Detection of Nano Eu$_3$O$_5$ in Cells and Study of its Biological Effects

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Abstract
The biological effect of rare earth represents the dual natures of promoting cell proliferation and apoptosis. The research on the biological effect of rare earth compound has aroused wide concerns, but it remained unknown for the transmembrane and distribution under the action of rare earth oxide nanoparticle with cell as well as its biological effects. In the present data, it was firstly observed that the nano Eu$_3$O$_5$ entered the living HeLa cell by endocytosis using Laser Scanning Confocal Microscope. The distribution of nano Eu$_3$O$_5$ was in the cytoplasm around the nucleus. Moreover, we studied the effect of nano Eu$_3$O$_5$ on living cells under the condition of in vitro culture. The result showed that within the low concentration range (<1.0 mg/mL), the nano Eu$_3$O$_5$ had no obvious effects on the apoptosis and the cell cycle, although the morphology appeared changes. When the concentration gradually rose, it had dramatic biological effects. 1.0 mg/mL nano Eu$_3$O$_5$ caused the cellular damages and led to the vacuolation on the cell surface. Meanwhile, it obviously promoted the apoptosis of Hela cells, which suggested that 1.0 mg/mL nano Eu$_3$O$_5$ induced a necrotic cell reaction with respect to the nature of cytotoxin.

Keywords: Nano Eu$_3$O$_5$; HeLa cells; Biological activity


1. Introduction
China is rich in rare earth resources and application, and rare earth mining, smelting, preparation and application are also growing and widespread. Rare earth is a non-life element, while our country has widely applied rare earth to agriculture and stock breeding, and even medicine, making rare earth increasingly enter the environment, food chain, and human body. Therefore, the research on human security to rare earth, i.e. physiology, toxicity, and pharmacology, has aroused wide concern from many aspects [1-5]. In recent years, nanotechnology has been applied to every field in biology and medicine [6-10]. However, there are few reports about the activity and effect of the nano materials of the rare earth oxide in biological medicine.

The biological security is always an important factor in inhibiting the development of nano science and technology. Firstly, we should focus on the security evaluation to the people engaged in the rare earth extraction, smelting and application, i.e. the effect of rare earth and its compound dust to the practitioners’ security, do these dusts enter the practitioners’ body, and if they do, what kind of effect do they cause in physiology and toxicity? Secondly, does the rare earth nanoparticle have medical value? And what function and effect does it have to the survival, growth, metabolism, and apoptosis of the normal and cancer cell. These are obvious questions need to be solved.

Eu$_3$O$_5$ is a kind of material with red fluorescence, and there has been already the report about the nano Eu$_3$O$_5$ material [11], but no report about the research on its biological effects on cell. For example, whether the nano Eu$_3$O$_5$ can enter cell like other nano materials, and where is it distributed after entering cell? How about the toxicity to cell? In this paper, we evaluate the effect of nano Eu$_3$O$_5$ on cell morphology and viability. The result showed that the low nano Eu$_3$O$_5$ concentration (<1.0 mg/mL) has less biological effects, while the relative high-concentration (≥1.0 mg/mL) has obvious toxic effects to the cell.
The nano Eu$_2$O$_3$ entered cells through endocytosis and localized just out the nuclear membrane. Finally, we studied the nature of cytotoxic reaction caused by 1.0 mg/ml nano Eu$_2$O$_3$ with respect to apoptotic or necrotic features.

