Engineering small MgAl-layered double hydroxide nanoparticles for enhanced gene delivery

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Abstract
In this paper we report an approach for engineering small MgAl-layered double hydroxide (sLDH) nanoparticles with the Z-average diameter of about 40 nm. This method first requires co-precipitation of magnesium and aluminum nitrate solution with sodium hydroxide in methanol, followed by LDH slurry collection and re-suspension in methanol. The methanol suspension is then heated in an autoclave, followed by separation via centrifugation and thorough washing with deionized water. The nanoparticles are finally dispersed in deionized water into homogeneous aqueous suspension after 4-6 day standing at room temperature. In general, sLDH nanoparticles have the Z-average size of 35-50 nm, the number-average size of 14-30 nm and the polydispersity index (PdI) of 0.19-0.25. The prepared sLDH suspension is stable for at least 1 month when stored at fridge (2-8°C) or ambient (22-25°C) temperature. Moreover, sLDH nanoparticles are found to carry higher payloads of small double stranded DNA (dsDNA). More excitedly, sLDH nanoparticles transfect dsDNA into HEK 293T cells with a 5 to 6-fold greater efficiency compared to the larger LDH particles (Z-average diameter of 110 nm).

Keywords: Small layered double hydroxide nanoparticles; Non-aqueous precipitation and heat-treatment; Ostwald ripening; Gene loading and delivery; Transfection
1. Introduction

In recent years, layered double hydroxide (LDH) nanoparticles have demonstrated good therapeutic-carrier properties as well as highly efficient delivery vehicles for anionic drugs and nucleic acids, due to their favorable physical properties and low cytotoxicity (Choy et al. 2004; Oh et al. 2009a; Ladewig et al. 2010). LDHs, also known as anionic clays or hydrotalcite-like compounds (HTlcs), are a broad family of layered inorganic materials, whose composition can be expressed using the general formula \[ [M^{2+}_{1-x}M^{3+}_x(OH)_2] (A^{m-})_{x/n} \cdot yH_2O, \] where \( M^{2+} \) can be typical divalent ions and \( M^{3+} \) typical trivalent ions; \( x \) represents the charge density of the hydroxide sheets, typically in the range of 0.2 to 0.33; \( A^{m-} \) represents hydrated anions in the interlayer and can be of any types of anions, such as various inorganic and (bio)organic anions (Braterman et al. 2004). The lamellar architecture alongside a well-distributed, condensed array of positive charges renders LDH an excellent carrier for a wide range of negatively charged therapeutics (Nakayama et al. 2004; Costantino and Nocchetti 2001; Constantino et al. 2008; Chakraborti et al. 2011). Moreover, once the therapeutics are intercalated between LDH interlayers, they are physically protected from degradation. For example, LDH has been used as a DNA vector where intercalation has inferred protection of the gene from DNase-mediated degradation (Choy et al. 2000; Kwak et al. 2002; Masarudin et al. 2009; Li et al. 2011).

Given that negatively charged therapeutics do not readily transcend cellular membranes (which are also negatively charged), attempts to overcome this barrier can be effectively addressed by incorporating these anionic therapeutics within the interlayers of LDH, leading to efficient cellular uptake \textit{in vitro} (Ladewig et al. 2010; Wong et al. 2010). The high cellular delivery efficiency is also benefited from the innate ability of LDH to actively escape from
endosomes (considered a major barrier to effective gene/drug delivery) through LDH-mediated endosomal buffering, which ultimately leads to counterions and water ingress, rupture of endosomal vesicles, and release of intact therapeutics into the cytoplasm (Khan et al. 2001; Hussein et al. 2002; Gu et al. 2008; Gasser 2009). This process thus circumvents the endo-lysosomal pathway (Ladewig et al. 2010; Choi and Choy 2011) and leads to substantial enhancements in delivery efficiency.

Aside from these highly desirable inherent properties of LDH that benefit cellular delivery, the particulate size is another key property that impacts the delivery efficiency. A few reports claim that nanoparticles with size ca. 50 nm are more efficiently internalized by a range of cell types, such as HeLa, Caco-2 and HT-29 cells (Chithrani et al. 2006; Maurice et al. 2009; Choi and Choy 2011). In particular, Choy et al. reported that LDH nanoparticles with a number-average size of 50 nm were the most efficient cellular delivery vehicles (Oh et al. 2009b; Choi and Choy 2011).

