

Observation of Positively Charged Magnetic Nanoparticles Inside HepG2 Spheroids Using Electron Microscopy

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Magnetic resonance imaging (MRI) using magnetic nanoparticles has been used to diagnose vascular diseases as well as to monitor transplanted cells and tissues. In this study, we synthesized magnetic iron oxide nanoparticles (TMADM-03), electrically charged by the presence of a cationic end-group substitution of dextran, and observed these nanoparticles inside three-dimensional models of HepG2 spheroids, which mimic tissues. Patterned cell array glass disks were prepared to visualize the presence of TMADM-03 uptaken by HepG2 spheroids using transmission electron microscopy (TEM). The HepG2 cells (2×10^5 cells) were inoculated onto Cell-able™ 12-well plates. After 48 h of culture, the cells were incubated with 75 µg Fe/ml TMADM-03 in culture medium for 24 h. To investigate the cellular function of the HepG2 spheroids, the albumin secretion was evaluated by an ELISA. The albumin secretion after incubation for 24 h was reduced compared with the secretion prior to the addition of TMADM-03. TEM image samples were prepared in a planar direction or a vertical direction to the HepG2 spheroids on patterned cell array glass disks. The incorporation of TMADM-03 inside the HepG2 spheroids was confirmed. In addition, TMADM-03 could be observed in the deeper layers of the spheroids, and this was localized in the lysosomes. These data suggest that the novel magnetic iron oxide nanoparticles invade three-dimensional HepG2 spheroids.

Key words: Magnetic iron oxide nanoparticle; Cationic dextran; Human hepatocellular carcinoma cells (HepG2); Spheroids; Electron microscopy

INTRODUCTION

Magnetic resonance imaging (MRI) employing magnetic nanoparticles has been used to diagnose vascular diseases as well as to monitor transplanted cells and tissues. For example, while the engraftment of transplanted pancreatic islet cells can be indirectly evaluated by measuring the blood pressure of the portal vein and the amount of secreted insulin after transplantation into congenital type 1 diabetic patients (20,25,26), the transplanted cells are not easy to evaluate, and few such indirect evaluation methods are available for the monitoring of success at early stages of engraftment.

In previous reports, we described several different types of magnetic iron oxide nanoparticles, which are electrically charged by the presence of a cationic or anionic end-group substitution of dextran (18,21,22). Each of magnetic iron oxide nanoparticles consists of a small monocrystalline superparamagnetic iron oxide (SPIO) core that is stabilized by a cross-linked aminated dextran coating. We showed that positively charged nanoparticles [trimethylamino dextran-coated magnetic iron oxide nanoparticles (TMADM-03)], in which exists the cationic end-group substitution of dextran, could be transduced into the mouse insulinoma 6 (MIN6) β-cell line, but

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that three commercially available nanoparticles could not (22). In this study, we synthesized TMADM-03 nanoparticles and observed them inside three-dimensional (3D) human hepatocellular carcinoma cells (HepG2; liver hepatocellular carcinoma-derived cell line) spheroids, which mimic an environment close to natural tissues using Cell-able™ plates.

A 3D cell culture system, the “cell-array system,” having attracted increasing attention in the fields of cellular medicine and drug discovery research (10,13,16), was established to create a 3D model (7,17,23). Otsuka and Kataoka et al. demonstrated that multicellular spheroids comprising a combination of primary hepatocytes and endothelial cells (JCRB0099: HH) maintained stable liver-specific functions (23). In the present study, we used a cell array system of HepG2 spheroids and examined their uptake of TMADM-03 nanoparticles.

MATERIALS AND METHODS

Chemicals

Dulbecco's modified Eagle's medium (DMEM) and antibiotics (penicillin, streptomycin) were purchased from GIBCO BRL, Life Technologies (Grand Island, NY, USA). Fetal bovine serum (FBS, BIO-WEST) was obtained from Funakoshi Co., Ltd. (Tokyo, Japan). Phosphate-buffered saline (PBS), paraformaldehyde (PFA), and glutaraldehyde (GA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Photosensitive polymers were obtained from Toyo Gosei Co., Ltd. (Chiba, Japan). Osmium tetroxide was purchased from Nisshin EM Co., Ltd. (Tokyo, Japan). Uranyl acetate was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other materials and chemicals not specified above were of the highest grade available.

