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Layered double hydroxide nanomaterials as potential cellular drug delivery agents*

Zhi Ping Xu‡ and G. Q. (Max) Lu

ARC Centre for Functional Nanomaterials, School of Engineering and Australia Institute of Bioengineering and Nanotechnology, The University of Queensland, Brisbane, Queensland 4072, Australia

Abstract: This paper briefly reviews the recent progress in using layered double hydroxide (LDH) nanomaterials as cellular delivery agents. The advantages of LDHs as cellular delivery agents are summarized, and the processes of interaction/de-intercalation of anionic drugs (genes) into/from LDH nanoparticles are discussed. Then the cellular delivery of LDH-drug (gene) nanohybrids and subsequent intracellular processes are presumably proposed. At the end, some challenges and remarks for efficient delivery of drugs (genes) via LDH nanoparticles are provided to the best of our knowledge.

Keywords: layered double hydroxide (LDH); hydrotalcite; drug delivery; gene therapy; endocytosis; endosome escape.

INTRODUCTION

Layered double hydroxide (LDH) nanomaterials as cellular delivery agents have been widely investigated only recently [1]. Although organic-type delivery agents have been developed for more than two decades [2,3], inorganic nanoparticles have been examined for similar applications just since the last decade [1]. In particular, LDH nanomaterials, naturally existing but readily synthesized in the laboratory, show a great potential as effective cellular delivery agents, as reviewed in the following sections.

General cellular delivery agents

Cellular delivery involving the transfer of various drugs and bioactive molecules (peptides, proteins, and DNA, etc.) through the cell membrane into cells via some delivery vehicles has attracted increasing attention due to the difficulty and inefficiency for bioactive molecules and drugs to go across the cell membrane. Therefore, searching for efficient and safe transport vehicles (agents) to cellularly deliver biomolecules and drugs has been a challenging yet exciting task for scientific researchers.

In the past few decades, many efforts have been devoted to designing and investigating various viral and nonviral organic cationic agents [2,3]. In viral agents, part of the original gene segment is eliminated to leave space for the genes (or drugs) to be inserted and delivered. Agents consisting of cationic compounds mainly include cationic lipids, cationic polysaccharides, and polycationic polymers, where the positive charges usually result from protonation of various amino/imino groups. A par-

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‡Corresponding author

ticular type of DNA delivery agents receiving attention recently is recombinant proteins [4]. These proteins mimic the various viral properties by combining diverse peptide segments into a single molecule through the protein engineering technique. These peptide segments are required for efficient gene (drug) delivery, including antibodies, antibody segments for target delivery, and/or some short peptide sequences acting as nuclear localization signals.

Inorganic nanoparticles have emerged as new nonviral agents only recently [1]. Many inorganic materials, such as calcium phosphate, gold, carbon materials, silicon oxide, iron oxide, and LDH, have been extensively investigated since the last decade. This is due to recognition of their versatile features suitable for cellular delivery, such as wide availability, rich surface functionality, good biocompatibility, potential capability of target delivery, and controlled release of the drug (gene) from these inorganic nanomaterials.

In brief, viral agents are to date the most effective while the successful application in cellular delivery is limited by severe side effects (e.g., immune response and insertional mutagenesis). Cationic carriers (lipids and polymers) may avoid such problems yet are often highly toxic to the cells. In contrast, inorganic nanoparticles, much less toxic, show promise for controlled delivery properties, and thus present a new alternative to viral carriers and organic cationic carriers. However, the cellular transfer efficiency with existing inorganic nanoparticles, including LDH nanomaterials, is relatively low at the moment.