To date, LDH particles reported in most literature have a Z-average (or intensity-average) diameter ≥50 nm, even when hydrothermal treatment has been used in an attempt to tailor and control the LDH particle size (Oh et al. 2002; Xu et al. 2006b; Oh et al. 2009b). Li et al. (2013) reported the so-called smallest CO$_2$-LDH nanoparticles with a number-average size of ~20 nm (observed in TEM) and an intensity average of ~60 nm (measured with DLS). On the other hand, using a non-aqueous precipitation method, Gardner et al. (2001) prepared a very well dispersed LDH suspension, and further Gunawan and Xu (2009) found that the number-average size of their as-prepared LDH particles was 30 nm, while limited knowledge in relation to the growth mechanism has been reported.
Given the present shortfalls in engineering of miniaturized sLDH, this research aimed to (1) decipher how small LDH nanoparticles can be prepared using non-aqueous methodologies through systematic investigation of effects of the experimental conditions on the size and the size distribution; and (2) demonstrate that the sLDH particles are able to more efficiently deliver dsDNA (21 bp) *in vitro*. We successfully demonstrated in this work that a reproducible approach towards generation of sLDH nanoparticles with a Z-average diameter of 35-50 nm and a number-average range of 14-30 nm. The sLDH nanoparticles (Z-average size of ~40 nm) were also found to deliver dsDNA into HEK 293T cells with a 5-6 fold greater efficiency in comparison with traditional LDH nanoparticles that have a Z-average particle size of ~110 nm.

2. Experimental

2.1. Materials Preparation

Small LDH (sLDH) nanoparticles were prepared by non-aqueous precipitation, followed by heat-treatment, purification and dispersion in water (refer to Supporting data Figure S1), which was modified from Pinnavaia’s method (Gardner et al. 2001). Typically, methanol solution (methanol, Fluka, ≥99.0%, 10 mL) of magnesium nitrate hexahydrate (Mg(NO₃)₂·6H₂O, Fluka, ≥99.0%, 6 mmol) and aluminum nitrate nonahydrate (Al(NO₃)₃·9H₂O, Fluka, ≥98.0%, 2 mmol) was added dropwise to a methanol solution containing sodium hydroxide (NaOH, Fluka, ≥97.0%, pellets, 16 mmol) under vigorous stirring. The mixture was stirred for 30-40 min, the precipitate slurry was then collected through centrifugation, redispersed in fresh methanol (40 mL) and transferred to a Teflon®-lined autoclave, followed by heat-treatment at 60 to 100°C for 0 to 144 h. After cooling the
sample to room temperature, the precipitate slurry was collected through centrifugation, and
washed twice with deionized water. The final collected slurry was manually dispersed in 40
mL of deionized water, followed by standing at room temperature with occasional hand-
shaking. This dispersion became transparent, resulting in a homogeneous LDH suspension
after 4-6 days with the LDH mass concentration determined to be 6 to 7 mg/mL (~50% yield).
A range of experimental factors, such as LDH mass concentration in the aqueous suspension
(Conc6.5 to Conc29), methanol (MW0 to MW2) and water washing (WW0 to WW2), the
heat-treatment temperature (HT60 to HT100) and duration (HD4 to HD144), co-precipitation
temperature with (CPT0HT to CPT50HT) and without hydrothermal treatment (CPT0 to
CPT50), were varied to examine their influences on the average particle size and distribution,
as summarized in Table 1. Note that all experiments were conducted in triplicates, and the
standard deviation of triplicate experiments is shown.

Large LDH particles were synthesized by vigorously mixing 10 mL of aqueous solution
containing magnesium chloride hexahydrate (MgCl₂·6H₂O, Sigma-Aldrich, 99.0-102.0%, 3.0
mmol) and aluminum chloride hexahydrate (AlCl₃·6H₂O, Fluka, ≥99.0%, 1.0 mmol) with 40
mL of sodium hydroxide (NaOH, Fluka, ≥97.0%, pellets) solution (0.15 M) for 10 min at
room temperature (Wong et al. 2010; Xu et al. 2006a). The LDH slurry was collected by
centrifugation and then washed twice with deionized water (40 mL), and resuspended in
dionized water (40 mL), after which the suspension was transferred to a Teflon®-lined
autoclave and hydrothermally treated at 100°C for 16 h. The suspension contained
approximately 4 mg/mL of homogeneously dispersed Mg₂Al-LDH nanoparticles, with a Z-
average particle size of 110 nm (Wong et al. 2010).
2.2. Materials Characterization

