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Subcellular compartment targeting of layered double hydroxide nanoparticles

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ABSTRACT

Current investigations show that layered double hydroxide (LDH) nanoparticles have high potential as effective non-viral agents for cellular drug delivery due to their low cytotoxicity, good biocompatibility, high drug loading, control of particle size and shape, targeted delivery and drug release control. Two types of Mg_2Al–LDH nanoparticles with fluorescein isothiocyanate (FITC) were controllably prepared. One is morphologically featured as typical hexagonal sheets (50–150 nm laterally wide and 10–20 nm thick), while the other as typical rods (30–60 nm wide and 100–200 nm long). These LDH_{FITC} nanoparticles are observed to immediately transfect into different mammalian cell lines. We found that internalized LDH_{FITC} nanorods are quickly translocated into the nucleus while internalized LDH_{FITC} nanosheets are retained in the cytoplasm. Inhibition experiments show that the cellular uptake is a clathrin-mediated time- and concentration-dependent endocytosis. Endosomal escape of LDH_{FITC} nanoparticles is suggested to occur through the deacidification of LDH nanoparticles. Since quick nuclear targeting of LDH_{FITC} nanorods requires an active process, and although the exact mechanism is yet to be fully understood, it probably involves an active transport via microtubule-mediated trafficking processes. Targeted addressing of two major subcellular compartments by simply controlling the particle morphology/size could find a number of applications in cellular biomedicine.

1. Introduction

It has been reasoned that naturally occurring minerals are the prerequisite to prebiotic life [1] and although it is difficult to find direct evidence that enables us to relate minerals to the origin of life, they did undoubtedly sustain prebiotic life. For example, natural minerals can catalyze the synthesis of various basic biological materials such as amino acids, nucleic acid bases and sugars from simple chemicals (CO_2, NH_3, HCHO, HCN etc.) under natural conditions, or even initiate condensation of these biomolecular units into oligomers (i.e., peptides, oligonucleic acids) and polymers (i.e., proteins, RNA and DNA) [1–3]. A possible example of these natural minerals, layered double hydroxide (LDH), has been found to catalyze the production of various prebiotic biological materials (i.e., diaminomaleonitrile) from HCN and CN^- at room temperature [4]. A more important feature is that this mineral can concentrate polar and ionic biomolecules, store and protect these prebiotic biological molecules from degradation under sunlight and/or under some severe conditions [5,6]. In this work we found that this mineral, when made in nanometer scale, like many nanostructured inorganic materials [7], may act as an efficient vehicle for the transport of biomolecules into living cells and nuclei, sustaining the cells to grow well and function properly or even forcing the cells to make changes according to the nature of the particular biomolecules.

Layered double hydroxides (LDHs) are a family of anionic clay materials, exemplified by the natural mineral hydrotalcite $[Mg_6Al_2(OH)_{16}CO_3{\cdot}4H_2O]$ [8,9]. Most LDH minerals can be described using the general formula $[M^{II}{}_nM^{III}(OH)_{2+2n}]^+(A^{m-})_{1/m}{\cdot}xH_2O$ (n=2–4), where M^{II} represents a divalent metal cation, M^{III} a trivalent metal cation and A^{m-} an anion. Structurally, cationic brucite-like layers ($[M^{II}{}_nM^{III}(OH)_{2+2n}]^+$) are bound together by the interlayer counter-anions as well as water molecules $[(A^{n-})_{x/n}\ mH_2O]$ [8,9]. LDH is found to be an effective non-viral agent for cellular drug and gene delivery due to its unique and versatile properties [10–13]. For example, two recent papers reported that LDH can effectively transport antisense oligonucleotides to HL-60 cancer cells [10] and GFP-encoding PCR-fragments (1632 nt) to various types of cells [11,14]. Intrinsically,

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LDH materials possess a number of advantageous properties for this purpose [12,15,16], such as good biocompatibility, low cytotoxicity [14,16], high loading of anionic/polar drugs, control release depending on pH, protection of drugs in the interlayer [15,17], and controllable particle size [12,16].

