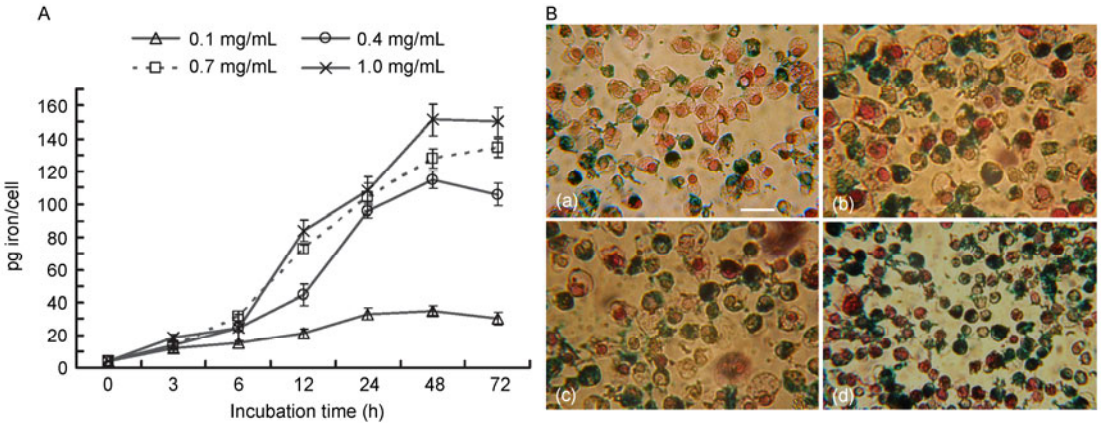

Figure 5 Time and concentration-dependent uptake of GSSG@Fe_3O_4 by SPC-A1 cells. A. Quantitative analysis of intracellular iron uptake by ICP-OES. Co-cultured SPC-A1 cells with GSSG@Fe_3O_4 at an iron concentration of 0.1, 0.4, 0.7 or 1.0 mg/mL for different periods of time (3, 6, 12, 24, 48 or 72 h), respectively. B. Qualitative analysis of iron uptake by Prussian blue iron stain essays (200×). Co-cultured SPC-A1 cells with GSSG@Fe_3O_4 at different iron concentration for different time periods: (a) 0.4 mg/mL for 6 h; (b) 0.4 mg/mL for 24 h; (c) 0.1 mg/mL for 24 h; (d) 1.0 mg/mL for 24 h. Scale bar=50 μm.

phagocytotic and pinocytotic [25]. Specifically, the pinocytotic pathway and phagocytotic pathway are inhibited by colchicine and cytochalasin B pretreatments, respectively [19,20]. We examined the impact of these inhibitors on these endocytotic pathways and noted the cellular uptake. The results are presented in Figure 6.

As shown in Figure 6, the amount of iron in SPC-A1 cells (18.7±1.9 pg) was apparently reduced compared with the control (82.6±5 pg) when pretreated with colchicine (P<0.05), but this reduction did not occur with the treatment of cytochalasin B (69.3±6.5 pg). These observations were in agreement with TEM observations (Figure 6B). Internalized GSSG@Fe_3O_4 in the cytoplasm of SPC-A1 cells was greater in cells treated with cytochalasin B compared with colchicine. Colchicine, known as a functional inhibitor of microtubule assembly that causes microtubule disruption, prevented nanoparticle uptake by inhibiting the pinocytotic pathway. These results suggest that pinocytosis appears to be the internalization pathway for GSSG@Fe_3O_4 in SPC-A1 cells.

TEM was then used to visualize the uptake process of GSSG@Fe_3O_4 and the intracellular compartmentalization in SPC-A1 cells. After co-culture of the SPC-A1 cells with GSSG@Fe_3O_4, the uptake process of nanoparticles from coated pits (Figure 7(a), black arrows) to endosomes (Figure 7(a), white arrows) and then lysosomes (Figure 7(c),(d)) was observed. We believe Figure 7(b) depicts the amalgamation process of endosomes (white arrow) and lysosomes (black arrow).