The LDH nanoparticle size distribution in aqueous suspension was measured by photon correlation spectroscopy (PCS) (also known as dynamic light scattering, DLS) using a Zetasizer Nano ZS (Malvern instrument) with three runs, from which an average of the Z-average size and the polydispersity index (PdI), also from three runs were calculated. Powder X-ray diffraction (XRD) patterns were recorded on a Rigaku Miniflex X-ray diffractometer with Co Kα source (λ = 0.1789 nm) at a scanning rate of 2.00°/min (2θ) from 2θ = 2° to 80°. Some XRD patterns were recorded on a thin film of LDH formed by dropping a few droplets of the LDH nanoparticle suspension and drying on a glass slide. FT-IR spectra were obtained on a Nicolet 6700 (Thermo Electron Corporation) by scanning 200 times from 4000 cm⁻¹ to 400 cm⁻¹ at a resolution of 1 cm⁻¹. The morphology and size of some typical LDH nanoparticles were examined by Transmission Electron Microscopy (TEM, Tecnai 20 FEGTEM) at 160 kV with a magnification of 100,000 to 700,000. The concentrations of sLDH and large LDH suspension were calculated from magnesium and aluminum concentrations determined by ICP-OES (Varian VISTA AX Pro) in digested LDH nanoparticle solutions. The carbon, hydrogen and nitrogen composition in powdered LDH nanoparticles were analyzed by CHON-S analyser (FLASH EA 1112 series, Thermo Electron Corporation).

2.3. Nucleic Acid Intercalation/adsorption

The intercalation/adsorption of nucleic acid (dsDNA) into sLDH/large LDH was conducted by mixing nucleic acids with pristine small and large LDH suspension using slight orbital shaker agitation (350 rpm/min). To optimize dsDNA loading, the intercalation
experiments were carried out with different mass ratios of dsDNA:LDH (from 1:0.67 to 1:10) for different periods of time (10 min to 18 h) at 37°C. Agarose gel electrophoresis was carried out by mixing the samples with DNA loading buffer and running on a 3.0% agarose gel at 80 V for 45 min. DNA was visualized using ethidium bromide and imaged using a GelDoc UV illuminator (Bio-Rad Laboratories, CA, USA). Normally, complementary strands of dsDNA (HPLC purified, Geneworks, Australia) were annealed at 37°C for 1 h. In some instances, both strands of the duplex were covalently coupled to the 6FAM fluorophore at the 5’ end. The sense strand dsDNA sequence was 5’-GCAATTTGCTCATCTCTAATT-3’.

2.4. Cell Culture and Transfection

Human embryonic kidney (HEK 293T) cells were cultured in L-glutamine containing RPMI-1640 medium (GIBCO, NY) with 10% fetal calf serum, penicillin (10 U/mL) and streptomycin (10 µg/mL, Gibco) in 5% CO₂ at 37°C. Cells were seeded at a density of 5×10⁵ cells/well in 6-well plates for 24 h. The transfection medium was then replaced with fresh culture medium containing sLDH or large LDH intercalated with dsDNA (the concentration of dsDNA in the medium was 0.1, 1.0 and 10 µg/mL; prepared at the dsDNA:LDH mass ratio of 1:5) for a further 24 h incubation.

2.5. Flow Cytometry Analysis

dsDNA-LDH uptake was determined by fluorescence-activated cell sorting (FACS). After incubation with the nanoparticles, HEK 293T cells were washed with PBS and detached using 0.05% trypsin-EDTA. The non-viable cells were excluded with 7-aminoactinomycin D (7AAD, 20 µg/mL, Molecular Probes, OR). Extracellular fluorescence was quenched by
treatment of 0.02% trypan blue. Flow cytometry analysis was undertaken on a BDTM LSR II Flow Cytometer with BD FACSDiva software. Fluorescence emissions from 6FAM and 7AAD were collected by 530/30 and 660/40 nm bandpass filter, respectively. The control cells (untreated cells and cells stained with 7AAD only) were used to determine the intrinsic cellular fluorescence baseline. In all analyses, 5,000-10,000 cells were analyzed. Data were presented as the mean ± SEM. Two-way ANOVA was used to assess statistical significance. *p<0.05.