2. Materials and Methods
2.1 Materials and instruments

Eu$_2$O$_3$ with the granularity of 60 nm (purity 99.91 %) was from Beijing Nachen Technology Development Co., Ltd. HeLa cell was provided by the Gene Engineering Center of Shanxi University. DMEM cell culture medium were purchased from Thermo Fisher Biological and Chemical Product Beijing Co., Ltd. The other materials used in this experiments listed as following: calf serum (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd), Trypsin (Sino-American Biotechnology Company), EDTA (Solarbio), PI (Sigma), RNase (Sigma), Triton X-100 (Sigma), 4 % paraformaldehyde (Solarbio), DIO (Beyotime Reagent), water-jacketed CO$_2$ cell incubator (Shanghai Lishen Scientific Instruments Co., Ltd), FQ-100DE numerical control ultrasonic wave washing machine (Kunshan Ultrasonic Instrument Co., Ltd), Laser Scanning Confocal Microscope (Olympus Fluoview FV1000), full automatic microplate reader (Bio-Rad Model 550), and fluorescence microscope (Olympus IX71).

2.2 Cell culture

Put the HeLa cells into the DMEM culture solution containing 10 % fetal calf serum, 37 °C, 5 % CO$_2$, and cultivate in the saturated humidity incubator. The cells pass one generation every two days after monolayer growth, and take the cells in the exponential phase of growth for use in the experiments.

2.3 Transmembrane of Nano Eu$_2$O$_3$ to HeLa cell

2×10$^5$ HeLa cells seeded into the 35 mm culture dish with the cover glass on the bottom. The cells were grown at 37 °C, 5 % CO$_2$. After 16 h, culture medium was replaced by culture solution containing the nano Eu$_2$O$_3$ after the ultrasonic shocking for 1.0 h. And the cells were incubated for 7 h. In the time, culture dishes were washed once with cold PBS and fixed with the 4 % paraformaldehyde for 8 minutes.

For the images shown in Figure 1. The nucleus and cell membrane were labeled with H33258 and green fluorescence indicator DIO, respectively. Fluorescence images were obtained using a Laser Scanning Confocal Microscope (Olympus Fluoview FV1000).

2.4 Cell viability measurement

5×10$^3$ healthy HeLa cells were plated into 96-well microtiter plates (Nunc). After 12 h, culture medium was replaced by 100 μL medium containing 0, 50, 200 and 400 μg/mL of nano Eu$_2$O$_3$, respectively. The cells were then incubated for 7, 24, 48 and 72 h. When the time was up, nano Eu$_2$O$_3$ solutions were aspirated and replaced by 180 μL DMEM. 20 μL MTT solution (5 mg/mL) were added into each well reaching a final concentration of 0.5 mg/mL MTT. After 4 h unreacted dye was removed by aspiration, the insoluble formazan crystals were dissolved in 200 μL dimethylsulfoxide (DMSO)/well with shaking for 10 minutes and measured spectrophotometrically in an ELISA reader at a wavelength of 490 nm. Each group has 6 samples, and the data obtained from two independent experiments. The spectrophotometer was calibrated to zero absorbance using culture medium without cells. The relative cell viability (%) related to control wells was calculated by $\frac{[A_{490} \text{ (sample)}]}{[A_{490} \text{ (control)}]} \times 100$ %

Where $A_{490}$ (sample) indicated the absorbency value using Eu$_2$O$_3$ to treat cells, and $A_{490}$ (control) indicated the absorbency value using DMEM only containing 10 % FBS to treat cells.

2.5 Cell morphologic observation

Cells (1.5×10$^5$) were seeded in a 35 mm petri dish and treated with media alone or with of 200, 400, 800, and 1000 μg/mL Eu$_2$O$_3$ for 7, 24, 28 h respectively. Following treatment, representative images were taken with the laser scanning confocal microscope and 40× objective.

2.6 Apoptosis (AnnexinV/PI assay)

To evaluate whether cell death induced by the Eu$_2$O$_3$ has apoptotic or necrotic features, 4×10$^5$ HeLa cells were seeded in a 60 mm Petri dish and treated with 200 μg/mL Eu$_2$O$_3$ for 7, 24, 48, and 72 h, respectively. Following treatment, cells were washed three times with cold PBS, and detached with trypsin-EDTA. The collected cells were applied to the apoptotic analysis with the Annexin V/PI cell apoptotic detection kit. The procedure followed the standard instruction and the cell suspension was subsequently analyzed by flow cytometry.

