Expert Opinion

Layered double hydroxide nanoparticles in gene and drug delivery

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Layered double hydroxides (LDHs) have been known for many decades as catalyst and ceramic precursors, traps for anionic pollutants, catalysts and additives for polymers, but their successful synthesis on the nanometer scale a few years ago opened up a whole new field for their application in nanomedicine. The delivery of drugs and other therapeutic/bioactive molecules (e.g., peptides, proteins, nucleic acids) to mammalian cells is an area of research that is of tremendous importance to medicine and provides manifold applications for any new developments in the area of nanotechnology. Among the many different nanoparticles that have been shown to facilitate gene and/or drug delivery, LDH nanoparticles have attracted particular attention owing to their many desirable properties. This review aims to report recent progress in gene and drug delivery using LDH nanoparticles. It summarizes the advantages and disadvantages of using LDH nanoparticles as carriers for nucleic acids and drugs against the general background of bottlenecks that are encountered by cellular delivery systems. It describes further the models that have been proposed for the internalization of LDH nanoparticles into cells so far and discusses the intracellular fate of the particles and their cargo. The authors offer some remarks on how this field of research will progress in the near future and which challenges need to be overcome before LDH nanoparticles can be used in a clinical setting.

Keywords: cellular uptake mechanism, gene and drug delivery, layered double hydroxides, nanoparticles

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1. Introduction

The delivery of therapeutic and bioactive molecules (e.g., peptides, proteins, nucleic acids) to mammalian cells *in vitro* and *in vivo* with the aim of transferring them across the cell membrane into the cytoplasm is an area of research with increasing importance to medicine. Direct delivery of drugs and biomolecules is generally inefficient and suffers from problems such as enzymatic degradation, poor bioavailability, poor circulation stability, undesirable accumulative effects of the carrier, and many others. Therefore, the search for efficient and safe transport vehicles/carriers has been a challenging yet very exciting area of research in recent years and is going to be of interest for many years to come.

Whilst traditionally much effort was devoted to the development of viral and non-viral transport vehicles [1-10], innovative approaches are inspired by the exciting new findings in the field of nanotechnology. Gold, carbon nanotubes, fullerenes, layered double hydroxides and several oxide nanoparticles have all been shown to facilitate cellular delivery of drugs and/or genes. However, most inorganic nanoparticles require chemical and/or biological pre-modification(s) in order to

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obtain desirable properties for cellular delivery, such as good biocompatibility, suitable charge densities, strong affinity between carrier and payload, cell targeting, suspension stability and long circulation time. Silica nanoparticles are usually modified with silane species [11,12], whereas gold nanoparticles are modified with thiol groups [13]. Carbon materials can have many surface functional groups, but usually free COOH groups are used to anchor the payload to the carrier [14]. In recent years it has become apparent that layered double hydroxides (LDHs), also known as hydrotalcite-like materials or anionic (more properly speaking, anion exchanging) clays, form an exception to this rule. Their anion-exchange property allows for the direct loading of anionic drugs/biomolecules into their interlayer galleries; and although this intercalation decreases their usually positive surface charge, they remain sufficiently positively charged to facilitate cellular uptake [14-19].

This review aims to report and examine the latest developments in LDH nanoparticles as efficient cellular delivery vectors for both *in vitro* and *in vivo* applications, and to provide an outlook on possible future progress in this research area as well as some likely challenges that could be encountered.

2. Background of layered double hydroxides

2.1 Structure and general aspects

Layered double hydroxides are a family of anionic clay materials, exemplified by the natural mineral hydrotalcite $[Mg_6Al_2(OH)_{16}CO_3{\cdot}4H_2O]$ [20,21]. Most LDH materials can be described using the general formula $[M^{II}_{1-x}M^{III}_x(OH)_2]^{x+}(A^{m-})_{x/m}\cdot nH_2O$ ($x = 0.2 - 0.4$; $n = 0.5 - 1$), where M^{II} represents a divalent metal cation, M^{III} a trivalent metal cation and A^{m-} an anion. Structurally, LDH is closely related to brucite, $Mg(OH)_2$. In a brucite layer each Mg^{2+} ion is octahedrally surrounded by six OH^- ions, and different octahedra share edges to form an infinite two-dimensional layer [21]. Partial replacement of Mg^{2+} ions by Al^{3+} ions gives the brucite-like layers positive charges, which are balanced by anions located in the interlayer gallery between two brucite-like layers. Hence, within the LDH crystal, cationic brucite-like layers, $[M^{II}_{1-x}M^{III}_x(OH)_2]^{x+}$, are bound together by interlayer counter-anions (which balance the overall charge) and water molecules $[(A^{m-})_{x/m}\cdot nH_2O]$ [20,21]. The electrostatic interactions and hydrogen bonds between layers and contents of the gallery hold the layers together, forming a three-dimensional structure, as shown in **Figure 1**.

There are several different ways for brucite-like layers to stack on top of one another [22,23]. LDH minerals are known in both hexagonal (2H) and rhombohedral forms (3R), whereas synthetic LDHs are usually assigned to the rhombohedral polytype only. There are several combinations of divalent and trivalent cations that can form LDHs. For these ions, the only requirement is that their radii are not too different from those of Mg^{2+} and Al^{3+} [20,24-27]. There is almost no limitation to the nature of anions in the interlayer gallery as long as the anions do not abstract the metal ions from the hydroxide layers and have a sufficient charge density in one cross-section. Interlayer anions in LDH are exchangeable, with the order of preference being, for example, NO_3^- < Halides < OH^- < SO_4^{2-} < HPO_4^{2-} < Naphthol Yellow^{2-} < CO_3^{2-} [24,28,29]. This order is the consequence of hydrogen bonding, charge and charge density. A very useful consequence is that a more weakly held anion can be quantitatively replaced by a more strongly held one, and this is simply accomplished by stirring the LDH materials containing the anion that is to be replaced in a solution containing an excess of the replacement. LDH nitrate and chloride are commonly used as starting materials in this procedure.

To date, many kinds of anions have been reported to be present in the interlayer gallery, such as common inorganic anions (carbonate, nitrate, phosphate, etc.), organic anions (carboxylates [30-32], glycolates [33], organic dyes [34], etc.), polymeric anions (PVS [35], PSS [36], PA [37], etc.), complex anions ($NiCl_4^{2-}$[38,39], $Fe(CN)_6^{3-/4-}$ [40-42]), macrocyclic ligands and their metal complexes [43,44], iso- and hetero-polyoxalates [45] and biochemical anions (amino acids [46-50], CMP, AMP and ATP [51,52], etc.). More recently, it was indicated that LDH nanoparticles (50 – 300 nm) can also incorporate and effectively transport antisense oligonucleotides to HL-60 cancer cells [17], GFP-encoding PCR fragments to various types of cultured cells [53,54], and siRNA molecules to HEK293T and NIH3T3 cells [55]. These applications will be discussed in more detail later in this review.