Cells

HepG2 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA).

Magnetic Iron Oxide Nanoparticles

Trimethylamino dextran-coated magnetic iron oxide nanoparticles (TMADM-03) were kindly provided by MEITO Sangyo Co., Ltd. (Kiyosu, Japan).

Preparation of Patterned Cell Array Glass Disks

Patterned cell array glass disks were prepared to visualize the presence of iron oxide nanoparticles uptaken by the HepG2 spheroids using transmission electron microscopy (TEM). The procedures were basically performed according to the previously reported protocol (23). Glass disks (21 mm in diameter, 0.1 mm in thickness; Matsunami Glass, Osaka, Japan) were treated with

an aqueous solution of photosensitive polymers, and the polymers were fixed by ultraviolet irradiation under patterned occlusion masks with 100- μ m diameter circles at 100- μ m intervals. After washing off all unconjugated polymers, the cell array disks were obtained with a regular dot pattern where cells can adhere. The viability and albumin secretion of the cultures were assessed to ensure that there were no significant differences between the cells cultured on cell array glass disks and those cultured on the Cell-able™ 12-well plates.

Culture of HepG2 Cells or Spheroids

HepG2 cells (2×10^5 cells) were inoculated onto Cell-able™ 12-well plates (Transparent Inc., Chiba, Japan) and cultured in 1 ml of DMEM containing 10% FBS, 100 U/ml penicillin, and 100 U/ml streptomycin (culture medium). As a control, HepG2 cells were cultured in 12-well plates (Falcon3043, Becton Dickinson, Franklin Lakes, NJ, USA) as monolayers. The medium was changed 24 h after cell seeding. After 48 h of culture, the cells were incubated with 75 μ g Fe/ml TMADM-03 in culture medium for 24 h. The cells were further incubated for 72 h at 37°C in an incubator with a humidified 5% CO₂ atmosphere. The viability of cells was determined by the trypan blue dye exclusion test (Trypan Blue Stain, Gibco BRL).

Quantitation of Albumin Secreted by HepG2 Cells and Spheroids

The albumin secretion by HepG2 cells was measured with a sandwich enzyme-linked immunosorbent assay (ELISA) using a human albumin ELISA quantification kit as instructed by the manufacturer (Bethyl Laboratories, Inc., Montgomery, TX, USA).

Transmission Electron Microscopic Observation of HepG2 Cells and Spheroids

TEM was used to visualize the presence of iron oxide nanoparticles inside the HepG2 cells and their spheroids. The HepG2 cells or spheroids labeled with TMADM-03 were fixed with 2% PFA and 2% GA in 0.1 M phosphate buffer (PB) pH 7.4 at 37°C and then cooled down to 4°C for 30 min. Thereafter, they were fixed with 2% GA in 0.1 M PB at 4°C overnight. Afterward, these fixed samples were rinsed three times with 0.1 M PB for 30 min each, followed by postfixation with 2% osmium tetroxide (OsO₄) in 0.1 M PB at 4°C for 1 h.

The samples were dehydrated through a series of graded ethanol concentrations (50%, 70%, 90%, and 100%). The schedule was as follows: 50% and 70% for 15 min each at 4°C, 90% for 5 min at room temperature, and three changes of 100% for 5 min each at room temperature. The

samples were then transferred to a resin (Quetol 812; Nisshin EM Co., Tokyo, Japan) and polymerized at 60°C for 48 h. The obtained blocks were ultrathin sectioned at 70 nm with a diamond knife using an ultramicrotome (ULTRACUT UCT; Leica, Tokyo, Japan), and the sections were placed on copper grids. They were stained with 2% uranyl acetate at room temperature for 15 min and then rinsed with distilled water followed by being secondary stained with lead stain solution (Sigma-Aldrich Co.) at room temperature for 3 min.

