Comparison of Biodistribution and Biocompatibility of Gelatin-Coated Copper Nanoparticles and Naked Copper Oxide Nanoparticles*

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In this study, we investigated the biocompatibility of gelatin-coated copper nanoparticles. To estimate their cytotoxicity, the coated copper nanoparticles were exposed to osteoblastic cells. The cell proliferation remained above 80% even when the particles concentration increased. When uncoated copper oxide nanoparticles were exposed to the cells, the proliferation ratio rapidly decreased with the concentration reaching 20% under the same conditions. To determine their biodistribution, the nanoparticles were administered to mice through their tail veins. The particles were subsequently found in some organs using an energy-dispersed X-ray spectrometer and inductively coupled plasma-atomic emission spectroscopy. The polymer-coated nanoparticles were observed in the lung, liver and spleen. They were also detected in the urine at higher concentrations than the copper oxide nanoparticles. Thus, the polymer coating is expected to improve biocompatibility by virtue of the excellent cytocompatibility and acceleration of the excretion process. [DOI: 10.1380/ejssnt.2012.33]

Keywords: Copper; Scanning Electron Microscopy; Nano-particles, quantum dots, and supra-molecules

I. INTRODUCTION

Recently, nanotechnology has developed drastically in several fields, and strong interest among many researchers and engineers has drive a wide range of nanotechnological applications [1–3]. Especially, inorganic nanoparticles have attracted interest because of their unique size-dependent properties. By virtue of this nanosizing effect, they are expected to show superior optical, electrical, and physical properties or chemical reactivity. For example, magnetite nanoparticles have been investigated for medical applications such as in hyperthermic oncology [4]. Gold, silver, and copper nanoparticles can be applied to highly sensitive biosensors based on the localized surface plasmon resonance and surface-enhanced Raman spectroscopy [5–7]. Copper nanoparticles have also received much attention for their use in electroconductive pastes for wiring or thin electrodes [8] or for multilayered ceramic capacitors [9] because the sizing effect induces their melting point depression. On the other hand, those nanoparticles are potentially biotoxic because of their high reactivity. When particles reach the nanolevel, some of them exhibit toxicity in vitro even if they are considered biocompatible at the macro level. Karlson et al., have reported that the cytotoxicity of metal oxide (including copper oxide) particles depended on the size decrease [10]. Tarantola et al. have reported that gold nanoparticles exhibited cytotoxicity depending on their shape [11]. In a previous study, we suggested that even biocompatible materials such as Ti and TiO₂ cause inflammation as their particle size decreases [12]. Therefore, it is important to understand the distribution of particles in the body of the materials and to elucidate their biotoxicity. To improve the biocompatibility of nanoparticles, we synthesized copper nanoparticles with a gelatin-modified surface. The size of the copper nanoparticles was controlled by the amount of gelatin, and the coating protected the particles from oxidation. In this study, we exposed the obtained polymer-coated particles to osteoblast cells. After 48 hrs cultivation with exposure, we estimated their cytotoxicity. The particles also were administered through the tail veins of mice to determine their biodistribution. After the administration, some organs were excised at several post-injection times, after which their biodistribution was observed using energy-dispersed X-ray spectroscopy and inductively coupled plasma-atomic emission spectroscopy.

II. EXPERIMENTAL

A. Preparation of copper nanoparticles

Copper oxide was purchased from Nisshin Chemco Co. (Osaka, Japan). Hydrazine monohydrate and ammonia aqueous solution (28%) were purchased from Kanto.
Copper nanoparticles coated with gelatin were synthesized with a hydrazine-reduction system similar to that used in our previous study [13]. CuO powders and gelatin were dispersed in 1 L of water and reduced by hydrazine at 80°C for 2 h. The obtained fine copper particles had a uniform diameter of ca. 100 nm. The obtained polymer-coated nanoparticles were characterized with an X-ray diffractometer (XRD: Rigaku Multi-flex, Tokyo, Japan). The morphology of the particles was determined with a scanning electron microscope (SEM: Hitachi S-4800, Tokyo, Japan). CuO nanoparticles used for the cytotoxicity measurements were also obtained from Sigma-Aldrich (St. Louis, MO, USA).