2.7 Cell cycle analysis by FACS

Cell cycle analysis and quantification of apoptosis was carried out by flow cytometry. Cells were seeded in 60 mm tissue culture dishes (2.0×10$^5$ cells/dish), allowed to attach overnight, and treated for 72 h with 200 μg/mL Eu$_2$O$_3$. At the end of the incubation period, detached cells were collected in 15 mL polypropylene centrifuge tubes along with the medium; culture dishes were washed once with PBS, adherent cells scraped off and combined in the same tube. After centrifugation (1000 rpm, 5 min) cells were fixed (ice-cold 70 % ethanol for 48 h) followed by incubation in a 500 μLmixture with 50 μg/mL of propidium iodide, 10
μg/mL of RNase and 1 % Triton X-100 for 20 min at room temperature in the dark. Flow cytometric analysis was performed on FACSScan. Ten thousand cells were analyzed for each sample.

3. Results

As an initial approach to evaluation of biological effects of nano Eu₂O₃, cell transmembrane experiment was carried out cells incubated with nano Eu₂O₃. It has been known that the nano Eu₂O₃ can be excited at 543 nm by preliminary experiments, thereby nano Eu₂O₃ is the measurable particle with the red fluorescence. In order to know whether the nano Eu₂O₃ particle can enter the living cells by endocytosis, we use the Laser Scanning Confocal Microscope to record the images of the distribution of nano Eu₂O₃ in the cells at different positions on Axle Z. Figure 1A (a) and 1A (c) indicated the localization of the particles of nano Eu₂O₃ in HeLa cells with red fluorescence excited by the 543 nm He-Ne laser. These two images implicated the relative position of nano Eu₂O₃ in the intact cells with ×40 object lens. Figure 1A (b) showed the nucleus with blue colour obtained by the 405 nm excitation and stained with H33258. Image 1A (d) is the merge of (b) and (c). It clearly indicated the nano Eu₂O₃ is round the nucleus, but can not enter it.

In order to further confirm that the nano Eu₂O₃ is in the cytoplasm, rather than the nucleus, we further stained the cell membrane with the green fluorescence indicator DIO, as figure 1B shows. Figure 1B (h) is the cells in the control group and the cell membrane was labeled with the green fluorescence. After treating the cells with the 400 μg/mL nano Eu₂O₃, Figure 1B (e, f ) showed green fluorescence DIO for the cell membrane and red fluorescence for the particles of nano Eu₂O₃. Figure 1B (g) is the merge of 1B (e) and (f). The images clearly indicated that the nano Eu₂O₃ particles with red fluorescence are in the cytoplasm range packed by the green cell membrane, and the red Eu₂O₃ particles are mainly distributed on the nucleolus surface, but can not enter the nucleolus. Hence, the experiments showed that nano Eu₂O₃ can certainly enter the living cells through endocytosis.

![Figure 1](image-url)

**Figure 1.** Distribution of the nano Eu₂O₃ in cells. A, (a) the imagine with bright field ; (b) the nucleolus stained with H-33258; (c) the nano Eu₂O₃ particles with red fluorescence; (d) the merge of b and c. B, (e) the cell membrane dyed with green fluorescence DIO; (f) the nano Eu₂O₃ with red fluorescence; (g) the merge of e and f; (h) the control group. Bar, 10 μm.
To determine effects of nano Eu$_2$O$_3$ on cell viability, the MTT test was used. After incubating the cells with the nano Eu$_2$O$_3$ at the concentration of 50-400 μg/mL for 24 h, the cell activity almost did not vary (figure 2A). In order to further confirm the effects of the low-concentration nano Eu$_2$O$_3$ on the cell growth at different time, cells were incubated with 200 μg/mL nano Eu$_2$O$_3$ for 7, 24, 48, and 72 h, respectively. The result as figure 2B showed. The data indicated that after incubating cells with Eu$_2$O$_3$ for 48 h, the cell viability did not change in comparison with control cells. When the treating time reached 72 h, the cell activity showed slight decreasing trend, and the cell activity decreased by about 16% at 72 h. The experiment results implicated that there was no obvious effects on the cell activity in the selected Eu$_2$O$_3$ concentration range and time.