The grids were observed by transmission electron microscopy (JEM-1200EX; JEOL Ltd., Tokyo, Japan) at an acceleration voltage of 80 kV. Digital images (2,048×2,048 pixels) were taken with a CCD camera (VELETA; Olympus Soft Imaging Solutions GmbH, Münster, Germany).

RESULTS

Cell Labeling With Magnetic Iron Oxide Nanoparticles

We developed novel magnetic iron oxide nanoparticles, electrically charged by the presence of a cationic end-group substitution of dextran (TMADM-03) (Fig. 1). In order to label cells with TMADM-03, the HepG2 cells and spheroids (2×10^5 cells) were seeded into each culture plate and incubated for 72 h at 37°C in a humidified

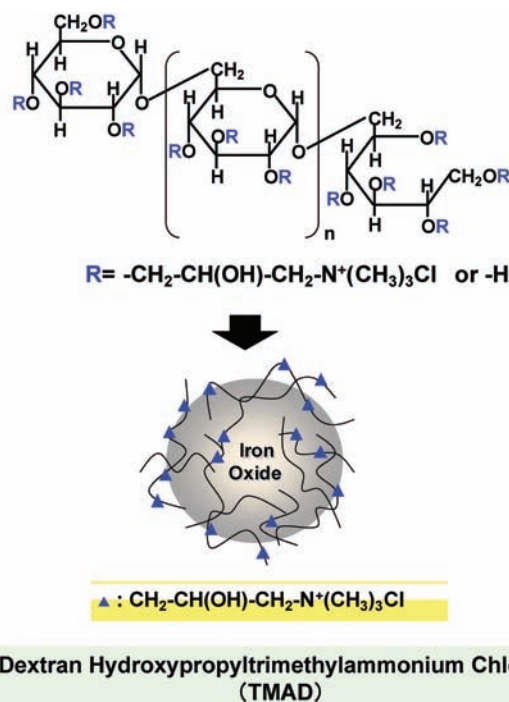


Figure 1. A schematic illustration of the magnetic iron oxide nanoparticles used in this study. The trimethylamino dextran-coated magnetic iron oxide nanoparticles (TMADM-03).

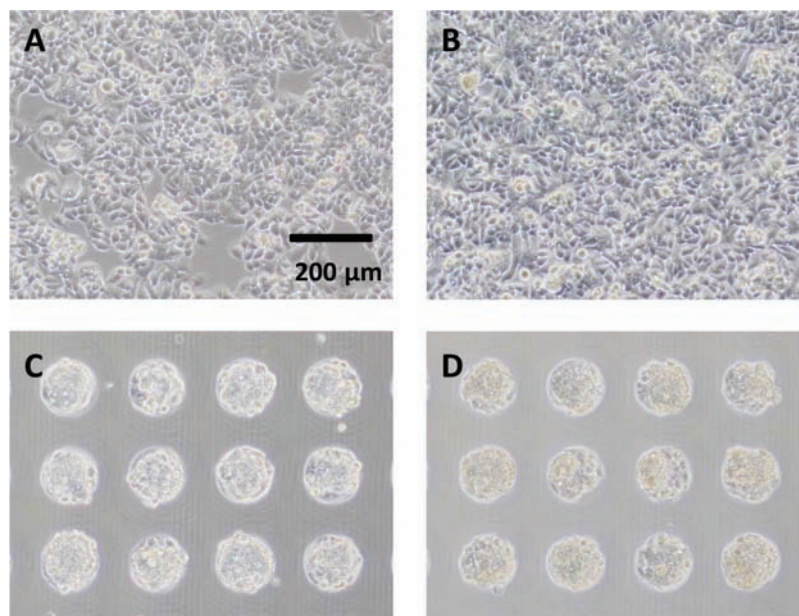


Figure 2. Phase-contrast photomicrographs of HepG2 cells and spheroids. The human hepatocellular carcinoma (HepG2) cells cultured on a standard 12-well plate (A, B) and HepG2 spheroids formed on a Cell-able 12-well plate (C, D) 72 h after inoculation. After 48 h of cell culture, the HepG2 cells were incubated with 75 μg Fe/ml TMADM-03 in culture medium for 24 h. The HepG2 cells labeled with TMADM-03 (B) and HepG2 spheroids labeled with TMADM-03 (D) are shown. Scale bar: 200 μm .