Layered double hydroxides

LDHs, also known as hydrotalcite-like materials or anionic clays, can be found in nature as minerals and readily synthesized in the laboratory. In nature, they are formed from the weathering of basalts or precipitation in saline water sources [5]. All LDH minerals found in nature and synthesized in the laboratory have a structure similar to that of hydrotalcite or its hexagonal analog, manasseite, and the majority adheres to the general formula $[M^{II}_{1-x}M^{III}_{x}(OH)_2]^{x+}(A^{n-})_{x/n}\cdot mH_2O$, where M^{II} represents a divalent metal cation, M^{III} a trivalent metal cation, and A^{n-} an anion. However, LDH materials are not limited to divalent M^{II} and trivalent M^{III}, but extended to monovalent M^{I} (such as Li^{I}) and tetravalent M^{IV} (such as Ti^{IV}, Si^{IV}) cations [5–7]. Structurally, LDHs consist of cationic brucite-like layers and interlayer anions as well as water molecules. In the brucite-like layer $[M^{II}(OH)_2]$, the substitution of M^{II} by M^{III} leads to a positive charge ($[M^{II}_{1-x}M^{III}_{x}(OH)_2]^{x+}$), which is neutralized by the interlayer exchangeable anion $[(A^{n-})_{x/n}]$. In general, the interlayer region also contains various amounts of water (mH_2O), hydrogen-bonded to the hydroxide layers and/or to the interlayer anions [5].

LDHs can be readily synthesized in the laboratory. The commonly used method is coprecipitation at varied or constant pH, followed by aging at a certain temperature [5–7]. However, as-prepared LDH materials in aqueous suspension are severely aggregated with size of 1–10 μm [8], comparable with the cell size (10–100 μm). Therefore, it is necessary to disperse the aggregates into individual LDH nanocrystallites in a stable suspension for cellular drug/gene delivery. As reported elsewhere, a few attempts have been made to disperse LDH aggregates into homogenous LDH suspensions [8–10] while with limited success. On the other hand, LDH particle size is an important factor for cellular drug (gene) delivery. For an effective endocytosis, the particle size is preferably under 150–200 nm [11]. We note that Choy et al. employed a hydrothermal treatment to tailor the MgAl–CO_3–LDH particle size in 85–340 nm, while the LDH materials are seemingly aggregated [12]. Luckily, we have found a simple yet effective hydrothermal treatment method to prepare stable homogeneous LDH suspensions with monodisperse particles between 50 and 300 nm in lateral size [13] and patented the process [14], meeting the basic requirement for the effective cellular delivery.

Recent research of LDHs as delivery agents

There are a number of examinations on the drug (gene) delivery application based on LDH materials in recent years, showing that LDHs can be used as effective drug (gene) carrier and controllable release system [15–30]. These examinations involve some basic issues, including the intercalation (loading) of drugs or biomolecules into the LDH interlayer, the release behaviors of drugs or biomolecules from the LDH interlayer under different physiological conditions [15–30], the toxicity tests of LDH materials to the cells [16,17,19,23] and to the animal [20], the cellular uptake of LDH nanoparticles [19,21,22], the cellular drug delivery tests [22,23], and furthermore, the gene transfaction in vitro tests in different cell lines [15–17,23] as well as the test for target delivery by conjugating the antibody protein [17].

ADVANTAGES OF LDHs AS DELIVERY AGENTS

Apart from the ready control of LDH particle size, versatile compositions and easy preparation in the laboratory [13] as mentioned above, LDH materials possess a number of advantages in various properties as the cellular delivery agents. The first advantage is the good biocompatibility. It is well known that MgAl–CO_3–LDH is a weak base, which makes itself a very good neutralizing agent, for example, as a pharmaceutical antacid (talcid) for ulcers with high antipeptic activity [5,31]. In fact, MgAl–LDH nanohybrids can undergo the following reaction very slowly under physiological conditions (pH = 7.4 or smaller) to release some by-products that are friendly to the cells and tissues:

$$\text{MgAl–Cl–drug–LDH} + H^+ \rightarrow Mg^{2+} + Al^{3+} + Cl^- + \text{drug} + H_2O$$

This reaction, on the other hand, can buffer the pH falling in case of the acidic situation, such as in the later endosome and lysosome after LDH–drug nanohybrids are taken up by cells. Moreover, this reaction aids LDH nanopaticles to escape from the endosome [32].