**Figure 2.** The effect of the nano Eu$_2$O$_3$ on the cell viability by MTT assay. (A) HeLa cells treated with different concentration of Eu$_2$O$_3$ for 24 h; (B) Effects of 200 μg/ml Eu$_2$O$_3$ on HeLa cells at different times. Each column represents the mean ± SD of six samples. Visible absorption spectra of (a) the nanoparticles and (b) pure FITC.

**Figure 3.** Nano Eu$_2$O$_3$ induces morphology changes and apoptosis in Hela cells. (A) Cells treated with different concentration of Eu$_2$O$_3$ cells for 24 h; (B) Cells treated with different concentration of Eu$_2$O$_3$ for 28 h. Bar, 50 μm.
Next, we examined the effects of relative high concentration of nano Eu₂O₃ on the cellular proliferation and morphologies. Initially, cells were treated with different concentration of nano Eu₂O₃ (200, 400, 800, and 1000 μg/mL) for 24 and 28 h. Interestingly, the cell membrane was vacuolated after nano Eu₂O₃ treatment, even the cells treated with the 200 μg/mL nano Eu₂O₃, but there is no obvious cell number reduction in this concentration (figure 3A). On the other hand, there are obvious effects of the nano Eu₂O₃ on the cell apoptosis when the nano particles concentration is up to 1.0 mg/mL (figure 3B).

By contrast, cells showed dramatically apoptosis treated with the concentration of 1.0 and 4.0 mg/mL Eu₂O₃ for 28 h (Figure 3B). Figure 4C indicated that the apoptosis rate at the concentration of 1.0 mg/mL reached 98%.

The above-mentioned results indicated that the damage to the cells from the nano Eu₂O₃ particles increased obviously along with the concentration increasing. Moreover, we studied the effect of the nano Eu₂O₃ particles on the cell cycle. Figure 5 showed the expression pattern characteristics of the analysis of each cell cycle on the flow cytometry.

When the cells do not enter the fission process, they are on the position of G1 period of the cell cycle. Therefore, the quantity of G1 cells absolutely forms the highest signal peak on the flow expression pattern. In the cells of G1 period, there is a group of cells specially silent, not entering any biological features of the cellular circulation, and these cells are called the cells in G0 period. Similarly, the cells in G2 and M period have the DNA content twice as the normal cells in G1 period, and form the signal peak twice as in G1 period on the column diagram. Actually, the ratio of G2/G1 is...
always less than 2.0, maybe because the DNA-protein (chromatin) of the cells in G2 period gathers more closely or is more concentrated, there when the DNA dye is coloring, the ability of combining with the DNA locus is weakened. It is analyzed from figure 6 that after the 200 μg/mL Eu$_2$O$_3$ treating the cells for 72 h, compared with the control group, the ratio of G1/G0 decreased by about 3.44 %, the ratio of G2/M increased by about 0.33 %, and the ratio to the cells in S period increases by about 3.11 %. Therefore, it is concluded that the cells treated with the 200 μg/mL Eu$_2$O$_3$ had no obvious effect on the cell cycle. In this experiment, we also tried to measure the effect of high-concentration Eu$_2$O$_3$ on the cell cycle, however the high-concentration Eu$_2$O$_3$ (1.0 mg/mL) particles covered the monolayer cells in the culture dish, influenced the detachment and collection of the cells, and it was hard to obtain adequate cell quantity to make the cellular flow experiments.