B. Cytotoxicity

To estimate the cytotoxicity of the CuNPs, we exposed the particles to mouse osteoblast-like cells (MC3T3-E1). To cultivate cells, alpha minimum essential medium (MEM) was used. MC3T3-E1 cells were seeded on 12-well plates at a density of $1 \times 10^4$/well and incubated at 37°C in a humidified 5% CO$_2$ atmosphere for 24 h. After this 24 h incubation, the culture medium was aspirated and another fresh medium including CuNPs was added. The cells were incubated for 48 h. The morphology of the cultured cells was observed using SEM. As a pretreatment for SEM observation, the non-adherent cells on the dishes were washed out with phosphate-buffered saline. The adherent cells were fixed on the dish with glutaraldehyde solution, and then the samples were critical-point dried. The cells were counted using a CellTiter-Glo assay kit (Promega, Madison, WI, USA), which measured intracellular ATP levels. CellTiter-Glo reagent was added to the culture medium and cell lysis was induced by shaking for 2 minutes. The luminescent signal was quantified using a luminometer (GloMax96 Microplate Luminometer, Promega). These procedures are described in detail in Ref. [14].

C. Biodistribution

Male mice (Jcl:ICR) were obtained from CLEA Japan, Inc. (Tokyo, Japan). The mice used ranged in age from 8 to 12 weeks. The polymer-coated nanoparticles were dispersed in saline. Their concentration was adjusted to 10 mg/mL and 0.6 mL of the dispersion was injected into the tail vein of each mouse. The organs (lung, liver, kidney, and spleen) were excised and subjected to fluorescence X-ray spectral analysis. The fluorescence X-ray spectra of the organ surfaces were measured by an energy-dispersed X-ray spectrometer (EDS) (Genesis, EDAX Japan, Tokyo, Japan) under the condition of 25 kV. To evaluate the concentration of particles in the organs, we calcined each organ at 800°C for 10 hrs in air and dissolved the samples in nitrohydrochloric acid. Their solutions were quantitated with inductively coupled plasma-atomic emission spectroscopy (ICP-AES, Hitachi, P-4010, Tokyo, Japan) and the concentration in each organ was estimated. Details of these measurements are described elsewhere [15]. All operations on animals were performed in accordance with the institutional animal care and use regulations of Hokkaido University.

III. RESULTS AND DISCUSSIONS

A. Sample preparation

Figure 1 shows typical SEM images of the polymer-coated copper nanoparticles in this study. The gelatin-coated particles were spherical shape and homogeneous in size. The diameters were almost 70 and 100 nm, respectively (as shown in Figs. 1(a) and (b)). For a counterpart, we selected naked CuO nanoparticles, because naked copper nanoparticles are readily oxidized in air into CuO. The TEM observation revealed that the obtained nanoparticles were covered by a gelatin layer of a few nanometers thickness [16]. To determine the chemical state, X-ray deflection analysis was carried out for the gelatin-coated copper nanoparticles (hereafter denoted as CuNPs(1) and (2)). Figure 2 shows the XRD profiles of CuNPs(1) and copper oxide nanoparticles (denoted as CuO NPs, 50.90 nm in diameter [17]). The observed XRD profile of
FIG. 2: XRD patterns of (a): polymer-coated copper nanoparticles (CuNPs (1)) and (b): copper oxide nanoparticles.

FIG. 3: Proliferation of MC3T3-E1 cells exposed to CuO and CuNPs (2), (n = 3).

CuNPs was completely different from that of CuO. It was assigned to that of metal copper. This result indicates that the nanoparticles consist of metal copper without copper oxide. In other words, the coated polymers protected against surface oxidation of the copper nanoparticles. In fact, we previously reported that the CuNPs showed no oxidation for months.

B. Cytotoxicity

To estimate the cytocompatibility of CuNPs, the particles were exposed to osteoblast-like cells (MC3T3-E1). The proliferation behavior was compared with that of CuO nanoparticles. As shown in Fig. 3, the proliferation ratio of MC3T3-E1 exposed to CuO decreased as the concentration increased. The value reached ca. 20% at 1 ppm. On the other hand, the ratio exposed to CuNPs (2) remained above 80% even at the highest concentration. When human hepatocytes, which are a type of human liver cell, were exposed to CuO or CuNPs, they showed similar proliferation behavior. Exposure to CuO NPs rapidly decreased the proliferation ratio depending on the concentration. In comparison, CuNPs exposure induced moderately decreasing behavior. These results suggested that the gelatin coating definitely improved cytocompatibility compared with bare CuO nanoparticles. In addition, the cell morphology also changed drastically when these cells were cultured with CuO NPs. Figure 4(a) shows a typical SEM image of non-exposed MC3T3-E1 cells as a control. They were spindle-shaped and elongated in one direction. In contrast, MC3T3-E1-exposed CuO particles showed a shrunken shape. Sometimes the edges of the cells were rolled up and many cracks appeared on their surfaces, as shown in Fig. 4(b).