Choy et al. recently suggested that the cellular uptake of hexagonal LDH particles is in principle a clathrin-mediated endocytosis and internalized LDH particles are mainly distributed within the perinuclear cytoplasm [18]. In this work, we hybridized LDH nanoparticles with the organic dye fluorescein isothiocyanate (FITC) to prepare a new type of LDH_{FITC} nanoparticles (rod-like), identical to well known hexagonal LDH particles (sheet-like) in chemical composition while distinct in morphology. We then comprehensively studied the cellular uptake behaviour of these particles in three different mammalian cell lines, i.e., Chinese hamster ovary (CHO-K1), mouse embryonic fibroblasts (NIH 3T3), and human embryonic kidney (HEK 293T) under the influence of various inhibitor drugs and dominant-negative (d/n) mutants. We found that both types of LDH hybrid nanoparticles are quickly taken up by these cells, and moreover the new rod-like LDH hybrid specifically targets the nucleus, while the sheet-like ones retain in the cytoplasma. Processes possibly involved in the uptake and nuclear localization of rod-like LDH particles are outlined in Fig. 1, i.e., quick adhesion to the cell membrane (step *I*), clathrin-mediated endocytosis (step *II*), endosomal escape (step *III*) and nuclear translocation of rod-like LDH nanoparticles (step *IV*). Such a shape effect of nanoparticles on the delivery destination has never been reported yet, though the shape effect of micrometer particles on the cell uptake has been reported in a few cases [19,20].

2. Experimental section

2.1. Preparation of LDH_{FITC} nanoparticles

The two forms of LDH_{FITC} nanoparticles were prepared in two different ways. For the preparation of LDH_{FITC} nanorods ($LDH_{FITC\text{-}ROD}$) 10 ml salt solution containing $MgCl_2$ (2.0 mmol) and $AlCl_3$ (1.0 mmol) was quickly added to 40 ml NaOH solution (0.15 M) under vigorous stirring, followed by 10 min stirring. The LDH slurry was collected via centrifugation and then well dispersed in 40 ml deionized water, 4.0 ml of which was reacted with 0.12 ml FITC (0.025 M) while gently shaking for 1 h. After separating the LDH_{FITC} slurry and washing it once, it was redispersed in 8.0 ml deionized water and subjected to hydrothermal treatment at 100 °C for 16 h in a stainless steel autoclave with a Teflon lining. The resultant stable suspension of $LDH_{FITC\text{-}ROD}$ nanoparticles contains approximately 2.0 mg/ml LDH and 140 μg/ml FITC with a nominal chemical formula of $Mg_2Al(OH)_6(Cl)_{0.9}(FITC)_{0.05}{\cdot}2H_2O$ (named as $LDH_{FITC\text{-}ROD}$), assuming all added $FTIC^{2-}$ ions replace the corresponding Cl^- ions in the LDH nanoparticles during the exchange reaction. Likewise, $Mg_2Al(OH)_6(Cl)_{0.5}(FITC)_{0.25}{\cdot}2H_2O$ with 50% of Cl^- ions replaced by $FITC^{2-}$ ions was prepared.

For the synthesis of hexagonal LDH_{FITC} nanosheets ($LDH_{FITC\text{-}HEX}$), the pre-prepared LDH slurry was washed twice and redispersed in 40 ml deionized water, followed by hydrothermal treatment at 100 °C for 16 h. The resultant stable homogenous LDH suspension (i.e. $LDH_{HEX\text{-}Cl}$, 0.4 wt.%) was then reacted with $FITC^{2-}$ at 60 °C for 15 min with light agitation, giving rise to $LDH_{FITC\text{-}HEX}$ nanoparticles. Note that nominal chemical formula as well as LDH and FITC concentrations of $LDH_{FITC\text{-}HEX}$ and $LDH_{FITC\text{-}ROD}$ suspensions are identical.

