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Intracellular release of fluorescein anion from layered double hydroxide nanoparticles indicating endosomal escape

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Abstract. In recent years, layered double hydroxide (LDH) has been attempted to be applied to a molecular container due to their anion exchange ability, low cytotoxicity and good biocompatibility. In this paper, we investigated the intracellular behaviour of LDH particles in mammalian cells after internalization. Nanoparticles of fluorescein (Fluo) intercalated LDH, Fluo/LDH, were prepared by the coprecipitation followed by subsequent hydrothermal treatment. As-prepared Fluo/LDH particles have the LDH structure and morphology of hexagonal sheet of 100 nm on the average. In addition, Fluo/LDH also exhibited high green fluorescence and low cytotoxicity. By a confocal laser scanning microscopy, the dim green fluorescence was observed throughout cells, including the nucleus. This result indicated that Fluo/LDH released guest anion (Fluo) from LDH structure inside cells. Furthermore, because the fluorescence was observed throughout the cell, Fluo was not retained within endosome structure, i.e., Fluo/LDH was dissolved to release Fluo from endosome.

1. Introduction

The layered double hydroxide (LDH) is widely known as an anionic clay, host-guest material and anion-exchanger. The chemical composition of LDH is represented by the general formula of $[M^{2+}_{1-x}M^{3+}_{x}(OH)_2](A^{n-}_{x/n}\cdot yH_2O)$, where M^{2+} is a divalent cation, such as Mg^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, and M^{3+} is a trivalent cation, such as Al^{3+}, Fe^{3+}, Cr^{3+}, and Co^{3+}, and A^{n-} is an exchangeable anion, such as OH^-, Cl^-, NO_3^-, CO_3^{2-}, SO_4^{2-}, and various organic anions. The LDH has a positively charged hydroxide basal layer, $[M^{2+}_{1-x}M^{3+}_{x}(OH)_2]$, due to the trivalent cation substituted for the divalent cation in the hydroxide basal layers, and an intercalated anion for charge compensation with water molecules into the occupying interlayer space.

In recent years, interesting researches have focused on a cellular uptake efficiency of the biomolecule/LDH composites. When some biomolecules, such as gene, DNA, peptide, are intercalated to the interlayer of LDH [1][2][3], the biomolecule/LDH composites have positive zeta potential [4][5][6]. On the other hand, cellular membrane, which consists of phospholipid groups has negative surface charge. Thus, biomolecule/LDH can approach to a cellular membrane easier than biomolecule alone. In fact, some anticancer drug/LDH composites exhibited higher cytotoxicity than drug alone [7][8][9]. Besides, fluorescein disodium salt (Fluo), an anionic dye, was transported into cells when

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Fluo was composited with LDH, nevertheless, Fluo was not transported when cells were incubated with Fluo alone [10]. This result also suggests that a positive LDH layer encourages the cellular uptake of negative guest ion. Moreover, the particle size of LDH can be controlled from nano-order to micro-order [11][12][13][14][15], the morphology of the secondary particle can also be controlled [16][17][18], and some functional groups can be added on the surface of the LDH basal layer [8]. Therefore, the LDH has a high potential as effective non-viral agents for the cellular drug delivery.