A very comprehensive discussion of the structure of layered double hydroxides, including type and orientation of intercalated species, superlattice formation within the interlayers, lattice parameters, and the different polytypes possible can be found in reference [21].

2.2 Synthesis

LDH materials can be found in nature as minerals or readily synthesized in the laboratory. In nature they are formed from the weathering of basalts or precipitation in saline water sources. Unlike silicate-based clays, however, LDHs are not found in large or commercially useful deposits. Direct synthesis is the most widely used method for LDH preparation. This method involves nucleating and growing the metal hydroxide layers by mixing an aqueous solution containing the salts of two metal ions with a base solution in the presence of the desired anion (also called *co-precipitation* method) [21]. One inherent limitation of this method is that it can be used only if the desired interlayer anion is at least as tightly held as the counter-anion in the metal salts used. For this reason, metal chlorides or nitrates are widely used, whereas sulfates are generally avoided. Variations of this method include titration at constant or varied pH and buffered precipitation [21,28]. The simplest buffer of all is an excess of divalent metal during a titration at varied pH. One advantage of this method is

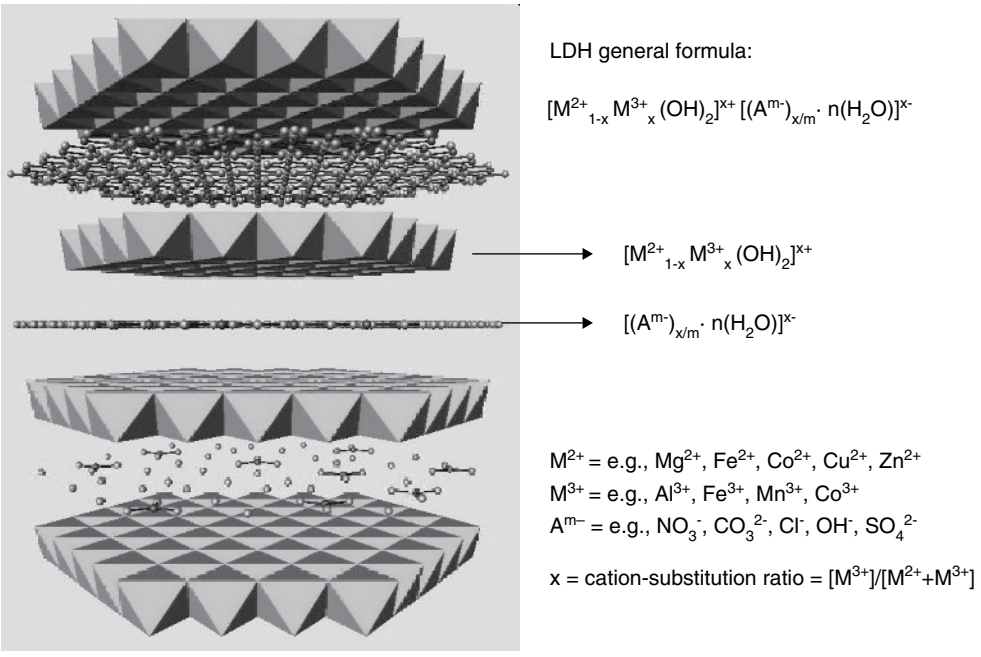

Figure 1. Layered double hydroxides have a brucit-like layered structure with negatively charged ions in the interlayer gallery for charge neutralization.

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that precipitation will occur at a lower pH than in the absence of excess M^{2+}, thus there is less risk of the incorporation of unwanted hydroxide anions and less uptake of CO_2 by the reaction mixture. Such uptake of carbon dioxide is a major nuisance in LDH chemistry because it gives rise to carbonate, which is the most strongly held anion within the LDH lattice [14,21,56].

Although most co-precipitation reactions have been carried out in aqueous solution, co-precipitation in alcohols is also an option. Gardner *et al.* described the formation of mixed alkoxide/inorganic anion–LDH materials [57]. This method gives rise to transparent LDH thin films that – after hydrolysis and anion exchange – can be used to form anionic pillared clays.

As mentioned previously, anions present in the LDH interspaces are exchangeable, and thus anion exchange is the second most widely used method for the synthesis of LDH hybrids. Anion exchange is simply accomplished by stirring previously formed LDH materials in solutions that contain the replacement anion species. Crepaldi *et al.* described a variation of the anion exchange reaction that is very promising for the incorporation of bulky anions [58]. In this particular method, an anionic-surfactant–LDH is treated with the salt of a cationic surfactant containing the desired anion. The cationic and anionic surfactants subsequently interact to form micelles, leaving the LDH free to capture the desired anion.

LDH materials can also be formed from metal oxides and/or hydroxides [50-61], or they can be prepared by sol-gel techniques [62]. Other methods include homogeneous preparation, precipitation via aluminate [63], preparation from metals [64] and preparation by oxidation [65,66].

Post-preparative treatments, for example, heating at gentle reflux or hydrothermal [24,31,56,67] and microwave treatments [68], are used mainly to improve crystallinity and uniformity of the obtained LDH materials.

2.3 Applications

Historically, LDH materials were of interest as catalysts, catalyst and ceramic precursors, traps for anionic pollutants, catalyst supports, ion exchangers and additives for polymers (**Figure 2**). Readers are referred to the many excellent reviews on the topic of hydrotalcites for a more comprehensive discussion of their many industrial applications [69-76].

The earliest application of hydrotalcites in relation to human health was their use as antiacids and antipeptic reagents [77-79], whereas in the last decade nanometer-sized LDH materials (50 – 300 nm) were increasingly explored as drug and gene carriers and controlled release delivery systems [14,15,17,18,55,80-94]. The following sections summarize and discuss the progress made so far in this area.