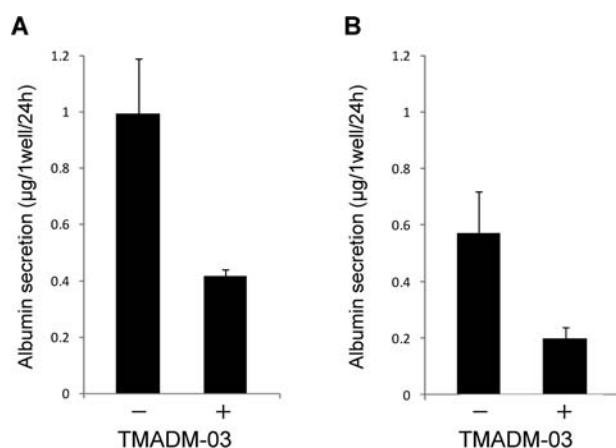


Figure 3. Albumin secretion from HepG2 cells and multicellular spheroids. (A) The HepG2 cells were incubated with 75 μg Fe/ml TMADM-03 (+) for 24 h. (B) The HepG2 spheroids were incubated with 75 μg Fe/ml TMADM-03 (+) for 24 h. As control experiments, HepG2 cells or spheroids were incubated without TMADM-03 (-) for 24 h. The data shown represent the means and SD of three independent experiments.

5% CO₂ atmosphere (Fig. 2). After incubation for 48 h, the HepG2 cells and spheroids were incubated for 24 h at 37°C in a humidified 5% CO₂ atmosphere with TMADM-03 (75 μg Fe/ml) reconstituted in culture medium (Fig. 2B and D). The morphology of both HepG2 cells and spheroids was similar to that of untransduced cells (Fig. 2A–D). However, the color of HepG2 spheroids labeled with TMADM-03 was a little darker compared with the control. The colors of HepG2 spheroids before and after labeling with TMADM-03 are shown in Figure 2C and D.

We also measured the cell viability by trypan blue exclusion assay under the conditions used for the experiments shown in Figure 2. There were very few dead cells after 72 h of cell culture (for Fig. 2A and C, the cell viability was about 95.3% and 93.6%, respectively). After the addition of TMADM-03 to the cells and incubation for 24 h, the viability was reduced compared to a 24-h incubation without TMADM-03 (for Fig. 2B and D, the cell viability was about 93.1% and 89.6%, respectively).