However, the cellular transportation efficiency of biomolecule/LDH composites is still not enough. We attempted a visualization of the intracellular behavior of LDH particles in order to clarify the cellular uptake mechanism and explore the most essential function of LDH to achieve high efficiency. In our previous works, Fluo/LDH particles were encompassed by a cellular membrane and internalized by a vesicle including Fluo/LDH particles within 10 min after the addition of LDH particles [6]. This vesicle, which consists of cellular membrane is called endosome, and this transportation mechanism of extracellular materials via endosome is called endocytosis. Thus, it is certain that the Fluo/LDH particles internalized by endocytosis within only 10 min incubation. Furthermore, after incubation for 30 min, Fluo/LDH particles were located not only in the endosome structure but on the cytoplasm. This result indicated that LDH particles were released from endosome. Although the Fluo/LDH particles were located on the periphery of the nuclear membrane, there were no particles inside the nucleus even after incubation time for 24 h. On the other hand, some reports described the transportation mechanism of LDH particles by using cell entry inhibitors [5][12]. It is considered that LDH particles are basically transported into the cell by the clathrin-mediated endocytosis [19]. However, how LDH particles behave in the cell after internalization (transportation, degradation, release and exportation) has not been clarified yet. Thus, we investigated the intracellular fluorescence behavior by using a confocal laser scanning microscope (CLSM), where Fluo was used as a guest molecule because it exhibits the high green fluorescence, low cytotoxicity and high thermal stability [6][20].

2. Experimental procedures

2.1. Preparation of Fluo/LDH nanoparticles

First, the precursor chloride ion intercalated LDHs (Cl/LDH) were prepared by the coprecipitation followed by subsequent hydrothermal treatment as already reported [21]. 10 mL mixed solution of 0.4 M $MgCl_2$ and 0.2 M $AlCl_3$ (Mg/Al molar ratio = 2.0) was quickly added (within 5 s) into 40 mL of 0.15 M NaOH solution under vigorous stirring at room temperature, where the solution was mixed under a N_2 atmosphere to prevent contamination by atmospheric CO_2. After aging for 10 min, the Cl/LDH slurry was obtained via centrifuge separation, washed several times with distilled water and then manually dispersed in 40 mL of distilled water. This aqueous suspension was transferred into a stainless steel autoclave with a Teflon lining. The autoclave was then placed in a preheated oven, followed by hydrothermal treatment at a temperature of 100 °C for 16 h. After air-cooling, a stable homogeneous Cl/LDH suspension was given. The as-obtained suspensions generally have a Cl/LDH concentration of about 0.4 wt. %. The LDH yield of 60 ±5% has been calculated from AAS and TG-DTA data as well as the LDH mass collected from suspensions.

To prepare the Fluo/LDH suspensions, Fluo was intercalated by the ion-exchange method. The as-prepared Cl/LDH slurry was transferred into a Erlenmeyer flask under vigorous stirring at room temperature with the reactor isolated from air. Then, 1.2mL of 25mM Fluo solution was quickly added (within 5 s) into LDH slurry, followed by 1 h stirring. The Fluo/LDH slurry was heated by a hot stirrer to collect Fluo/LDH nanoparticles.

2.2. Characterization

Powder X-ray diffraction (XRD) measurements were performed using a Bruker D2 PHASER, with Cu Kα radiation of 10 mA, 30 kV and 2θ angle ranged from 2° to 70°. Fourier-transform infrared (FT-IR) spectra were recorded on a Jasco FT/IR-4200 by the KBr disk method. For each spectrum, 16 scans

from 4000 to 400 cm^{-1} were performed at a resolution of 4 cm^{-1} to measure the FTIR absorbance of KBr discs containing 1–2 wt.% of LDH sample. Morphology of Fluo/LDH particles was observed by a JEOL JSM-6510LA scanning electron microscope (SEM) at an acceleration voltage of 10 kV and a JEOL JSM-2100 transmission electron microscope (TEM) at an acceleration voltage of 200 kV. For TEM imaging, the LDH slurry was treated with ultrasonication for 10 min, and then a droplet was dropped on a copper grid coated with carbon film. The fluorescence of Fluo/LDH particles was obtained on a Nikon Confocal Microscope C2 si.