Low cytotoxicity is the other virtue of LDH nanomaterials. Giannelis et al. reported that there is not any inhibiting effect of LDHs on the 9L Glioma cell growth at the dose they applied [16,17]. Choy et al. did the cytoxicity tests and showed that HL-60 cells live well without obvious death at the level of 1000 μg/mL of MgAl–NO_3–LDH for up to 4 days [19,23]. Our recent tests indicate that the viability of HEK 293T cells can be over 50 % upon exposure to 200–300 μg/mL of MgAl–Cl–LDH for 3 days. These results suggest that MgAl–LDH is about 10 times less cytotoxic than the commonly used polymer transfection agents, such as polyethyleneimine [33]. In addition, in vivo testing of adult male Sprague–Dawley rats showed that the MgAl–NO_3–LDH nanomaterials have little systemic effect on the rat growth at doses up to 200 mg/kg of rat weight [20].

The third advantage is that the framework of LDH structure provides full protection for the loaded drugs (genes) in the interlayer. Since most drugs (genes) are interlaminated between hydroxide layers, the hydroxide layers can stop most other materials (such as enzymes and oxygen) on the external surface of LDH particle. This is sometimes critical for the gene transfection since some genes are prone to degradation in the endosome, lysosome, and cytoplasm. In a similar way, LDH can protect some easily oxidizable drugs, like vitamins, from oxidation [28]. The property, on the other hand, can be also used to suppress the toxicity of drugs by slowly releasing drugs from the interlayer in a limited amount within a unit time.

Physically, the zeta potential of LDH–drug hybrid nanoparticles can be adjusted by loading various amounts of anionic drugs [34]. Normally, the inorganic LDH nanoparticles are naturally positively charged [34]. This is a good feature for LDH nanoparticles to approach the negatively charged cell membrane in an in vitro test. However, this may become a hindrance in an in vivo test as the nanoparticles may interact with any cells and hardly reach the target cells. Therefore, it is ideal that the surface charge can be adjusted. Fortunately, our titration experiments show that the zeta potential of LDH nanoparticles decreases when more organic anions are loaded [34]. Thus, this provides a controllable way to adjust the surface charge. For example, the zeta potential can even be changed to negative [34],

which produces electrostatic repulsion between LDH particles and cells and thus provides a chance for the nanoparticle to recognize specific cells via antibody–antigen interactions if the antibody is conjugated onto the particle surface.

STORAGE OF DRUG (ANIONIC SPECIES) INTO LDHs

There are various ways to intercalate organic anion species into LDHs [35]. Similarly, if the drug is of anion or zwitterion, then it can be also included in LDH. The most simple and direct method is one-pot preparation of LDH–drug nanohybrids. This method involves a coprecipitation of cations, such as Mg^{2+} and Al^{3+}, in an alkaline solution containing the anionic drug to be loaded, followed by post-treatment [36]. Desigaux et al. used this method to prepare various LDH–DNA nanohybrids that show their own characteristic XRD patterns [23b]. During the precipitation, intercalation of the anionic drug is competitive to that of inorganic anions, such as Cl^- or NO_3^-, which generally leaves some drug molecules in the solution and causes some waste.

One other often used method is anionic exchange. A precursor LDH with Cl^- or NO_3^- as the counter-anion is first prepared. The LDH precursor is then exchanged with the anionic drug to form LDH–drug hybrids. Giannelis et al. [16] and Choy et al. [23] used this method to prepare LDH–DNA and other LDH–drug hybrids. A modified method is to calcine the precursor LDH at a mild temperature into mixed oxide and then to reconstruct LDH from the mixed oxide to intercalate the anionic drugs. Aisawa et al. employed this method to include amino acids into the LDH structure [37].