2.2. Characterization

Photon correlation spectroscopy (PCS, Nanosizer Nano ZS, MALVERN Instruments) was used to analyze the particle size distribution of LDH suspensions. The same instrument was also used to measure the Zeta potential of LDH nanoparticles in as-prepared suspensions. Fourier transformed infrared (FTIR) spectra were collected on a Nicolet 6700 FTIR spectrometer. For each spectrum 40 scans from 4000 to 400 cm^{-1} were performed at a resolution of 4 cm^{-1} measuring the FTIR absorbance of KBr discs containing 1–2 wt.% of LDH sample.

X-ray diffraction patterns were collected on a Rigaku Miniflex X-ray diffractometer with variable slit width at a scanning rate of 2°/min with 2θ ranging from 5° to 80° using Co K_α radiation ($\lambda = 0.17902$ nm). Mg and Al concentrations in LDH_{FITC} samples were determined by inductive coupled plasma atomic emission spectroscopy (ICP-AES) on a Varian Vista Pro instrument. Transmission electron microscopy (TEM) images of LDH and LDH_{FITC} nanoparticles were obtained on a JEOL JSM-2010 transmission electron microscope at an acceleration voltage of 200 kV.

2.3. Cell culture and uptake of LDH-FITC nanoparticles

Chinese hamster ovary (CHO-K1), HEK 293T or NIH 3T3 cells were seeded in 6-well plates (Greiner) at a density of 5×10^5 cells/well and cultivated to semi-confluency in HAM's F-12 medium (BioWhittaker) in a humid atmosphere supplemented with 5% CO_2 at 37 °C. Cells were washed 3 times with PBS and twice with OptiMEM, before 1.0 ml of OptiMEM containing 10 μl of as-prepared LDH_{FITC} suspension was added to each plate (final concentration of LDH and FITC in cell culture media: 20 μg/ml and 1.4 μg/ml, respectively) and cells were incubated for a given period of time at 37 °C/5% CO_2.

We worked with three standard mammalian cell lines to demonstrate that clathrin-mediated endocytosis and fast nuclear translocation of LDH nanorods are intrinsic characteristics of the LDH nanomaterials, and are not due to cellular factors. For ease of presentation, almost all results discussed in this paper refer to CHO cells, but virtually identical results were obtained for the other two cell lines unless stated otherwise.

In order to differentiate between extra- and intracellular fluorescence, extracellular fluorescence from LDH_{FITC} nanoparticles was quenched by briefly lowering the medium pH below 4.5, upon which LDH_{FITC} particles attached to the surface were dissolved and thus extracellular FITC lost its fluorescence. LDH_{FITC} nanoparticles already internalized within the cells were protected by the cell membrane from the extracellular pH change and therefore remained fluorescent. The acidified medium was removed after 1 min and cells were washed twice with PBS before being returned to the normal cell culture medium. As confirmed by bright-field microscopy, cell viability was not affected by the pH change during the time-course of the analysis.

The effect of LDH_{FITC} concentration on the cellular uptake was investigated by adding various amounts of LDH_{FITC} suspension (0.1 to 10 μl) to 1.0 ml of OptiMEM and subsequent monitoring of the cellular uptake. For quantification of intracellular FITC, extracellular LDH_{FITC} was removed by washing the cells with a medium with a pH below 4.5, after which cells were collected in and lysed with PBS containing 1% TritonX-100. FITC fluorescence in the PBS solution was measured on a Shimadzu scanning UV/Vis spectrophotometer at $\lambda_{Ex} = 494$ nm and $\lambda_{Em} = 520$ nm.

Confocal images of cells before and after LDH_{FITC} nanoparticle uptake were taken with an Andor spinning-disk confocal microscope using a 60× oil-immersion objective and a standard FITC filter set. For confocal microscopy, cells were seeded on glass-bottom Petri dishes at 5×10^5 cells per dish.

2.4. Cellular fractionation

CHO cells were fractioned into nuclear, cytosolic and membrane fractions by means of differential centrifugation using a protocol adapted from Evans [21]. In brief, CHO cells were exposed to LDH_{FITC} for a given period of time before extracellular LDH_{FITC} was washed away with PBS (pH was adjusted to 4–5). This was followed by