2.3. Intracellular fluorescence of cells with Fluo/LDH particles

The cellular uptake of the Fluo/LDH for mammalian cell, L929 (mouse, connective tissue), has confirmed by the observation of intracellular fluorescence. The cells were grown to semi-confluency in Eagles's minimum essential medium (MEM) supplemented with 10% heat inactivated fetal bovine serum without antibiotics and incubated at 37°C in a 5% CO_2 humid incubator. Cells were washed once with a phosphate buffered saline (PBS) and treated with Trypsin-EDTA for 1 min. Whole cells were collected by centrifugation at 1000rpm for 3 min and re-dispersed to 10 mL of MEM. The cell suspension was dropped to a glass bottom dish and pre-incubated for 24 h at the same condition. After pre-incubation, the nucleus of cell was stained using DNA-specific fluorescent dye, Hoechst 33342. Hoechst 33342 was diluted to 2 μg/mL with PBS and incubated with cells for 30 min. After the nucleus stains, the Fluo/LDH was added to the cell. The sterilized Fluo/LDH particles were dispersed for the MEM as 100 μg/mL. The MEM with LDHs was added to a glass bottom dish, and then cells were incubated with the samples for a given period of time under the same condition. Fluorescence images were taken using a Nikon Confocal Microscope C2 si at the excitation wavelength of 405 nm (Hoechst) and 488 nm (Fluo).

3. Results and discussion

3.1. Characterization of Fluo/LDH nanoparticles

A homogeneous Cl/LDH suspension was obtained after hydrothermal treatment of the pre-Cl/LDH slurry. As-prepared suspension was stable for 6 months, and these Cl/LDH particles consisted of hexagonal sheet of 100–150 nm of the average size as observed with TEM as shown in our previous report [21]. he XRD pattern of Fluo/LDH is shown in Figure 1. The intensive diffraction peaks with the LDH structure were observed at d_{003} = 0.75 nm and d_{006} = 0.38 nm. The diffraction peak of (110) was also supported the assignment to LDH structure, indicating the existence of hydroxide layer as a two dimensional crystal [22]. There is no significant expansion in the interlayer distance of Fluo/LDH, because when low content of Fluo is in interlayer, Fluo anion is arranged in horizontal arrangement in the interlayer spacing or adsorbed on the surface of LDH [10]. Small peaks of impurity phase are also detected. These diffraction peaks can be indexed to Gibbsite $Al(OH)_3$ (JCPDS No. 07-0324). Furthermore, there are some rod-like particles in TEM observation. It was found that the rod-like particles contained a lot of Al element by EDS analysis (inset of Figure2). This contamination was also reported in a previous report for the fluorescein isothiocyanate intercalated LDH [5].

Fluo/LDH nanoparticles showed the high green fluorescence by fluorescence microscopy in Figure 2 (a). Furthermore, Fluo/LDH nanoparticles exhibited very low cytotoxicity as a result of the colony formation assay as same as our previous report [6]. Moreover, after ion exchange with fluorescein aqueous solution, the morphology of hexagonal sheet of 100–150 nm in diameter was still retained (Figure 2 (b)). These particles seem to be efficient for cellular uptake, because it was reported that LDH possessing a particle size of less than 200 nm showed excellent efficiency for cellular uptake. LDH particles with a size range of 50 to 200 nm could be primarily internalized into cells through the clathrin-mediated endocytosis, although the 350 nm-sized LDH was not selected in endocytic pathways. This is highly correlated with the efficient cellular uptake of the present Fluo/LDH samples observed by Fluorescence-Activated Cell Sorter (FACS) analysis [12]. Tus, we consider that this Fluo/LDH nanoparticle can be used as an efficient probe for the visualization of cellular uptake.