3. Layered double hydroxides in gene and drug delivery

Although the number of publications discussing LDH materials has more than doubled during the last decade (~ 120 – 140 annually in 2000/01; > 300 annually in 2008), the number of

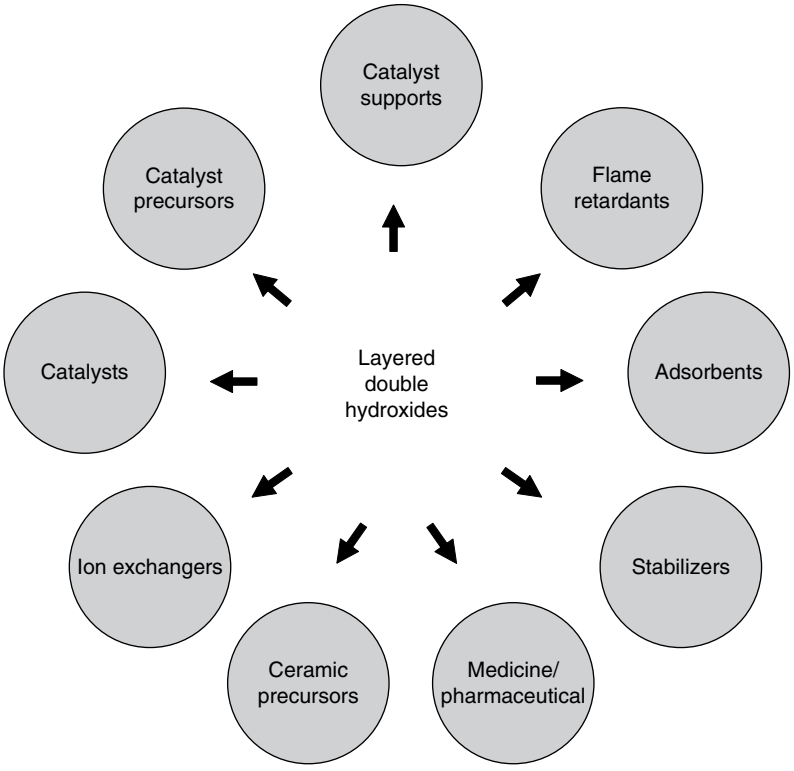

Figure 2. Main industrial applications of layered double hydroxides and their derivatives.

publications discussing LDH materials as potential gene and drug carriers has increased even more significantly (~ 1 – 2 annually in 2000/01; ~ 30 in 2008/09). A similar trend can be seen in the number of filed and successful patent applications in the area (1 in 2000/01; 5 annually in 2007/08). In the meantime the proportion of reports that describe successful delivery of genes and drugs using LDH (nano)particles has soared from ~ 1.5 to ~ 8% (**Figure 3**), which shows it is an extensively researched area. These rapid changes have thus mandated a review of research achievements so far and of course an outlook on challenges that lie ahead.

3.1 LDH nanomaterials in drug delivery

Initially, most research reported the successful intercalation and delivery of molecular drugs not genes; and hybrids of LDHs with many different molecules of biological interest, such as amino acids and peptides [95,96], nucleotides [93], porphyrins [97], biocatalysts [98,99], ATP [19,100], anti-inflammatory drugs [101-107] and anticancer drugs were described [108-111]. Tronto *et al.* intercalated a variety of anions of pharmaceutical use, for example, salicylate, citrate, glutamate and aspartame, using two different synthesis methods (co-precipitation and anion exchange) [112]. The first to report the use of LDHs in controlled release formulations were Doi *et al.* in 1985 [113]. They described the synthesis and application of sustained-release pharmaceuticals containing nifedipine and LDHs. LDHs are regarded as biocompatible materials [20], hence they have found wide application as drug supports or matrices in the pharmaceutical industry [80]. LDH hydroxide layers provide a well-defined, relatively hydrophilic microenvironment for anionic drugs of any kind and have been shown to inhibit photodecarboxylation of intercalated aptopril and aminosalicyclic acid [114], or racemization of L-tyrosine that usually occurs on exposure to sunlight, high temperature or ultraviolet light [115]. Therefore, LDH materials could potentially be used as 'molecular containers' [116]. Another spin on the 'molecular container' concept, although not primarily a drug delivery application, are new sunscreen formulations with LDH materials, as proposed by Rossi *et al.* [117], who intercalated ferulic acid into LDH by a simple anion-exchange process. Ferulic acid acts as a free radical trap and is an excellent UV absorber, but can degrade quickly and might cause skin irritation or allergies on direct contact with skin. LDH intercalation protected ferulic acid from degradation and improved absorption of UV-B irradiation, with the hybrids being successfully formulated into a silicon cream without leaching of the active compound into the silicon phase.

The beneficial effects layered double hydroxides can have on anti-inflammatory therapy are threefold. Intercalation of anti-inflammatory drugs in LDH materials leads to different drug release profiles compared with traditional/commercial formulations [102], improves the solubility of poorly water-soluble drugs [103,118], and probably reduces the side effects of drugs,

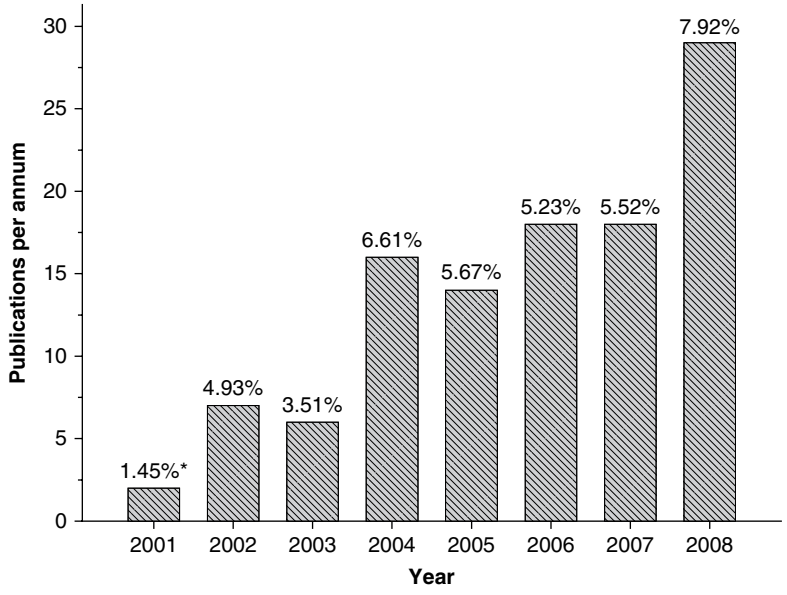

Figure 3. Publications on the topic of LDH materials as gene and drug delivery agents.
*Publications on LDH nanoparticles in gene and drug delivery as percentage of total publications.
Source: SciFinder Scholar.

for example, the ulcerating damage of indomethacin [119]. Owing to their alkalinity, however, their use as orally available drug delivery systems could be difficult. Li *et al*. described a promising approach to improving the passage of LDH hybrids through the gastrointestinal tract by coating fenbufen-intercalated LDHs with enteric polymers, for example, Eudragit® S 100 (Röhm GmbH & Co. KG, Darmstadt, Germany) [120]. Under *in vitro* conditions these core–shell materials showed a controlled rather than the burst release profile for the incorporated drug that one would expect for the untreated, basic LDH hybrids passing through the gastrointestinal tract.