Generally, intercalation of anionic drugs (genes) via exchange method is very efficient. We did a test for LDH to adsorb fluoresceine 5-isothiocyanate (FITC) with exchange method and noted that less than 0.1 % FITC was left in the solution when 10 % of Cl^- in LDH was designed to be exchanged with FITC. The high exchange efficacy is not only attributed to electrostatic interactions between the anionic drugs and cationic hydroxide layers, but also to hydrophobic interactions between the drug molecules. The latter one is more pronounced when close packing of drug molecule chains is achieved in the interlayer [36]. In particular for DNA double-stranded/single-stranded chains, the intercalation into the interlayer is even more efficient since numerous anionic phosphate groups along the DNA chain produce a very strong binding between the DNA chains and the cationic hydroxide layers [15,16,18,19,21,23]. When DNA segments are intercalated, the interlayer spacing is expanded to 2.0–2.4 nm no matter how long the DNA segments are [15,18], indicating the chain is laid in parallel to the hydroxide layers. If a DNA chain has 1000 base-pairs or more, its chain length is over 300 nm, so we believe such a DNA chain is either partially intercalated in one LDH nanoparticle (50–300 nm) or co-intercalated in a few LDH nanoparticles.

CONTROLLED RELEASE OF INTERLAYER ANIONIC SPECIES

The release of interlayer anionic drugs may undergo two parallel pathways. As we mentioned in the previous section, the anionic drug can be intercalated into the interlayer via anionic exchange. Likewise, the anionic drug in the interlayer can be de-intercalated from the interlayer via the similar exchange process with the surrounding anions, such as Cl^- and/or phosphates. Choy et al. noted that part of FITC was de-intercalated from LDH with Cl^- via this exchange process [19]. For example, 35 % FITC was released after 8-h exchange at $[Cl^-] = 1 \times 10^{-1}$ M similar to that in body fluid [19].

The more possible release pathway is the acidic dissolution of hydroxide layers during the delivery process. The experiment by Tyner et al. showed that the weak base LDH nanocrystallites were completely dissolved upon exposure to a solution with pH 5.0 while kept almost unchanged at pH 7.2 for 1 h [17]. This could be the only pathway for the big anionic species to be released. For example, it seems very difficult for long-chain DNA (hundreds or even thousands of base-pairs) to exchange with Cl^- and/or phosphates.

No matter how the drug is released from LDH interlayer, the release of small anionic drugs can be controlled in a passive way by selecting a proper set of conditions, including the LDH system, the drug itself, pH, and ionic strength of the simulated body fluids. O'Hare et al. [26] chose $LiAl_2$–LDH as the drug carrier and found that various drugs (including diclofenac) in LDH were released quickly, to about 50 % within 1 min and 90 % within 35 min at pH = 4 and 7. In comparison, Grandolini et al. [25] used Mg_2Al–LDH as the carrier and found that the 90 % release of diclofenac took more than 9 h at pH 7.0 and 7.5. Hessein et al. [27] found the release of a plant growth factor (α-naphthaleneacetate) from Zn_2Al–LDH may take several days to reach 50–90 % at pH = 1–14. In general, the release follows a mode with two stages: initial burst release and the subsequent slow steady release. At pH = 7.0–7.5, the release via dissolution of LDH is very much limited and the anionic exchange is the major event. In the initial stage, the anionic drugs on the surface and the external part of LDH crystallites can be exchanged quite easily, leading to the release bursting. Following this step, the anionic drugs diffuse from the LDH crystallite internal part to the external part and then to the solution, which results in a steady slow release rate [25]. This implies that the LDH crystallite size is an important factor for the controlled release of drug molecules.

The release of DNA chains from LDH seems to undergo the dissolution pathway. Choy's test indicated that DNA chains (500–1000 base-pairs) were released only at pH below 3.0 upon incubation for 1 h at 37 °C [18]. This may imply that the DNA transfection via LDH carrier would be a slow process since the pH of endosome is usually about 6. We did observe the slow DNA transfection rate via LDH agents in comparison with the commercial polymeric carrier FuGENE®6 in our recent in vitro tests.