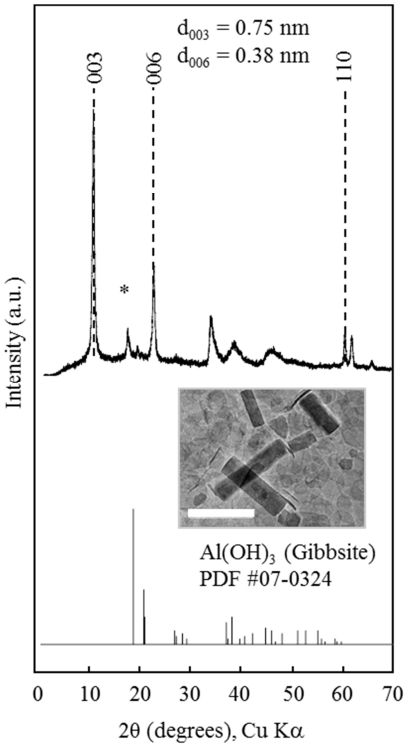


Figure 1. XRD pattern of Fluo/LDH (Inset: TEM image of impurity Gibbsite particles. The scale bar is 200 nm.).

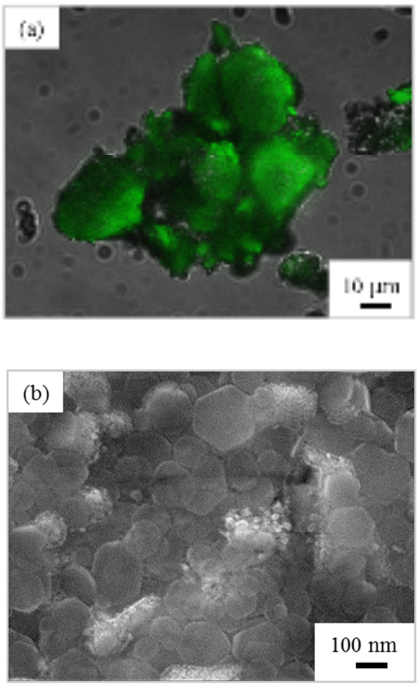


Figure 2. (a): Fluorescence image of Fluo/LDH. (b): SEM image of Fluo/LDH.

3.2. Investigation of intracellular behaviour of Fluo/LDH nanoparticles

In order to define the intracellular behavior of Fluo/LDH nanoparticles, we observed the intracellular fluorescence by using CLSM. Confocal images are shown in Figures 3 and 4. Figure 3 shows the cell after incubation with Fluo/LDH dispersion for 30 min. The blue color in Figure 3 (b) represents a stained nucleus by Hoechst 33342 and the green color in Figure 3 (c) represents the emission of Fluo. After 30 min of incubation, both of intercellular and extracellular Fluo/LDH particles showed green fluorescence. It indicated that some of Fluo/LDH particles were internalized in cells, and this result is consistent with the previous TEM observation [6]. Figure 5 shows the cells after incubation with Fluo/LDH dispersion for 1 h. In Figure 4 (c), there are two kinds of green fluorescence. First, local intense fluorescence are in the cells (white arrows). Besides, in the differential interference contrast (DIC) images (Figure 4 (a)), there are black ones at both ends of cell (black arrows). These black ones (Figure 4 (a)) are located at the same position of the intense fluorescence (Figure 5 (c)). These results indicate that the intense green emission was derived from Fluo/LDH nanoparticles. On the other hand, the dim green fluorescence was spread throughout the cell, nevertheless, Fluo/LDH particles were not located on whole cell. In addition, this fluorescence was quickly quenched, when the cell as exposed to laser. These results indicate that this dim green emission was derived from Fluo anion. It is suggested that this Fluo anion was released from Fluo/LDH particles, because Fluo anion was not transported to cells when Fluo solution was added to the cells [10]. Furthermore, Fluo anion seemed to be escaped from endosome after released from Fluo/LDH, and transported to the nucleus, because whole cells, including nucleus exhibited green fluorescence.