According to the World Health Organization, cardiovascular disease is globally the primary cause of death. Gu *et al*. recently showed that it is possible to intercalate the anticoagulant low-molecular-weight heparin (LMWH) a highly sulfated glycosaminoglycan, into layered double hydroxides [94]. Heparin with a molecular mass of 4 – 6 kDa is frequently used as an anticoagulant, but has some pharmaceutical limitations, for example, a short half-life (2 – 4 h), low efficiency of cellular delivery and lack of oral absorption, thus requiring generally twice-daily injections. The strongly negative charge of LMWH enables quantitative anion exchange and intercalation into the LDH interlayer, thus protecting the anticoagulant and extending its half-life in blood plasma. Further, it was shown that the LMWH is released in a sustained way.

Applications of hydrotalcite-like LDH materials in anticancer therapy are twofold. One approach, the one mostly explored, is the intercalation of anticancer drugs; the second approach is more of a gene therapeutic nature and will be discussed further below. Tyner *et al*. developed a new method to deliver the poorly soluble drug camphothecin by incorporating it first into micelles, and the drug-loaded micelles subsequently into an LDH host [121,122]. Camphothecin and its derivatives inhibit the enzyme DNA topoisomerase, hence inhibiting DNA replication in treated cells and causing (cancer) cell death. They showed that the intercalation did not diminish the effect of the drug, while providing a means of controlling the release. The intercalation of fluorouracil, an antimetabolite used to treat digestive system cancers, into LDH by reconstruction resulted in hybrids that showed an initially rapid release of the drug but later provided the desired sustained release of the drug [108,123]. A similar result was reported for the Keggin polyoxotungstate PM-19, a potentially antiviral agent [56].

3.2 LDH nanomaterials in gene delivery

With respect to an application of LDH nanoparticles as gene carriers, that is, for the delivery of nucleic acids, one needs to keep in mind the size of the actual nucleic acid delivered. Most of the reports focus on the delivery of molecular drugs, smaller (hence linear) nucleic acids as commonly used in gene therapy (antisense oligonucleotides, siRNA, etc.) [17,55,124], or PCR fragments [53], or sheared genomic DNA in some earlier studies [17]. Kwak *et al.* synthesized LDH hybrids containing *myc* antisense oligonucleotides [124], which on delivery to leukemia cells caused growth inhibition of HL-60 cells, a cell line that usually overexpresses the proto-oncogene c-*myc*. Taking this approach even further, the authors recently demonstrated the successful delivery of far more effective, but also more easily degraded siRNA molecules to mammalian cells using LDH materials. The delivery resulted in the efficient (but naturally transient) knockdown of a target gene, while maintaining high viability [55]. It was established that a 1:5 mass ratio of siRNA:$Mg_2Al(OH)_6NO_3$-LDH nanoparticles is

sufficient to complex fully the nucleic acid, whereby the negative charge of the backbone of intercalated/associated nucleic acids, which otherwise prevents them from freely crossing the cell membrane, is shielded by the positive surface charge of the nanoparticles. Cellular uptake of siRNA-loaded LDH was confirmed by flow cytometry studies using FITC-tagged siRNA duplexes. It was shown that naked siRNA cannot enter the HEK293T cells, whereas siRNA associated with LDH nanoparticles was taken up with an efficiency of 99%. On delivery of anti-MAPK1(ERK2) siRNA duplexes to HEK293T cells using LDH nanoparticles, a transient knockdown of ERK2 protein expression was observed [125]. In combination with LDH's low cytotoxicity and ready availability, this discovery is very promising for future *in vivo* applications.

Previously, Choy *et al.* [17] and Tyner *et al.* [53] intercalated sheared genomic DNA (500 – 1000 bp) and PCR fragments of 800 bp length, respectively, in the interlayer galleries of LDHs and used these hybrids as non-viral vectors. However, one must be careful when jumping to the conclusion that LDH nanoparticles can be used as versatile carriers in applications where larger nucleic acids are to be delivered, that is, the commercial-scale transfection of mammalian cells with plasmid DNA (pDNA). It seems that larger and sterically hindered biomolecules such as plasmid DNA, which in solution usually adopts a supercoiled structure, are not completely accessible to intercalation in the interlayer galleries by means of anion exchange [125]. Desigaux *et al.* reported the formation of DNA–LDH hybrids using a co-precipitation method whereby the DNA molecules were up to 8 kbp long [126]. However, they specifically noted the incomplete intercalation of DNA and the resulting negative zeta-potential. They hypothesized that this should not impede cellular uptake and/or protection from degradation, which is yet to be confirmed.

3.2.1 *Bottlenecks for in vitro and in vivo gene delivery systems*

The primary goal of gene delivery is to achieve high transfection efficiency. Chemical and physical methods were first used for *in vitro* gene transfer because of the high efficiency with which they carried DNA plamids into cells. These approaches became more important for *in vivo* applications after the discovery that these plasmids could also be efficiently delivered and expressed *in vivo*. Biological vector approaches use a natural cell receptor or other entry mechanism for the original biological agent (virus or bacteria). Therefore, they are more likely to achieve systemic infection (when using live vectors) or one-round transfection (when using inactivated or defective vectors). Contrary to this, physical methods involve completely different mechanisms that force cells or tissues to take up genetic material. These methods normally give rise to higher transfection efficiencies, but only in the local area subjected to the physical force. Biological approaches bring the most concern about safety because some vectors are similar to original pathogens and therefore pose an inherent threat of disease. In addition, the processes of their production add further concerns, for example, the potential for tumorigenicity. Furthermore, there is a possibility of allergic reactions towards vectors *in vivo* and there are also challenges related to pre-existing immune responses towards vectors in those applications.

Regardless of the specific delivery method applied, however, gene transfer into animal cells must accomplish three distinct goals: i) the exogenous genetic material must be transported across the cell membrane; ii) it must be released into the cell (cytoplasm/nucleus) from which it may need to be transported to the site of function; and iii) at some stage during these processes the genetic material must be activated, that is, released from its complex and rendered competent for expression and/or interaction with the host genome. Also, *in vivo* gene delivery methods need to facilitate transfer of DNA from the site of DNA administration to the surface of target cells.

Transfer across the cell membrane seems to be independent of the nature of the genetic material, which is inert and passive at this stage. In physical transfection methods, transport across the membrane is achieved by direct transfer, for example in microinjection or particle bombardment where the membrane is breached during delivery, or in electroporation where transient holes are formed through which DNA and RNA can diffuse. Meanwhile, in chemical methods a complex is formed between nucleic acid and synthetic compound that binds to the cell surface before internalization. In transduction methods this complex comprises nucleic acids packaged inside a viral capsid (**Figure** 4) [127].

In most transfection methods, DNA or RNA complexes are deposited in the cytoplasm, either directly under the plasma membrane or deeper in the cytosol following escape from the endosome. The DNA must then be transported to the nucleus, a process that depends on poorly understood, cell intrinsic DNA trafficking systems, whereas RNA can function directly in the cytoplasm [127]. In some physical methods, that is, particle bombardment and microinjection, it is possible to deliver DNA directly into the nucleus, hence intrinsic transport pathways are not required. In addition, many viruses deliver nucleic acids to the nucleus as part of the infection cycle.