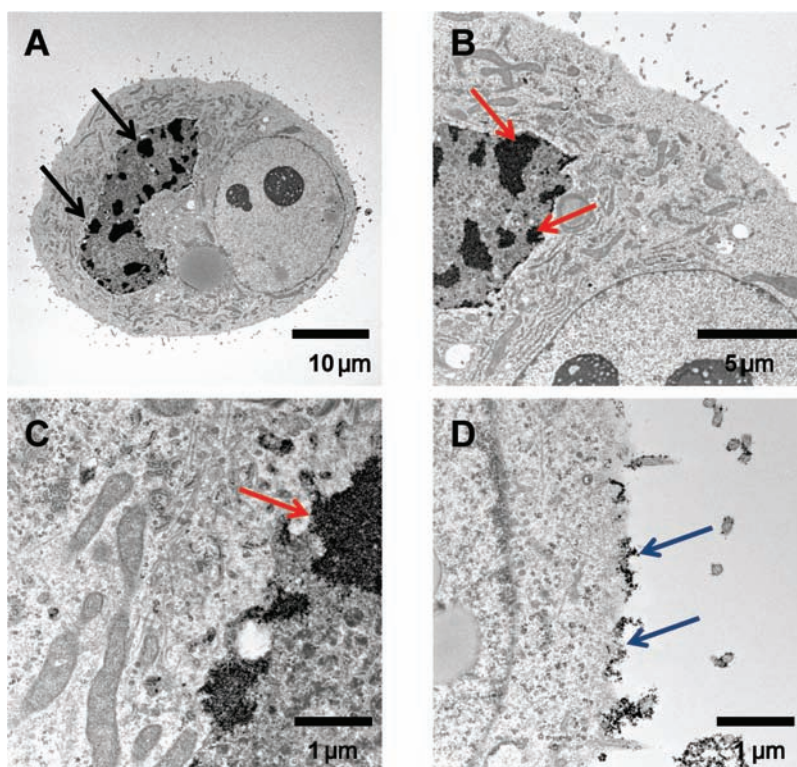


Figure 4. Transmission electron microscope (TEM) images of horizontal cross sections of HepG2 cells that had taken up TMADM-03 magnetic nanoparticles. (A) The morphology of HepG2 cells labeled with TMADM-03. The arrows point to lysosomes containing the nanoparticles (1,740×). (B) The nanoparticles (red arrow) were detected in the lysosomes (4,860×). (C) Another photograph of the nanoparticles (red arrow) in the lysosomes (18,400×) and (D) nanoparticles (blue arrows) attached to the cell membrane (18,400×).

Functional Examination of HepG2 Cells and Spheroids

To investigate the cellular function in the HepG2 spheroids, the albumin secretion was evaluated using an ELISA. The amount secreted by HepG2 cells and spheroids is shown in Figure 3A and B, respectively. The HepG2 cells and spheroids (2×10^5 cells) were incubated for 48 h at 37°C in a humidified 5% CO₂ atmosphere. After incubation for 48 h, the HepG2 cells and spheroids were incubated for another 24 h at 37°C in a humidified 5% CO₂ atmosphere with TMADM-03 (75 µg Fe/ml) reconstituted in culture medium. The albumin secretion after incubation for 24 h was reduced compared to a 24-h incubation without TMADM-03. The albumin secretion showed a decreasing trend in both the cell monolayer cultures and spheroid cultures.

TEM Images of HepG2 Cells That Had Taken Up Magnetic Nanoparticles

The incorporation of magnetic nanoparticles inside the HepG2 cells was confirmed by TEM (Fig. 4). Figure 4A shows whole images of HepG2 cells. Enlarged images of HepG2 cells are shown in Figure 4B–D. The presence of

iron was confirmed after 24 h by chemical fixation, and it was found that more iron was associated with HepG2 cells in the presence of TMADM-03 than without and that this was localized in the lysosomes (Fig. 4A–C). In addition, it was located closer to the cell membrane (Fig. 4D).

TEM Images of Horizontal Cross Sections of HepG2 Spheroids That Had Taken Up Magnetic Nanoparticles

After incubation for 48 h, the HepG2 spheroids were incubated for 24 h at 37°C in a humidified 5% CO₂ atmosphere with TMADM-03 (75 µg Fe/ml) reconstituted in culture medium. Each sample was prepared in a planar direction to HepG2 spheroids on patterned cell array glass disks. The presence of iron was confirmed by TEM (Fig. 5). The incorporation of magnetic nanoparticles inside the HepG2 spheroids was confirmed (Fig. 5). Figure 5A shows a whole image of HepG2 spheroids. The enlarged images of HepG2 spheroids are shown in Figure 5B–D. In addition, magnetic iron oxide nanoparticles could be observed in the deeper layers of the spheroids (Fig. 5A), and these were localized mainly in the lysosomes (Fig. 5B–D).