2.6. Imaging

To ensure that dsDNA-6FAM was not associated with the cell surface, cells were washed with acidic medium (pH = 3.5) for 30 sec at 4°C after incubation with 1 µg/mL of dsDNA-6FAM associated with sLDH and large LDH to remove any particles bound to the cell surface. Images of cells were captured on a Zeiss Axio Imager (Carl Zeiss, Germany).

3. Results and discussion

3.1. General Features of sLDH Nanoparticles

As shown in Figure 1A, sLDH nanoparticles typically possessed a narrow particle size distribution. All nanoparticles were exclusively in the size range of 15-120 nm, with a Z-average diameter of 42 nm and polydispersity index (PdI) of 0.21. The TEM image (Figure 1B) indicates that these sLDH nanoparticles maintained the well-established hexagonal nanosheet morphology, with the lateral dimension of most sheets in the range of 20-80 nm. Such sLDH nanoparticles were nearly half the size of those reported earlier, whose Z-average particle size was ≥ 80 nm (Xu et al. 2006b). Also of note is the fact that the number-average
diameter of as-obtained sLDH nanoparticles was 21.4 nm, which is less than half that of the
LDH particles (~50 nm) reported by Oh et al. (2009b). In comparison, the most widely
reported to-date LDH particles prepared in aqueous solution had a Z-average size of 110 nm
and a PdI of 0.22 (Chen et al. 2013; Xu et al. 2006b), with an empirical formula of
\[ \text{Mg}_{1.9}\text{Al(OH)}_{5.8}\text{Cl}_{0.8}\text{(CO}_3\text{)}_{0.1}\cdot1.5\text{H}_2\text{O}, \]
as reported previously (Wong et al. 2012).

The XRD pattern (Figure 1C) shows that sLDH nanoparticles possessed the typical
lamellar structure, as featured by reflections (003), (006) and (009) in the thin film mode (Gu
et al. 2008). The d-value was 0.79 nm, close to the reported value for \(\text{Mg}_3\text{Al-NO}_3\text{-LDH} (0.81\)
nm) (Gu et al. 2008; Xu and Zeng 2001). Note that the (003) reflection had a full width at
half-maximum (FWHM) of 1.2°. This indicates that the thickness in the c-axis was \(~7\ nm,

This estimation has also revealed that the aspect ratio of as-prepared sLDH was \(~6\).

The FT-IR spectrum of sLDH (Figure 1D) is, as expected, largely identical to that of
traditional \(\text{Mg}_3\text{Al-LDH} \) (Xu and Zeng 2001), characteristic of the broad band at \(3440\ \text{cm}^{-1}\)
(stretching vibrations of O-H in brucite-like layer and interlayer H\(_2\)O molecules), the peak at
\(1637\ \text{cm}^{-1}\) (the bending vibration of interlayer and adsorbed H\(_2\)O molecules) and the bands at
around \(591\ \text{cm}^{-1}\) (M-O and M-O-H stretching vibrations) (Nakamoto 2009). Of particular
note is that the signal at \(1358\ \text{cm}^{-1}\), overlapping with the stretching vibration of NO\(_3^-\) (Xu and
Zeng 2001; Choy et al. 2004), is assigned to the stretching vibration of contaminant CO\(_3^{2-}\),
which was likely captured from air during the preparation and drying processes. The element
analysis (Mg/Al molar ratio = 2.9; 2.6 wt% N; 1.4 wt% C) gave an empirical formula of
\[ \text{Mg}_{2.9}\text{Al(OH)}_{7.8}\text{(NO}_3\text{)}_{0.5}\text{(CH}_3\text{O)}_{0.2}\text{(CO}_3\text{)}_{0.1}\cdot\text{OH}_{0.1}\cdot2.0\text{H}_2\text{O}. \]
In a typical non-aqueous preparation procedure, sLDH nanoparticles in aqueous suspension were obtained by the following procedure: (i) precipitation of mixed salts in basic methanol; (ii) collection and methanol-washing of the precipitate and hydrothermal treatment in fresh methanol at a range of temperatures and time periods; (iii) washing with deionized water; (iv) natural dispersion in deionized water for several days with occasional hand-shaking (Figure S1). As expected, the Z-average particle size and PdI of as-prepared sLDH are affected by the broad range of experimental parameters, including the dispersion duration and final sLDH concentration, methanol and water washing, heat-treatment temperature and duration, and co-precipitation temperature, as explored in detail below.