Gene delivery using LDH nanoparticles falls into the category of chemical transfection methods, which have to overcome several hurdles to deliver active DNA into the nucleus. Unlike physical gene transfer methods that mechanically breach the cell membrane and in many cases the nuclear membrane, chemical transfection methods have to persuade the cell to take up and process exogenous DNA, in order to deliver at least some intact DNA molecules to the nucleus. As discussed above, the first obstacle to gene transfer is the cell membrane, which is hydrophobic and negatively charged. DNA is hydrophilic and negatively charged, thus can interact with or cross the cell membrane only if it is either enclosed in a fusogenic capsule (e.g., liposomes) or

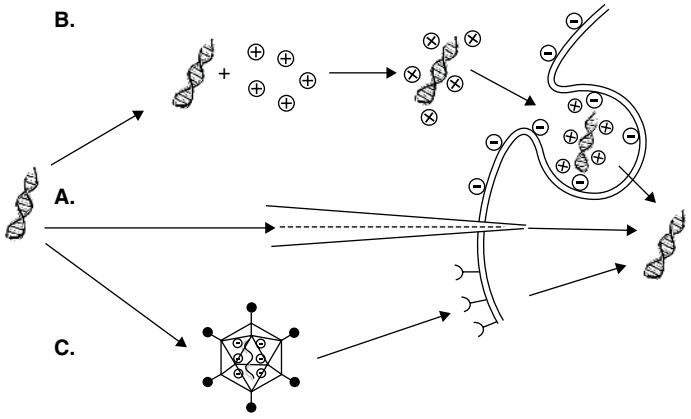

Figure 4. Summary of gene transfer mechanisms for introducing genetic materials into animal cells. A. DNA can be introduced directly into the cell, and in some cases the nucleus, by physical transfection methods. **B.** Alternatively, the DNA can form a chemical complex with a synthetic vector, which binds to the cell surface resulting in its uptake into the cell and the release of DNA into the cytoplasma from where it enters the nucleus. **C.** Or it may be encapsulated within a viral particle (or bacterial cell), which interacts with cell surface receptors and internalizes via either endocytosis or fusion with the plasma membrane.

is part of a complex with a net positive charge (all other chemical methods including LDH nanoparticles). The main function of the synthetic (or chemical) transfection reagent is therefore to form either a positively charged complex or a fusogenic particle in which the DNA is encapsulated. Drug/gene encapsulation into the interlayer galleries of LDH nanoparticles exhibiting a positive surface charge readily achieves this without necessitating any further surface functionalization, as is the case for silica [11,12], or gold nanoparticles [13], or carbon materials [14].

The second boundary to successful transfection, and still the one causing the most difficulties, is DNA's transport to the nucleus. Following membrane fusion, the fusogenic particles deposit their DNA into the cytoplasm just below the cell membrane, from where the DNA finds its own way to the nucleus by means of an intrinsic transport pathway, although the pathway itself is poorly understood. Contrarily, complexes taken up by endocytosis are transported in acidic endosomes, and eventually deposited in lysosomes and degraded. To achieve high levels of transfection efficiency, the DNA must escape from the endosome into the cytosol and find its way to the nucleus. Certain chemicals known to disrupt the endosomal transport pathway by bursting the endosomes can be included in the transfection protocol to aid DNA escape into the cytoplasm, but are being replaced more and more by tailored transfection agents with inherent 'proton-sponge' properties (e.g., protonation of the amine groups in polyethyleneimine (PEI) or dissolution of the carrier, e.g., LDH), which have a similar ability to assist endosomal escape [16,128,129]. Although the process of DNA transfer to the nucleus is poorly understood, it has been found that the DNA sequence itself [130], as well as the addition of some peptides carrying nuclear localization sequences to the transfection mixture, can promote the nuclear import of exogenous DNA [131].

The final hurdle to efficient gene delivery is the activation of the exogenous DNA by dissociation from the complex once within the cell, because only free DNA is available for expression or interaction with the host cells' genome (which in itself can be a major obstacle) [127]. This dissociation is thought to occur either by simple diffusion (as in $CaPO_4$ transfection) [132,133], or by interaction of the positively charged complex with negatively charged intracellular molecules and subsequent charge neutralization (as in DEAE-dextran and PEI transfection) [129,134]. LDH-NP-mediated transfection overcomes the last two hurdles mentioned above using one and the same mechanism: dissolution of the transfection agent in the endosome, which aids endosomal escape and releases the DNA simultaneously [16]. A positive side effect exclusively observed for LDH-NP-mediated gene and drug delivery is that this dissolution results in the disintegration of the transfection reagent into cytofriendly ions, which means no accumulation of the carrier inside the cells is observed as is the case for many polymeric or other inorganic carriers.

Depending on the intracellular place of function for a particular drug, similar obstacles may apply to drug delivery applications of LDHs; however, generally it is not required that the drug enter the nucleus, which negates one of the major bottlenecks discussed.

In terms of *in vivo* delivery, the main impediments for successful gene or drug delivery are means of administration (oral bioavailability – stability in acidic environment of stomach; subcutaneous or intravenous – blocking of capillaries), stability of the carrier in body fluids, circulation time and targeting of the particles to particular tissues/cells. Another challenge, especially for gene therapy applications that target neurodegenerative diseases, is to overcome the blood–brain barrier (BBB) and of course functional readout of the nucleic

acid delivered, that is, up- or downregulation of the gene of interest. Although no successful *in vivo* gene delivery using LDH nanoparticles has been reported yet, LDH-NPs are quite well positioned to overcome some of the hurdles mentioned above. Owing to their small size they should avoid renal clearance, which translates into a long circulation time and increases their chance of crossing the BBB. Their inherent positive surface charge enhances cellular uptake rates and may also increase serum stability. Their dissolution after internalization means that no accumulative effects should be observed, making them highly biocompatible.

3.3 Loading of drugs/genes into LDH nanoparticles

Co-precipitation in the presence of drugs is the most direct and quantitative route to LDH–drug conjugates, but drugs must be able to withstand post-preparative treatments such as hydrothermal treatment that are commonly used to improve uniformity and crystallinity of the materials obtained. Some anions, however, such as siRNA or antisense oligonucleotides, are not able to withstand these conditions and they are better incorporated by means of anion exchange with anions already present in the LDH interspaces, which is the second most widely used method for the synthesis of LDH–drug conjugates. Anion exchange is simply accomplished by stirring previously formed LDH materials (containing NO_3^- or Cl^- anions) in solutions that contain the replacement anion species. However, the replacement reaction is not always quantitative and in some instances is sterically/spatially hindered (also see below) [125]. In the case of poorly soluble drugs, one can either resort to working in non-aqueous solvents, for example, alcohols [57], or utilize a method developed by Tyner *et al.*, who first incorporated the drugs into micelles and subsequently the drug-loaded micelles into an LDH host [121,122].