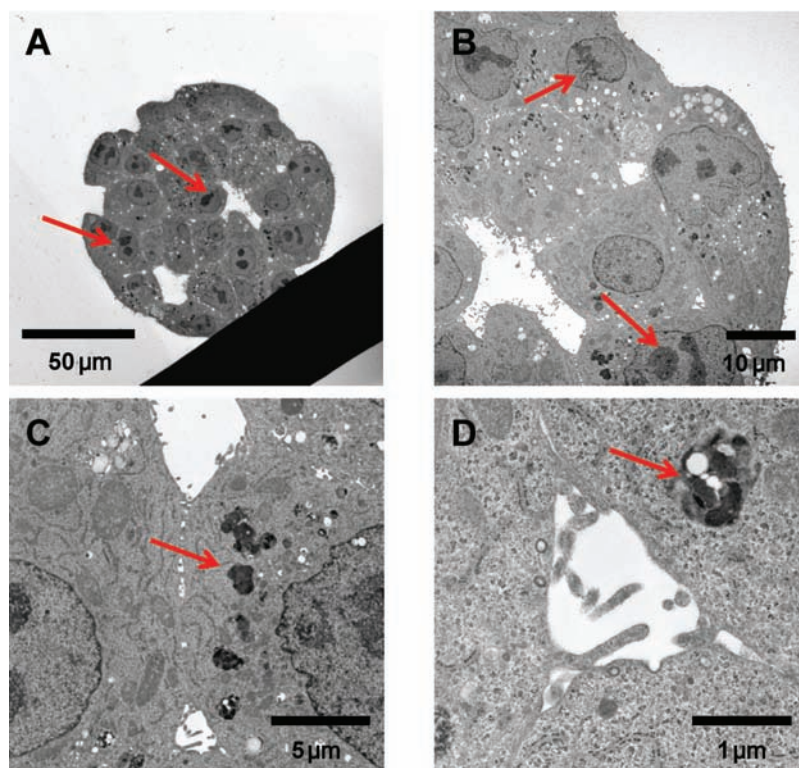


Figure 5. TEM images of horizontal cross sections of HepG2 spheroids that had taken up TMADM-03 magnetic nanoparticles. (A) Each sample was prepared in a planar direction to the patterned cell array glass disks. The morphology of HepG2 spheroids labeled with TMADM-03 is shown. The red arrows point to lysosomes containing the nanoparticles (579×). (B) A photograph showing the nanoparticles (red arrow) localized in the lysosomes (1,740×). (C) Another photograph of the nanoparticles (red arrow) in the lysosomes (4,860×). (D) A higher magnification photograph showing nanoparticles (red arrow) in the lysosomes (18,400×).

TEM Images of Vertical Cross Sections of HepG2 Spheroids That Had Taken Up Magnetic Nanoparticles

Each sample was prepared in a vertical direction to HepG2 spheroids on patterned cell array glass disks. Figure 6A shows a whole image of HepG2 spheroids. Enlarged images of HepG2 spheroids are shown in Figure 6B–D. Magnetic iron oxide nanoparticles could be observed in the deeper layers of the spheroids (Fig. 6A) by TEM. The iron was detected in the lysosomes (Fig. 6B–D).

DISCUSSION

To observe cells and tissues posttransplantation, they can be labeled with magnetic metallic oxides, such as SPIO before transplantation, and then tracked by MRI (1,2,4–6, 8,9,11,14,15,19,24,27). Techniques for visualizing therapeutic cells used for cell therapy is highly sought after since a patient's postoperative course can thus be monitored noninvasively. It has been proposed that cells can be labeled using a complex of polysaccharides, such as magnetic metallic oxides–dextran. For example, it was reported that magnetic metallic oxide nanoparticles covered with diethylamino-dextran (3) and surface-modified

iron oxide nanoparticles (IONs) were obtained by coprecipitating a pullulan and its derivatives in an ammonium aqueous solution of $\text{Fe}^{2+}/\text{Fe}^{3+}$ (12). However, commercially available magnetic nanoparticles are not efficiently transduced into cells. In previous reports, we described the novel magnetic iron oxide nanoparticles, which are electrically charged by the presence of a cationic end-group substitution of dextran (21,22).