The concentration of Ca$^{2+}$ in the cytoplasm must be in the strict control, and the Ca$^{2+}$ stability maladjustment must cause the severe cellular damage and death. Therefore, we further checked the effect of the Eu$_2$O$_3$ on the free Ca$^{2+}$ distribution in the cells. To adopt the Ca$^{2+}$ fluorescence indicator flour-3/Am to measure the variation of the intra-cellular free Ca$^{2+}$ level after the treatment of HeLa cells with 1.0 mg/mL nano Eu$_2$O$_3$, as figure 6 showed. The result indicated that, compared with the Ca$^{2+}$ level in the cells at quiescent stage, the addition of the nano particles made obvious Ca$^{2+}$ gathering in the cells, while in the control group, the Ca$^{2+}$ was well distributed in the cytoplasm. The experiments showed that the introduction of the nano particles made the Ca$^{2+}$ in the cells gathered and the local Ca$^{2+}$ concentration obviously rose. We therefore postulated the alteration of Ca$^{2+}$ distribution might lead to the Ca$^{2+}$ stability maladjustment in the cells, and further cause the cell damaging and the apoptosis.

The selected nano Eu$_2$O$_3$ particle diameter is 60 nm, while it is roughly estimated that the diameter of the nano Eu$_2$O$_3$ particle entering the cells is about 600 nm, observed with the Laser Scanning Confocal Microscope, i.e. the diameter turns larger by about 10 times. The reason is that the nano particles are in the DMEM (10 % FBS) suspending liquid, although dispersing them with ultrasonic wave, due to the gathering feature of the nano particle, factually, the diameter of the nano Eu$_2$O$_3$ particle in the suspending liquid we obtained is about 600 nm-1 μm, similar to the size of the particle entering the cells we observed. The size of the HeLa cell is 10-25 μm. It allows the gathered nano Eu$_2$O$_3$ entering the cells by endocytosis. In the present work, we firstly observed the nano Eu$_2$O$_3$ could enter the living HeLa cell through endocytosis. Our result showed that the cells were vacuolated with nano Eu$_2$O$_3$ treatment, even if the concentration of nano Eu$_2$O$_3$ is 200 μg/mL. When treating the cells with the high-concentration nano Eu$_2$O$_3$, there was obvious apoptosis effect on the cells. While quantitatively analyzed the effects of the low-concentration nano particles to the apoptosis and the cell cycle specificity with the flow cytometry, the result showed that the low-concentration nano Eu$_2$O$_3$ (<1.0 mg/mL) had no obvious biological effects, and the high-concentration nano Eu$_2$O$_3$ (≥1.0 mg/mL) had obvious toxic effects to the cells. Because the nano Eu$_2$O$_3$ cannot enter the cell through receptor-mediated, it may enter the cell through the nonspecific pinocytosis. During the serial layer scanning the cell with the Laser Scanning Confocal Microscope, it cannot found that the expected nano Eu$_2$O$_3$ particles gather on the inner wall of the cell membrane; but found that they gathered on the outer membrane of nucleolus, and meanwhile some part Eu$_2$O$_3$ particles was suspending randomly in the cytoplasm.

The nano Eu$_2$O$_3$ might be packed by the endosome, and this kind of existence and distribution status reduced their interference to the cell pathway and the cellular organ, which might be the reason that the low-concentration nano Eu$_2$O$_3$ has no obvious effect to the cellular biological activity. At the high concentration, after adopting the Ca$^{2+}$ fluorescence indicator flour-3/Am to measure the effect of with the 1.0 mg/mL Eu$_2$O$_3$, the free Ca$^{2+}$ level in the HeLa cells varied. The result indicated that, compared with the Ca$^{2+}$ level in the cells at quiescent stage, the addition of the nano particles result in the Ca$^{2+}$ in the cells gather, leading to the obvious rise of the local concentration. The inhibition of the high-concentration Eu$_2$O$_3$ to the cell proliferation maybe through the down-regulation of the Ca$^{2+}$-relying protein kinase C and the calmodulin, to inhibit the cancer cell growth, and its apoptosis mechanism still needs the further investigation.

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