For a detailed discussion of reaction kinetics and potential mechanisms, readers are referred to the excellent work by Williams and O'Hare, who studied the intercalation reaction of many different LDH materials using energy dispersive X-ray diffraction (EDXRD) [135].

3.4 Cellular uptake of LDH–drug/gene nanohybrids and their intracellular fate

At present, the most widely accepted mechanism of how LDH nanoparticles deliver their cargo such as DNA to cells is as follows: i) ion-exchange of interlayer anions such as NO_3^- or Cl^- with negatively charged biomolecules facilitates the formation of LDH-bio-nanohybrids; ii) the positive surface charge of the nanoparticles attracts them to the cell surface owing to electrostatic interaction; iii) the LDH-bio-nanohybrids are then taken up by means of receptor-mediated endocytosis [16,17,109]; and iv) owing to the lower pH in the endosome, the LDH particles dissolve, whereby they buffer the endosomal pH (hence protect their cargo) and subsequently facilitate escape of the LDH-biomolecule hybrids into the cytoplasm by endosome rupture. **Figure 5** illustrates this mechanism as hypothesized by Choy *et al.* [17], which was later shown to probably be clathrin-mediated by Oh *et al.*

Through the use of uptake-inhibiting drugs and dominant-negative mutants, Xu *et al.* finally confirmed that clathrin-mediated endocytosis, together with a minor portion of caveolae-mediated endocytosis, is the means by which LDH-NP enter the cells [16]. In this study it was further shown that endocytosed particles are subsequently stored in slowly acidifying vesicles of the endosomal pathway. This, however, results in their partial dissolution, which subsequently buffers the pH and causes the endosome to rupture, releasing LDH-biomolecule hybrids and free biomolecules into the cytoplasm. Other groups have recently come to similar conclusions [89,136]. However, we were not only able to confirm the uptake of LDH hybrids carrying FITC-tagged siRNA molecules by means of endocytosis, but also their perinuclear localization after release into the cytoplasm was identified and the buffering of the endosomal pH using a pH-sensitive probe was verified [55]. In further studies it was found that another possible release pathway could be ion-exchange with cytoplasmic anions (e.g., Cl^-, PO_4^{3-}, etc.) once the nanoparticles are internalized, but this seems to occur on a much slower timescale [94,137].

Despite these fundamental studies into the mechanism by which LDH nanoparticles deliver their payload, however, only limited success has been reported on the efficient delivery of DNA plasmids using LDH nanoparticles. Most of the reports focus on molecular drugs or smaller (hence linear) nucleic acids (PCR fragments, antisense oligonucleotides, siRNA, etc.), while it seems that larger and sterically hindered biomolecules such as supercoiled plasmids are not completely accessible to quantitative intercalation in the interlayer galleries via anion exchange [125]. The authors observed that there are differences in transfection efficiency when transfecting adherent or suspension cells with plasmids when using LDH nanoparticles as the carrier [125,138]. In combination with a rigorous characterization of pDNA–LDH complexes (i.e., aggregation of particles on plasmid addition), this suggests a different transfection mechanism for LDH nanoparticle-mediated transfection of mammalian cells with supercoiled plasmids. On addition to the LDH nanoparticle suspension, the plasmids do not intercalate as desired, but rather wrap around individual particles and in some instances interconnect the particles with one another, thereby forming aggregates. These aggregates settle on top of adherent cells when added to the cell culture medium and are subsequently taken up via a yet to be determined endocytotic pathway and may be released into the cytoplasm. Based on the size of these aggregates (> 500 nm), slower pathways, for example phagocytosis or macropinocytosis, may be involved during the uptake process. However, because of the constant agitation of suspension cultures, the particle aggregates cannot settle on the cell surface of suspension cells, hence no significant transfection is observed for these cells. LDH nanoparticle-mediated transfection is