Novel magnetic iron oxide nanoparticles improve the efficiency of their uptake into MIN6 cells (22). These nanoparticles could be used to observe transplanted cells inside the body (data not shown). In experiments using in vitro models, we observed the localization of nanoparticles in greater detail, specifically within 3D models, which mimic environments close to that of natural tissues using the Cell-able plates. Albumin secretion by HepG2 cells incubated with TMADM-03 was lower than by those incubated without TMADM-03 in 2D and 3D cultures. The decrease in albumin secretion might be due to the suppressed cell proliferation.

In the present study, TMADM-03 was efficiently incorporated into HepG2 spheroids. We observed TEM image

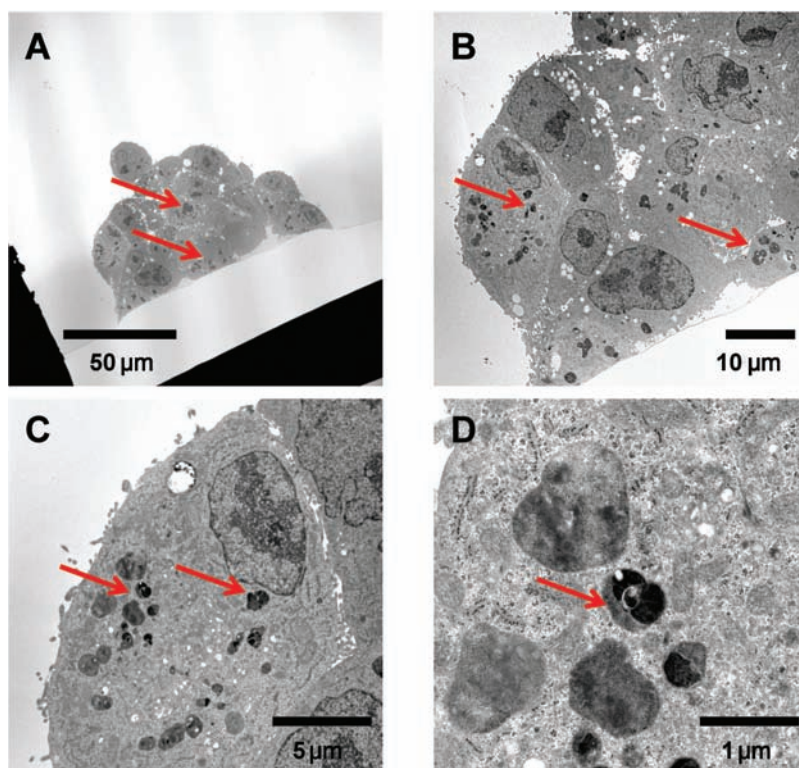


Figure 6. TEM images of vertical cross sections of HepG2 spheroids that had taken up TMADM-03 magnetic nanoparticles. (A) Each sample was prepared in a vertical direction to the patterned cell array glass disks. The morphology of HepG2 spheroids labeled with TMADM-03. The arrows point to lysosomes containing the nanoparticles (579 \times). (B) Another photograph of the nanoparticles (red arrow) localized in the lysosomes (1,740 \times). (C) An enlarged photograph showing the nanoparticles (red arrow) in the lysosomes (4,860 \times). (D) A high magnification photograph showing the nanoparticles (red arrow) detected in the lysosomes (18,400 \times).

samples that were prepared in a planar direction or a vertical direction to the HepG2 spheroids. The incorporation of TMADM-03 could be confirmed in the deeper layers of the spheroids, and the nanoparticles were observed on sections of both the planar direction and vertical direction. In addition, TMADM-03 nanoparticles were localized in the lysosomes of the HepG2 spheroids. Both HepG2 cells (2D cultures) and HepG2 spheroids (3D cultures) showed a tendency for the TMADM-03 nanoparticles to be introduced mainly into the lysosomes.

In conclusion, we observed HepG2 spheroids, which mimic an environment close to that of natural tissues, and examined, in detail, the localization of TMADM-03. Results obtained using this evaluation system are expected to be useful for drug development applications employing the Cell-able plates.

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