3.2. Effects of Preparation Conditions

**Dispersion.** Figure 2 shows the change of Z-average particle size and PdI of sLDH nanoparticles in suspensions with the duration of natural dispersion and the final sLDH concentration (Conc6.5-Conc29 in Table 1). After heat-treatment and water washing, the collected precipitate was manually dispersed in deionized water, which was then left to stand at room temperature with occasional hand-shaking. The particle size distribution of this suspension was then measured daily. As shown in Figure 2A, after 4-6 days of natural dispersion, the Z-average particle size of these sLDH particles was stabilized at ~45 nm. This size corresponds to the lateral size of the sLDH nanosheets (Figure 1B), thus revealing that individual sLDH nanosheets in suspension can be obtained after 4-6 days of natural dispersion. A shorter period of dispersion resulted in a Z-average particle size of 100-200 nm (at day 1) and 45-65 nm (at day 2). The gradual reduction in Z-average particle size over time (day 1 to day 4) indicates that the aggregated precipitate is gradually becoming individually
dispersed in deionized water. Similarly, the PdI of as-obtained sLDH nanoparticles in
suspension reduced in line with the dispersion time (day 1 to day 4) and remained constant
thereafter (Figure 2B).

Data in Figure 2 seemingly indicates that the final sLDH concentration, which was
achieved by dispersing water-washed sLDH slurry in a control volume of deionized water,
has some influence on the dispersion state (PdI), but not the Z-average particle size. For
example, the PdI was ~0.42 at an sLDH concentration of 29 mg/mL, double the value (0.21)
in the case of 6.5 mg/mL (Table 1 and Figure 2B). Although the Z-average sLDH particle
sizes were 42-44 nm (Table 1) over the entire concentration range (6.5-29 mg/mL), the
variation of the Z-average particle size at a higher sLDH concentration was greater (error bar
in Figure 2A and STDEV in Table 1). Both the higher PdI value and larger variation indicate
there is some, albeit minor aggregation when higher sLDH concentrations are employed. In
addition, the smaller number-average size at higher sLDH concentrations reveals that there is
a greater population of much smaller LDH particles (around 10 nm) (Table 1, Conc29
generated smallest number-average size) in the high-concentration suspension.

**Methanol- and water-washing.** Before and after heat-treatment, washing the collected
sLDH slurry is a crucial step, which significantly affects the Z-average particle size, PdI as
well as final product purity. We made three batches of sLDHs that differed merely in terms of
the methanol washing time (0, 1, 2) before heat-treatment (MW0-MW2 in Table 1). As
clearly shown in Figure 3, washing twice with methanol led to the best dispersed suspension
with the narrowest size distribution (44.8 nm, PdI of 0.20), while no washing gave a broader
distribution with a significantly larger Z-average particle size (70 nm) and PdI (0.27),
although the XRD patterns (Figure 3B) demonstrate the similar crystallinity of these sLDHs.
Similarly, the water-washing time (0, 1, 2) also significantly impacts the Z-average particle size and PdI (WW0-WW2 in Table 1). As shown in Figure S2, the size of sLDH particles washed twice had a narrower distribution (PdI 0.21) and a smaller Z-average particle size (42.3 nm). When water washing was omitted, only a small proportion of the collected sLDH aggregate was seen to disperse (Figure S2), which could be attributed to the presence of impurity salt NaNO$_3$ (Xu et al. 2006b). The presence of this salt was confirmed by the sharp reflections in the XRD pattern (ICDD PDF card no. 36-1474, WW0 in Figure 4A). Washing only once seemed to partially wash away the impure water-soluble salt NaNO$_3$ (although not detectable by XRD), which is likely responsible for the broad particle size distribution with a much larger Z-average size (Figure S2 and Table 1). The two small peaks around 2963 and 2850 cm$^{-1}$ in FT-IR spectra (Figure 4B), attributed to C-H stretching vibrations of $-CH_3$ in methoxide anion (Dijkstra et al. 1973), was weakened after water-washing, indicating that water-washing also assists the removal of methoxide from the sample, as is further explained below.