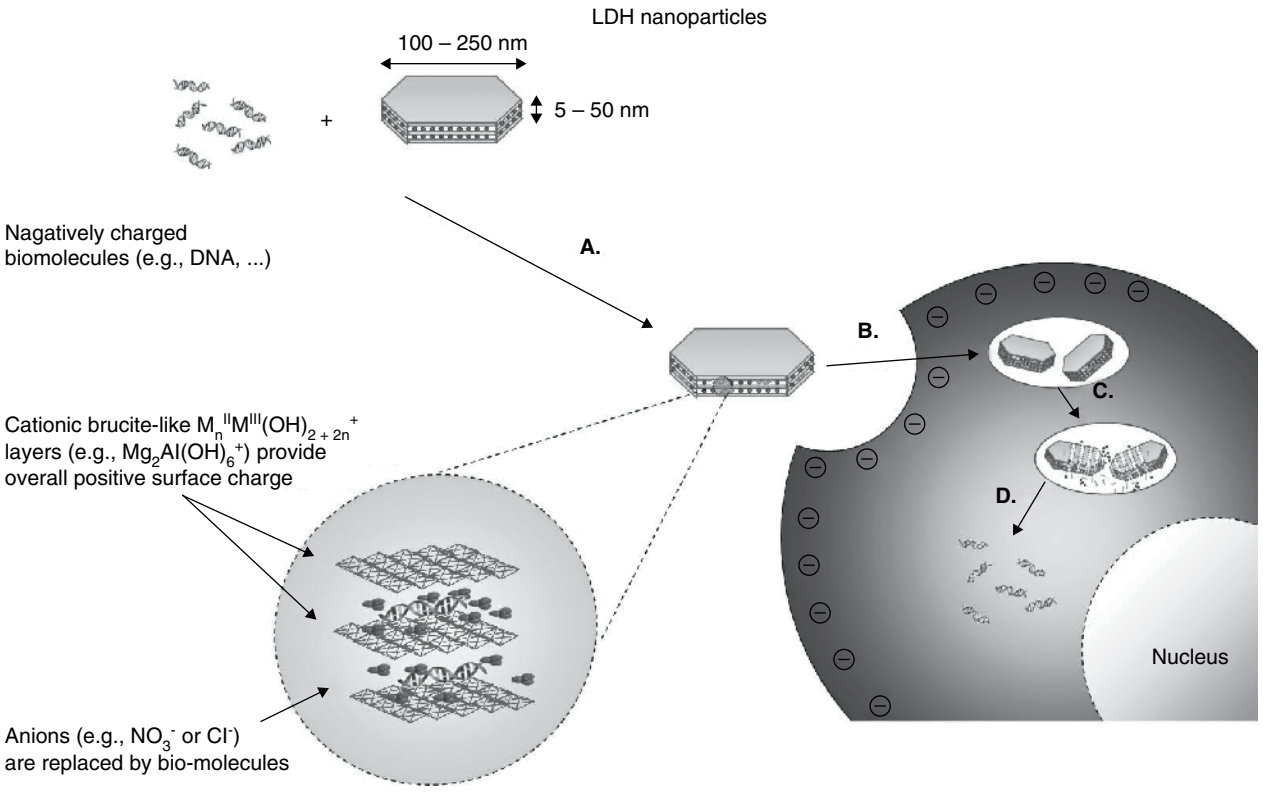

Figure 5. Currently accepted mechanism for LDH-mediated transfection of mammalian cells with, for example, DNA. A. Anion exchange between interlayer NO_3^- or Cl^- anions and negatively charged biomolecules, for example, DNA, leads to the formation of LDH-nanobiohybrids. **B.** Uptake via receptor-mediated endocytosis. **C.** Acidification of the endosome causes LDH particles to dissolve slowly, thereby buffering the endosomal pH ('proton-sponge effect') and releasing the biomolecule. **D.** Further influx of H^+ into the endosome and dissolution of LDH particles leads to an increase in ionic strength inside the endosome and causes the endosomal membrane to rupture, which liberates the payload (and undissolved particles).

therefore similar to $CaPO_4$-mediated transfection, which works well in adherent cell lines but results in poor transfection outcomes when suspension cells are to be transfected [125,138]. Furthermore, the proposed mechanism also explains another observation made during the transfection experiments. Owing to the wrapping-around, the plasmids-LDH hybrids are less likely to be taken up by cells due to their close to zero or even negative zeta-potential [126]; the uptake is much slower; and the plasmids are subjected to more degradation than would be the case if they were properly intercalated within the interlayer galleries of the LDH nanoparticles. This leads to less functional plasmid being delivered to individual cells, and hence the observation during our experiments that although determined positive by FACS analysis, transfected adherent cells were expressing far lower amounts of reporter proteins than the positive controls, which used commercially available transfection methods [125,138].

Contrary to gene delivery, delivery of molecular drugs (e.g., ibuprofen, fenbufen, etc.) using LDH materials can be far more efficient for two possible reasons. First, co-precipitation or anion exchange in solutions containing excess of the desired anionic drugs usually results in quantitative incorporation of the drug within the LDH nanoparticles. Second, most drugs do not require delivery to the nucleus. This means that as long as the drugs are aided in crossing the cell membrane and subsequently released from their respective LDH conjugates, they should have the desired effect.

Cellular uptake of LDH–drug conjugates of course also mandates consideration of intracellular fate of the LDH particles themselves. As discussed above, the particles are understood to dissolve partly during the uptake process, and in the case of $Mg_2Al(OH)_6$-LDHs, which many researchers use to deliver drugs and genes, this leads to the formation of free Mg^{2+}, Al^{3+}, and NO_3^- or Cl^- ions only. In concentrations as low as those generally used for delivery applications, none of these ions is known to have detrimental effects on cell viability or proliferation [14]. However, the alkalinity of LDH materials, which is desired in antiacid applications, could pose a problem when they are used as *in vivo* delivery agents.

3.5 LDH cytotoxicity and influence on cell proliferation

Although many research groups reported relatively low-to-negligible cytotoxicity of LDH nanoparticles towards mammalian cells, published results are far from being in agreement over a 'safe' LDH concentration (**Table 1**) [14-16,53,54,111,121,139-141]. First and foremost this can be attributed to the differences in LDH materials tested (e.g., nitrate versus chloride-based LDH precursors), the difference in sensitivity of individual cell lines towards changes caused by LDH addition to the cell culture medium (e.g., rise of pH, change in ionic strength), and the different cell culture conditions (e.g., 10% FCS or serum-free medium). However, it also has to be attributed to the adequacy or inadequacy of particular cytotoxicity assays for the purpose of answering the question of whether LDH materials are toxic and if so at which concentration.

Relying solely on morphological changes in order to determine the effect of LDH nanoparticles on mammalian cells is just as inadequate as sole reliance on MTT assays or similar, and leads to the huge variation in reported LDH cytotoxicity levels. It is advisable to take a more comprehensive approach by monitoring cell viability and proliferation using two independent assays [55,125], which shows that $Mg_2Al(OH)_6NO_3$, for example, causes ~ 50% growth inhibition of HEK293T cells when applied at concentrations of 0.125 mg/ml or more, whereas the viability itself is affected only to a negligible extent, with an impressive 94% viability after 3 days of continuous exposure (as compared with Lipofectamine where it drops to well below 70%) [55]. It results in a similar growth inhibition when added to CHO-S cells at concentrations of 0.05 – 0.100 mg/ml while maintaining the viability as high as 88 – 94% [125].

3.6 Targeting of LDH nanoparticles to specific organs/tissues and intracellular compartments

Surface functionalization with the aim of adhering targeting moieties such as antibodies to the surface of layered double hydroxides is complicated by their ionic nature. In this regard, LDH materials are very much contrasted to silica materials. The latter are readily functionalized using the OH-groups present on virtually any silica surface and an extensive chemistry based on converting this group to a range of functionalities exists. However, the ionic OH-groups that are present in the brucite-like layers of LDHs rarely undergo similar reactions and if they do these are easily reversed by hydrolysis [21]. Grafting reactions, that is, OH-groups being formally replaced by oxygen atoms of interlayer anions, are at present the closest approach to functionalization [142,143]. Pseudo-grafting reactions that occur on the surface of aminopropyltriethoxysilane-functionalized LDH particles have also been reported [136,144], but caution is advised when comparing cellular uptake of these particles with pristine LDH particles, as particle size and zeta-potential are strongly affected by the modification. Coating with SiO_2 or amine-functionalized SiO_2 layers also negates one of the major advantages of LDH materials for cellular delivery, that is, the dissolution into cytofriendly ions following cellular uptake.

Another approach to targeting – at least intracellularly – is based on the different subcellular localizations observed for LDH particles showing different morphologies. Xu *et al.* reported the astounding difference in intracellular localization when the morphology of usually hexagonal LDH nanoparticles was changed to a rod-like shape (**Figure 6**) [16].

4. Summary and conclusion

This paper has reviewed the application of layered double hydroxide nanoparticles, a family of anionic clay materials, in gene and drug delivery. In addition to pharmaceuticals and enzymes, a variety of anions of biological interest have been intercalated in LDH materials either through co-precipitation or anion exchange reaction and in some cases successfully delivered to mammalian cells. They range from amino acids and peptides [50,95,145], over vitamins [146], DNA and other nucleic acids/nucleosides [17,18,147,148], ATP [93], to polysaccharides, such as chitosan [149], and alginate [150].