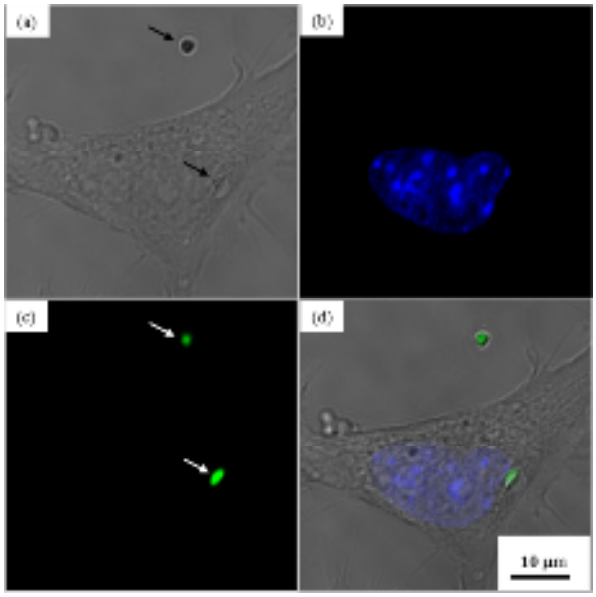


Figure 3. Confocal images of L929 cell after 30 min of incubation with Fluo/LDH dispersion. (a): DIC image, (b): Hoechst (nucleus) excited image, (c): Fluo excited image, (d): merged image.

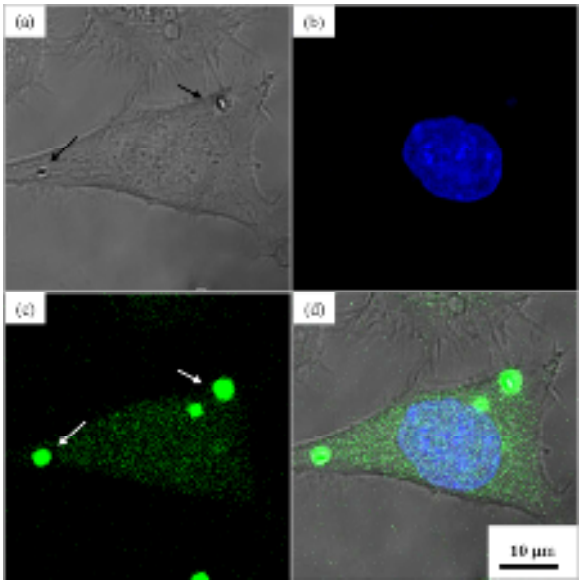


Figure 4. Confocal images of L929 cell after 1 h of incubation with Fluo/LDH dispersion. (a): DIC image, (b): Hoechst (nucleus) excited image, (c): Fluo excited image, (d): merged image.

We expect that the escape of Fluo anion and Fluo/LDH from endosome occurred as follows [6][23]. Generally, the extracellular materials internalized by endocytosis will be subjected to hydrolysis in lysosome. The proton and chloride ion are injected into the endosome structure in order to adjust the endosomal pH to mild acidic condition, because the lysosomal hydrolysis enzyme works in acidic solution. In the case of LDH, it is possible that LDH is dissolved by proton and release guest anion when proton enters inside endosome. Moreover, it is also conceivable that the endosome including LDH will be raptured, because the endosomal osmotic pressure will be raised by the release of constituent elements of LDH basal layer and subsequent entering of plenty of water. Thus, Fluo/LDH

can escape from endosome and release the Fluo anion. If this mechanism is correct, LDH has the ability to escape from the lysosomal degradation, which is a big advantage for delivery materials.

4. Conclusion

In summary, the intracellular behavior of Fluo/LDH after internalization was demonstrated by the CLSM observation. The Fluo/LDH nanocomposite with hexagonal sheets of 100 nm in diameter was synthesized by the coprecipitation followed by subsequent hydrothermal treatment, and could be transported into mammalian cells. After internalization, whole cells including the nucleus exhibited green fluorescence. This result indicated that Fluo anion was released from Fluo/LDH and endosome due to the dissolution of Fluo/LDH nanoparticles by proton. Because the endosomal escape leads to avoid the lysosomal degradation, it is a big advantage for delivery materials, indicating that LDH has a possibility as non-viral, non-toxic and high-efficiency delivery materials.

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