The effect of methanol washing seems to be largely masked by the subsequent water washing step. This is because the solubility of NaNO$_3$ in water (0.911 g/mL at 25°C) is much greater than that in methanol (0.00333 g/mL at 25°C) (Lide et al. 2011-2012). Therefore it is reasonable to infer that water washing twice removes all NaNO$_3$ from our LDH slurry and only in the case where methanol wash was omitted (sample MW0 in Figure 3) did traces of salt remain, which could be responsible for incomplete dispersion.

**Heat-treatment.** Figure 5 displays the effect of heating temperature (60-100°C) and duration (4-144 h) (over 15 days of natural dispersion) on the Z-average particle size and PdI of sLDH nanoparticles. As shown in Figure 5A and 5B, when the treatment temperature was
raised from 60 to 100°C, the Z-average particle size and PdI change was only slight, from 38 to 42 nm and 0.19 to 0.23 after 6 days of dispersion, respectively (HT60-HT100 in Table 1). Thus in general, the heat-treatment temperature had a limited impact on the sLDH particle size and distribution. Therefore in the context of these studies heat-treatment at 80°C resulted in the smallest Z-average particle size (~38 nm) and narrowest particle size distribution (PdI of ~0.19).

In contrast, prolonging the heat treatment duration from 4 to 144 h at 100°C increased the Z-average particle size from ~39 to ~47 nm while the PdI (0.19-0.22) remained unchanged after 6 days of dispersion (Figure 5C and 5D, HD4-HD144 in Table 1); this observation demonstrates that extending heating treatment time increased the particle size marginally.

The limited effect of heating temperature and duration on the average particle size and distribution using methanol as solvent is in sharp contrast to that when water is used as solvent. As reported early by Oh et al (2002) and Xu et al (2006b), the average LDH particle size can be tailored in a larger range (from 60-80 up to 300-400 nm) and the particle size distribution (PdI) increases from 0.2 to 0.5 during heating at 80-150°C over 2-144 h.

**Co-precipitation temperature.** The co-precipitation step was trialed at temperatures of 0, 23 and 50°C by mixing the salt methanol solution with the basic methanol solution. Both solutions were pre-cooled to 0°C or pre-heated to 50°C before mixing. Stirring was conducted throughout the co-precipitation process and continued for 30 min at the same temperature, followed by methanol washing, heat-treatment at 100°C for 18 h, and deionized-water washing twice (CPT0-HT to CPT50-HT in Table 1). As shown in Figure 6A and listed in Table 1, co-precipitation at 0 and 23°C yielded sLDH particles of the similar size with minor fluctuations in their size distribution (44.3 and 42.3 nm with a PdI 0.25 and 0.21), while co-
precipitation at 50°C generated a slightly larger particles (50.5 nm), indicating that the co-
precipitation temperature has a marginal effect on the sLDH nanoparticle size.

Furthermore, if heat-treatment was omitted (Figure 6B, CPT0 to CPT50 in Table 1), the
resultant LDH particles had a smaller Z-average size than those heat-treated. In particular, co-
precipitation at 0°C led to the smallest sLDH size (35.0 nm) with a narrower size distribution
(0.21) compared to those at 23 and 50°C (size of ~40 nm with PdI of ~0.24).

It is worth noting that once prepared the homogeneous dispersion remained stable for more
than one month, regardless of whether the sample was stored at fridge temperature (2-8°C) or
room temperature (22-25°C) (refer to Figure S3).

3.3. Mechanism of sLDH Formation

In summary, non-aqueous preparation, involving co-precipitation and heat-treatment in
methanol, and then dispersion in deionized water for 4-6 days, generally produces much
smaller LDH nanoparticles that can be individually dispersed into homogeneous aqueous
suspensions. Our investigations indicate that the co-precipitation temperature, heating
temperature and duration have marginal influences on the Z-average particle size of
homogenously dispersed sLDH nanoparticles (i.e. the sLDH crystals). Other factors, such as
methanol washing, water washing, the duration of natural dispersion and sLDH mass
concentration in the final aqueous suspension, merely affect the dispersity of sLDH
nanoparticles in suspensions.