Previous reports [12,23,26] and inhibition assays lead us

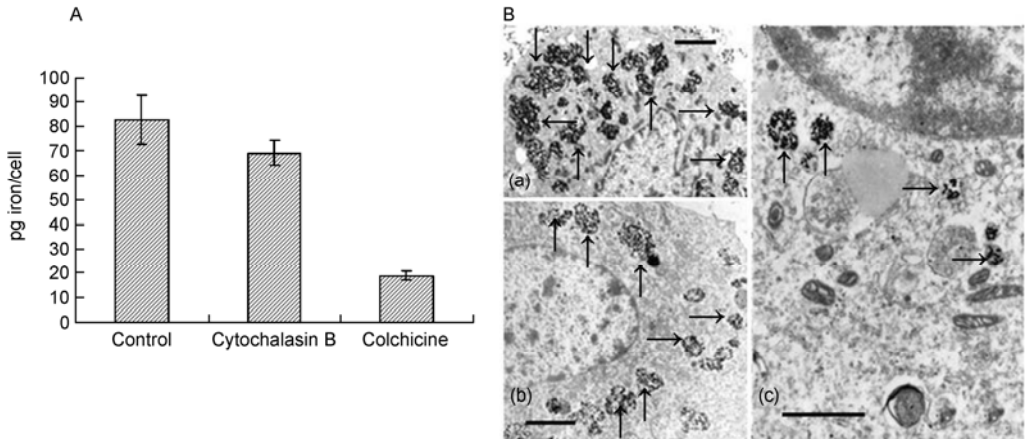

Figure 6 The effects of inhibitors on iron uptake. A, Iron uptake after cells were treated with inhibitors was determined by ICP-OES assays (mean±SD, n=3); B, TEM images of GSSG@Fe_3O_4 uptake by SPC-A1 cells after treatment with inhibitors ((a) control; (b) cytochalasin B; (c) colchicine). Scale bar=1 μm. SPC-A1 cells incubated with inhibitor-free medium served as controls.

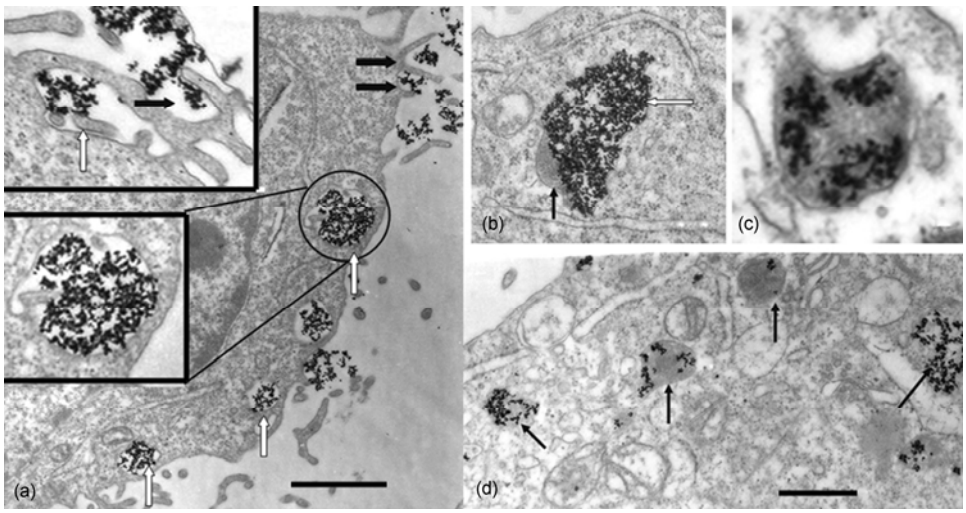

Figure 7 TEM images of the GSSG@Fe_3O_4 uptake process into SPC-A1 cells. After co-culture of SPC-A1 cells with GSSG@Fe_3O_4 for 1 h, GSSG@Fe_3O_4 entered the coated pits ((a) black arrows) and endosomes ((a) white arrows). After 3 h, the amalgamation process of the endosome ((b) white arrow) and the lysosome ((b) black arrow) was observed. After 6 h, many lysosomes contained entrapped GSSG@Fe_3O_4 ((c), (d) black arrows). Scale bar=1 μm.

to believe that these TEM images may show the process of pinocytosis, but further research is required for clear elucidation of this process. As in our previous research on uptake mechanisms, phagocytosis, instead of pinocytosis, may be involved in the internalization process of aminosilane-coated MNPs into SPC-A1 cells [26]. Our results showed the mechanism of endocytosis was dependent on the surface modification of nanoparticles.

3 Conclusions

In summary, GSSG@Fe_3O_4 showed high biocompatibility and high capture efficiency (up to 160 pg iron per cell) in SPC-A1 cells. Furthermore, GSSG@Fe_3O_4 nanoparticles were retained in the cytoplasm of SPC-A1 for six generations. The uptake of GSSG@Fe_3O_4 into SPC-A1 cells was energy, concentration and time-dependent. Pinocytosis might be involved in the internalization process of GSSG@Fe_3O_4, but remains to be elucidated.

The safe and efficient localization of GSSG@Fe_3O_4 to the cytoplasm may be critical in applications such as MRI, drug delivery systems for lung cancer therapy and intracellular hyperthermia. This study provides theoretical and experimental insight into the biomedical applications of GSSG@Fe_3O_4.

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