Intrinsically, LDH nanoparticles possess several advantageous properties for delivery applications [15,56,110], such as good biocompatibility and reportedly low cytotoxicity [15,54], high loading of anionic/polar drugs, pH-controlled release of the cargo, protection of drugs in the interlayer [110,122] and finely tunable particle size [15,56]. LDH materials are therefore well positioned to overcome some hurdles that usually impede successful delivery.

Despite several fundamental studies of the mechanism by which LDH nanoparticles deliver their payload, only limited success has been reported on the efficient delivery of large biomolecules so far. Most reports focus on intercalation and delivery of molecular drugs (anti-inflammatory drugs, anticancer drugs, etc.) and small nucleic acids (PCR fragments, antisense oligonucleotides, siRNA, etc.), which are quite promising for future bioapplications. Targeting of drug/gene-carrying LDH materials is difficult because of the ionic nature of their surfaces, but Oh *et al.* [136] and Xu *et al.* [16] present two ways to circumvent the targeting problem, either by functionalizing LDH nanoparticles through condensation of APS on LDH nanoparticle surface or by varying the morphology of the particles so that they target different intracellular compartments.

5. Expert opinion

5.1 Prospects and challenges for future developments in this field

LDH nanoparticles are certainly promising gene and drug delivery vehicles. They have been shown to be very effective in an *in vitro* setting in providing sustained release of drugs with an otherwise very short half-life *in vivo*, for example, heparin [94]. However, to further this application, detailed studies of the *in vivo* cytotoxicity as well as the biodistribution are

Table 1. Examples of cytotoxicity levels reported for hydrotalcite-like LDH materials.

LDH material	Cell line	Reported cytotoxicity level
$Mg_2Al(OH)_6NO_3$	HL-60	No cytotoxic effect observed up to maximum tested (1 mg/ml) [15]
$Mg_2Al(OH)_6NO_3$	Saos-2 and Mg-63	0.5 mg/ml [111]
$Mg_2Al(OH)_6NO_3$	9L glioma (LD_{50})	0.025 mg/ml [53]
$Mg_2Al(OH)_6Cl$	HEK 293T	0.5 mg/ml [54]
$Mg_{0.68}Al_{0.32}(OH)_2(CO_3)_{0.16}$ 0.1 H_2O	A549, L-132, HeLa, HOS	< 0.250 mg/ml [138,139]

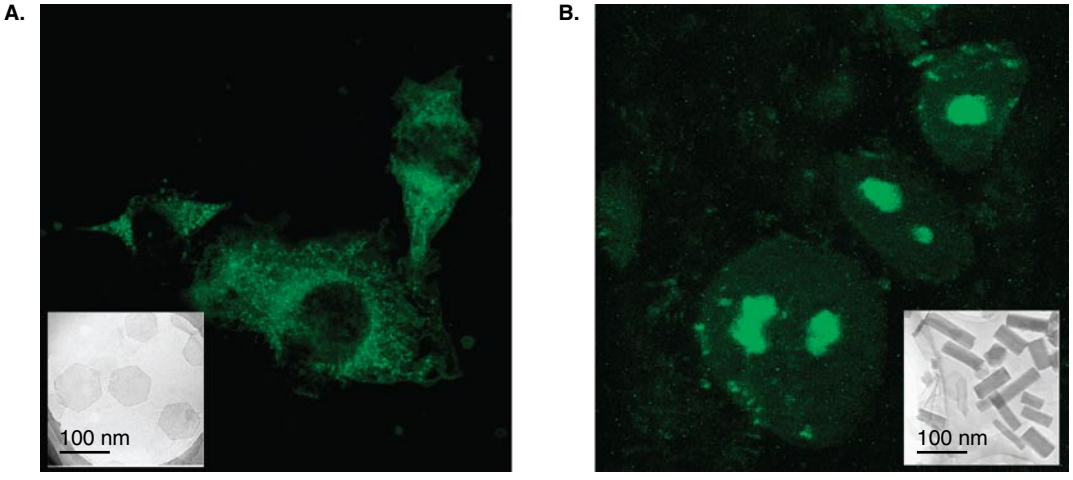

Figure 6. Morphology-dependent cellular localization of (A) hexagonal and (B) rod-like LDH nanoparticles. Inset: TEM, large: HEK 293T cells 5 h post-transfection.

required. It is not fully understood how these particles behave in the bloodstream and whether they are capable of crossing the blood–brain barrier. Achieving free circulation of LDH nanoparticles in the bloodstream without degradation and aggregation will be a short-to-medium-term goal for many research groups interested in LDH nanoparticles. Owing to the inherent problems in functionalizing purely ionic crystals such as the LDH nanoparticles discussed in this paper, new innovative approaches will have to be used in order to surface-functionalize the carriers with antibodies or other targeting surface groups. Some early work in this area was reported by Dey *et al.* [151], and others [136,144], but more research needs to be performed so as to maintain the original properties of the LDH nanoparticles that provide these carriers with an advantage over others (e.g., dissolution on endosomal acidification). A different approach to targeting, that is, intracellular targeting, is the synthesis of LDH nanoparticles with different morphologies [16]. With respect to the delivery of nucleic acid-based drugs to the brain for the treatment of neurodegenerative diseases, the ability of a carrier to cross the BBB is of utmost importance. Although it has been shown that this is possible for other nanoparticle-based systems [152-155], it is yet to be proven for layered double hydroxide nanoparticles, and we see this as one aspect of LDH research that will attract much attention in the medium-to-long-term future.

The use of LDH nanoparticles in an *in vitro* setting, that is, for the routine transfection of mammalian or insect cells with plasmids, will need to overcome the problems discussed in this review. The sensitivity of plasmid DNA towards high temperatures mandates an anion exchange approach (rather than a co-precipitation/hydrothermal treatment approach) for the synthesis of well-dispersed and highly crystalline plasmid–LDH nanohybrids, but the supercoiled structure of plasmids renders them too bulky for quantitative anion exchange with nitrate- or chloride-containing LDHs. Plasmids tend to wrap around the nanoparticles rather than completely intercalate and subsequently cause aggregation of the particles, which makes them less likely to be able to cross the cell membrane. They are also more prone to degradation than would be the case if they were properly intercalated within the interlayer galleries of the LDH nanoparticles where they would be protected from enzymatic degradation. This leads to less functional plasmid being delivered to individual cells, hence lower transfection efficiencies (especially in suspension cultures).

However, anion exchange with smaller nucleic acids, for example oligodinucleotides and siRNA, is a readily occurring process and LDH nanoparticles have been used successfully for the delivery of both [17,55,124]. This is very promising and given that the particles might be able to cross the BBB, we foresee many opportunities in the delivery of nucleic acid-based drugs to the brain using LDH nanoparticles.

Declaration of interest

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