It is suggested that when the mixed salts are added into basic methanol solution, the
following precipitation takes place to generate LDH nuclei, as in the aqueous case:

$$3\text{Mg(NO}_3\text{)}_2 \cdot 6\text{H}_2\text{O} + \text{Al(NO}_3\text{)}_3 \cdot 9\text{H}_2\text{O} + (9-y-2z)\text{NaOH} + x\text{CH}_3\text{OH} + z\text{Na}_2\text{CO}_3$$
In particular, CH$_3$O$^-$ is intercalated into the interlayer, which is evidenced by the characteristic CH$_3$ peaks in IR (Figure 4B), the expansion of the interlayer and the detection of higher amount of C% in the solid samples.

Following nucleation, nuclei are normally aged at a pre-determined temperature for a given period of time to enable them to disperse and grow into large crystallites. As reported elsewhere, in the case where water is employed as the solvent, the heat treatment can increase the LDH particle size by a factor of 3-5. For example, the particle size can grow from ~90 to ~280 nm (Z-average particle size) upon heat treatment at 100°C from 4 to 144 h, and from ~90 to ~190 nm when heated at temperatures for 16 h at 80 to 150°C (Xu et al. 2006a). In other reports (where the size was reported as a number-average value), LDH particle size can be tailored from ~85 to ~120 nm when heated at 100°C from 12 to 72 h, from ~115 to ~340 nm when heated for 48 h at 100 to 180°C (Oh et al. 2002) and from ~50 to ~350 nm when heated at 100-200°C for 12-48 h (Choi and Choy 2011). In this research, however, where methanol was employed as the solvent, the sLDH Z-average particle size was increased only slightly from 39 from 47 nm when heated at 100°C from 4 to 144 h, i.e. 20-30% increase. More strikingly, there was only a 10% variation (38-42 nm) in the Z-average particle size when heated from 60 to 100°C for 18 h. The sharp contrast in MgAl-LDH crystallite growth behaviors in methanol and water could be largely attributed to the solubility differences of MgAl-LDH in the two solvents.

During heat treatment, the so-called Ostwald ripening process occurs, i.e. large crystallites grow at the expense of small crystallites, primarily due to the higher solubility of small
crystallites in the same solvent as a result of their higher surface energy and specific surface area. Moreover, such a dissolution/recrystallization process is highly dependent on the solubility of LDH in that solvent. As reported previously (Choi and Choy 2011), the solubility of MgAl-Cl-LDH and MgAl-CO$_3$-LDH in water is 110 and 40 mg/L, respectively, which is consistent with our finding that as-prepared large MgAl-NO$_3$-LDH possesses a solubility of ~100 mg/L in water at 23°C. In contrast, we noted the solubility of MgAl-LDH in methanol is only 5-10 mg/L at 23°C. Therefore, as-formed LDH crystallites are considerably less soluble in methanol than in water. If we suppose that the solubility difference between large and small LDHs in methanol or water is proportional to the solubility in methanol or water, then the growth rate of LDH crystallites in methanol would be much slower than that in water under identical conditions, which would explain why the Z-average particle size is not very sensitive to variation in heating temperature and duration in methanol. This may mean that the size of as-prepared sLDH is largely dependent on the size of LDH nuclei formed in methanol. This hypothesis is further supported by the data in Table 1 that the Z-average size and PdI of sample CPT23 (without heat-treatment, 38.6 nm and 0.23) were very similar to those of samples HT60-HT100 (with heat treatment at 60-100°C for 18 h, 38-42 nm and 0.19-0.23). Nonetheless, 144 h heating treatment gave better crystallinity (sample HD144), as reflected by the smaller FWHM value [1.22° for (003) reflection, refer to Table S1] than that of CPT0 (FWHM = 1.54°) without heat-treatment.

Given this relationship, engineering sLDH without any heat treatment and with co-precipitation at 0 °C would be most desirable. This process indeed led to the smallest LDH with the Z-average particle size of 35.0 nm and PdI of 0.21 (CPT0 in Table 1) in this research.
The dispersion of as-formed sLDH nanoparticles in aqueous suspension can be tightly correlated to temperature as well as impure salt presence and concentration. As reported previously (Xu et al. 2006b; Xu et al. 2006a), hydrothermal treatment of manually dispersed LDH aggregates in water at 80-150°C results in a well-dispersed LDH suspension; this could be attributed to hydrothermal heating, which provides enough thermal energy for some LDH particles to detach from an aggregate and become suspended in the solution. As all LDH nanoparticles carry a positive charge (zeta potential 40-50 mV) (Ladewig et al. 2010; Wong et al. 2010), so the repulsion between LDH nanoparticles is then expected to keep them stably suspended in solution once disaggregation has occurred. As explained above, the existence of NaNO₃ in the current system prohibits complete dispersion of sLDH; this is primarily due to electrolyte ions reducing the thickness of electric double layers, which act as the ‘glue’ between particles (Vincent 2012; Fornasiero and Grieser 1991). We also gather from previous experiments that LDH particles will no longer disperse when the electrolyte concentration reaches a threshold value, for example, at 0.1-0.2 M of NaCl.