CELLULAR UPTAKE OF LDH–DRUG NANOHYBRIDS

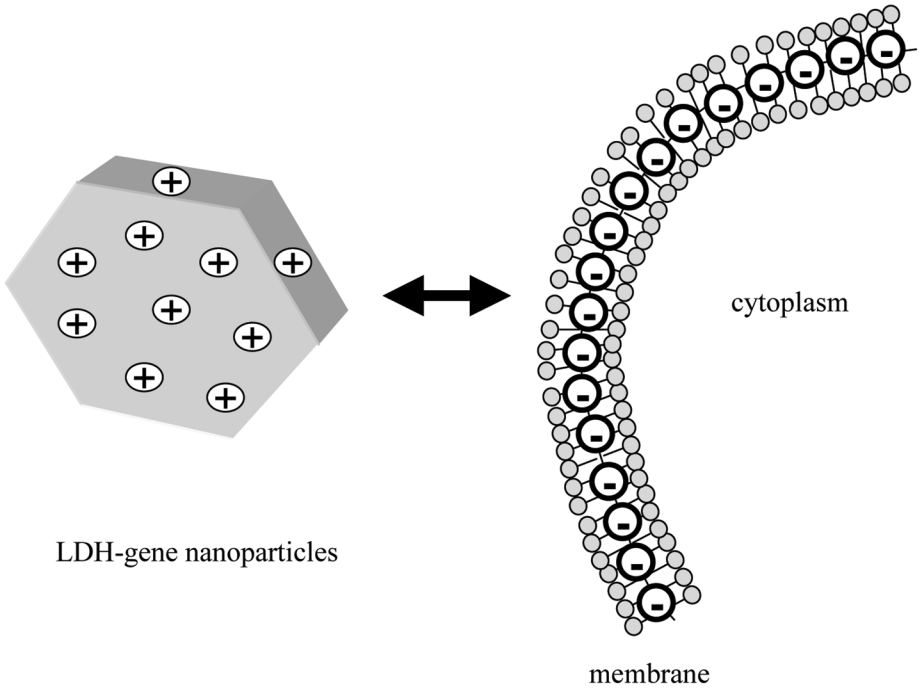
Our recent experiments show that the LDH–drug (biomolecule and gene) nanohybrids (~100 nm) have a positive zeta potential of 20–30 mV, therefore, the nanohybrid particles can approach and adhere to the negatively charged cell membrane via electrostatic interactions (Fig. 1A). As reported by Choy et al. [21,22] and observed in our experiments, the approach and adhesion to cells is a quick process (Figs. 1A and 1B).

Once LDH–drug particles are adhered to the cell membrane surface, some are internalized into the cell. The possible pathway for such nanohybrids to be internalized into cells is phagocytosis or endocytosis [38]. Phagocytosis generally involves the uptake of particles larger than 500 nm. In this connection, slightly agglomerated LDH aggregates (several hundreds of nm) may be taken up via phagocytosis while individual LDH crystallites (50–300 nm) via endocytosis. Obviously, two pathways give rise to a different internalization efficacy, and endocytosis can lead to a quicker uptake of LDH nanoparticles. Although endocytosis has not been well understood, microscopic observations indicate that cell morphology and cytoskeleton structure are gradually changed during the process, which is presumed to take place even more difficultly for phagocytosis. The endocytosis may be a receptor-mediated process [38] that may facilitate the uptake of LDH–drug nanohybrid particles (Fig. 1B).