Karlsson et al. reported that the cytotoxicity of different nanoparticles varies widely. They investigated the toxicity of several metal oxide nanoparticles (CuO, TiO$_2$, ZnO, CuZnFe$_2$O$_4$, Fe$_3$O$_4$, Fe$_2$O$_3$) and then mentioned that CuO nanoparticles were the most cytotoxic and did the most DNA damage. They also reported that CuO nanoparticles caused oxidative lesions and induced an increase in intracellular ROS (reactive oxygen species) [18]. They said that copper’s ability to damage mitochondria is a key mechanism in the toxicity of CuO nanoparticles, and that the release of Cu ions in the cell medium was not likely the main cause of the toxicity [10]. Heinlaan et al. reported that intimate contact between cells and particles...
seems to be more important for causing the toxicity of metal oxide particles and not necessarily for entering the cells. This may cause changes in the microenvironment in the vicinity of the organism-particle contact area and may either increase the solubilization of metals or generate extracellular ROS that may damage the cell membrane [19]. Those authors’ explanations are consistent with our results. The present results showed that the gelatin coating improved cell proliferation. The surface modification may prevented intimate contact between cells and particles, thereby reducing the toxicity of these particles compared with uncoated copper oxide nanoparticles.

C. Biodistribution

We administered copper particles through the tail veins of mice and then observed the time profiles of their body weight with post-injection time. As shown in Fig. 5, the body weight of CuNPs (1)-administered mice was maintained for more than 7 days. On the other hands the weight of CuO-administered mice decreased with post-injection time, and some of them passed away during the observation period. In other words, administered CuO NPs exhibit acute toxicity in mice, while CuNPs do not exhibit serious acute toxicity. This result suggests that the gelatin coating improved the biocompatibility of copper nanoparticles.

To investigate the biodistribution of the polymer-coated particles, the particles were administered to each mouse through the tail vein and some organs were excised at 24 hours layer. The specimens of the obtained organs were subjected to an energy-dispersed fluorescence X-ray spectrometer. Figure 6 shows typical fluorescence X-ray spectra of mouse organs after CuNPs (2) administration. The strong peaks around 8.1 and 9.0 keV were assigned to the copper element. The copper nanoparticles were also detected in the spleen and kidney. These results suggested that the administered copper nanoparticles were transported through the blood circulation and then arrived at these organs within 1 day post-injection. In our previous study, metal oxide nanoparticles such as TiO$_2$, CuO and In$_2$O$_3$ were temporarily trapped in the lung then re-transported to other organs [17, 20, 21]. On the other hand, metal particles, such as platinum or palladium, were quickly dispersed to some organs and the relative concentrations remained for several weeks at least. The biodistribution of CuNPs in this study showed a different tendency compared with metal oxide and was relatively close to that of metal particles. We speculated that the gelatin coating could promote the quick transport of the administered CuNPs to some organs. We also estimated the concentrations in some organs using an ICP-AES. In our previous study, CuO nanoparticles administered to mice accumulated mostly in the spleen, liver, lung, and kidney at 1 day post-injection [17]. The CuNPs indicated similar tendencies as shown in Fig. 7. However, the administered CuNPs were also detected in the urine at high concentrations. In the case of CuO administration, the particles were detected in urine at low concentrations only. This suggested that the gelatin-coated copper nanoparticles were quickly transported to some organs after administration and then induced the rapid excretion of CuNPs.
According to these results, the gelatin-coating modification reduced the cytotoxicity of copper nanoparticles compared with uncoated copper oxide nanoparticles. Also, this modification could induce quick circulation of nanoparticles after administration and then accelerate the excretion process. Since their congestion time in the body was shortened, rapid excretion would be expected to reduce their biotoxic risk.

IV. CONCLUSIONS

In this study, we investigated the biocompatibility of biopolymer-coated copper nanoparticles. This surface modification was found to reduce the cytotoxicity of the nanoparticles. To determine their biodistribution, the obtained particles were administered to each mouse through the tail vein. The polymer-coated nanoparticles were observed in the lung, liver, and spleen. They were also detected in the urine at high concentrations. Therefore, the polymer-coating modification is expected to improve biocompatibility by virtue of its excellent cytocompatibility and by the acceleration of the excretion process.

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