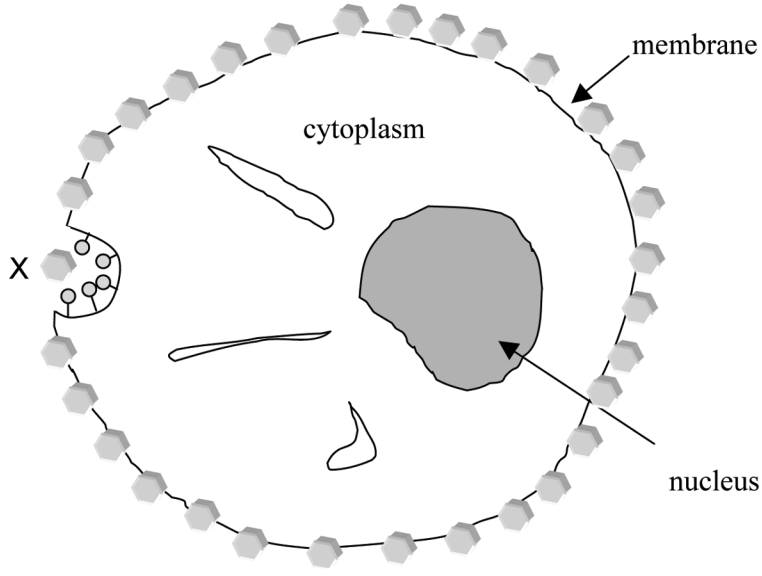
The cellular uptake is one key step for drug (gene) delivery. LDH nanoparticles, as potential delivery agents, provide the basic prerequisites to maximize the efficacy of the cellular uptake by tailoring the LDH particle size, adjusting the zeta potential, and conjugating the ligands to enhance the receptor-mediated endocytosis.

As discussed previously, the partial dissolution of LDH layers in endosome cannot only realize the passive control release of drugs (genes), but also buffer the excess protons. This may help drugs (genes) to escape from endosome [32], improve the viability of drugs (genes) in cytoplasm, and enhance the delivery efficacy (Fig. 1C).

(A)


cytoplasm
LDH-gene nanoparticles
membrane

(B)


membrane
cytoplasm
X
nucleus

(C)

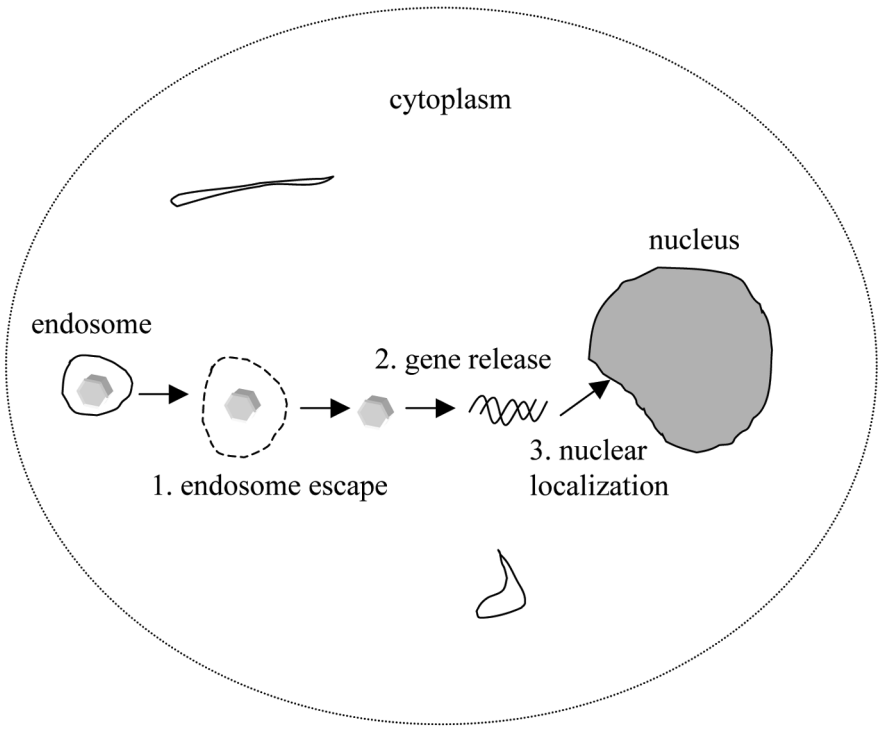


Fig. 1 Schematic of LDH-gene nanoparticle delivery processes. (A) LDH nanoparticle approaches the cell membrane due to the electrostatic attraction between the positively charged LDH nanoparticle and the negatively charged cell membrane. (B) LDH-gene nanoparticles adhere on the cell membrane exterior via the electrostatic attraction, which may interfere the cell function and cause some cells to die. Some particles are taken up via the mediated endocytosis, as pictured in area X. (C) After endocytosis, the endosome undergoes three critical processes: 1. endosome escape to release the LDH-gene nanoparticle, 2. gene release from the LDH-gene nanoparticle, and 3. nuclear localization of the delivered gene.

CURRENT CHALLENGES

In our opinion, the crucial challenge in efficiently delivering the LDH–drug (gene) nanohybrids is to stably suspend the LDH–drug (gene) nanohybrid particles in the cell growth medium. Although the agglomerated particles possess the so-called sedimentation effect [39] and thus promote the particles themselves to cross the cell membrane, the aggregation of LDH nanohybrids leads to a fast sedimentation so that some cells are transfected while others are not. Presumably, the agglomerated LDH–drug particles may undergo the phagocytosis that may be a slow delivery process. Anticipatively, the agglomeration of LDH nanohybrid particles loses the chance for target delivery since the sedimentation will hinder the particles to selectively combine with specific cells via antibody–antigen interactions. More seriously, the agglomeration of LDH particles will prevent particles from smoothly flowing in the blood circulation and inter-intestinal regions in the potential in vivo trials. It is lucky for us to find a way to make stable homogeneous LDH as well as LDH–drug aqueous suspensions [13,14]. However, LDH–drug nanohybrids tend to aggregate in the cell culture medium and thus the stabilization of such nanoparticles in the cell culture medium is still underway.

The second challenge is target delivery to specific cells and nuclei. Particularly for gene therapy, genes should be delivered into specific cells, or anticancer drugs to the cancer cells. Therefore, it is a

great advantage for the LDH nanoparticle vehicles to be able to target the specific cells. The LDH nanoparticles possess the potential by conjugating an antibody (peptide) to the particle surface to target specific cells and the nucleus. The real challenge in practice is how the antibody (peptide) is stably conjugated onto LDH nanoparticles to assure the targeting.

The third challenge is to reveal the details of cellular delivery processes, including the approach and adhesion to cell membrane surface, LDH particle endocytosis, and the release of drugs (genes), etc. How quickly the LDH–drug particles approach and adhere to the cell surface may provide some hints for the realization of target delivery. The complete understanding of endocytosis (such as the size effect and mediation effect of ligands) may help us improve the transfer efficacy of LDH–drug nanohybrids across the cell membrane.

It is worthwhile retesting the cytotoxicity of various LDH materials in various common cell lines. Choy et al. reported that MgAl–LDH up to 1000 μg/mL does not affect the HL-60 cell viability [19,23] while we found that MgAl–LDH at 100–200 μg/mL has already caused some death of HEK 293T cells. In addition, different LDH material systems, such as $LiAl_2$–LDH and ZnAl–LDH, having some controllability for the release of drugs (genes) from LDH interlayer as presented previously, need to be further tested in cytotoxicity for the potential delivery application.

CONCLUDING REMARKS

In conclusion, it is clear that LDH materials have been extensively investigated on a chemical basis, including elucidation of LDH structures, preparation of LDH nanoparticle suspension, intercalation/deintercalation of various drugs into/from the LDH interlayer (e.g., at 1-nm level). We have also reviewed some information on interactions between LDH particles and big biomolecules (such as genes). However, some details, such as how they form biomaterials (at the 10–100-nm level) and how stable LDH–gene (drug) nanohybrids, are unclear in most cases. The most deficient information is regarding interactions between LDH particles and cells, the cellular uptake process, the endosome escape, and nuclear localization (at the 0.1–1-μm level, see Fig. 1), which needs further intensive exploration. We anticipate that the understanding of the interactions at the 0.1–1-μm level may bring about a breakthrough in cellularly delivering LDH nanoparticles.

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