In this research, the dispersion of sLDH nanoparticles was conducted at room temperature, without the assistance of heat. Normally 4-6 days of standing with occasional hand-shaking gives rise to a complete dispersion of sLDH nanoparticles in solution. Achieving a relatively small particle size (40-50 nm) is a central aim of this research, and the thermal energy at room temperature is considered sufficient to de-aggregate sLDH nanoparticles from the aggregates. A plausible reason for the smaller particle dispersion could be related to the following exchange reaction occurring during the dispersion process:

\[
\begin{align*}
\text{Mg}_3\text{Al(OH)}_8(\text{CH}_3\text{O})_x(\text{NO}_3)_y(\text{CO}_3)_z(\text{OH})_{1-x-y-2z}\cdot m\text{H}_2\text{O} + (n+x-m)\text{H}_2\text{O} \\
\rightarrow \text{Mg}_3\text{Al(OH)}_8(\text{NO}_3)_y(\text{CO}_3)_z(\text{OH})_{1-y-2z}\cdot n\text{H}_2\text{O} + x\text{CH}_3\text{OH}
\end{align*}
\] (2).
This reaction likely takes place when the LDH slurry is washed with water. As shown in Figure 4B, the IR peak intensity of methoxide C-H vibrations decreased from sample WW0 to WW2, indicating that CH$_3$O$^-$ is gradually eliminated from the interlayer upon water washing. The replacement of a somewhat bulky methoxide by smaller OH$^-$/NO$_3^-$ ions is also reflected by decrease in the interlayer distance from WW0 (c/3, e.g. 0.92 nm) to WW2 (0.82 nm) (Table S1). This is also consistent with the reduction of carbon weight percentage from sample WW0 (2.50 wt %) to WW2 (1.41 wt %) when more CH$_3$OH is washed out. It is our belief that the occurrence of this exchange reaction facilitates sLDH dispersion, although this remains under close investigation.

3.4. dsDNA Association and Delivery Efficiency

The dsDNA loading capacity of sLDH and large LDH (Z-average size of ~40 and ~110 nm, respectively) was determined by agarose gel electrophoresis and visualized using ethidium bromide (Figure 7). When a current is applied, the native dsDNA (negative charge) readily migrates through the gel towards the positive electrode (cathode). Conversely, when dsDNA intercalates into the LDH interlayers or is adsorbed onto the LDH surface, the negative charge is effectively neutralized, and the dsDNA-LDH complexes are expected to remain in the loading well, unaffected by the current applied. Note that in all lanes the total amount of dsDNA was kept constant (100 ng in 20 μL) and the quantity of LDH varied according to the pre-set ratio. This approach allowed comparison of adsorption/intercalation between sLDH and large LDH at each mass ratio.

First, dsDNA was incubated with sLDH at 37°C using dsDNA:LHD mass ratios of 1:10 and 1:1 from 10 min to 18 h (Figure 7A). At a mass ratio of 1:10 the association occurred
rapidly and completely as nearly 100% dsDNA was retained in the loading well after just 10 min of incubation. In contrast, adsorption/intercalation at 1:1 was limited and probably increased only slightly after 18 h. Next, the dsDNA loading capacity of sLDH and large LDH at a range of mass ratios after incubation at 37°C for 10 min was determined. Strong fluorescence was observed in the loading wells when dsDNA was mixed with sLDH at ratios of 1:10 and 1:5 (Figure 7B), indicating that almost all dsDNA was associated with our miniaturized nanoparticles. In striking contrast, only weak fluorescence was seen using identical mass ratios for the larger LDH particles, demonstrating that they were effectively associating with only a small percentage of dsDNA. This comparison reveals that sLDH possesses a much higher dsDNA loading capacity than large LDH. On the basis of these results, intercalation/association of nucleic acids into LDHs was carried out with the mass ratio of 1:5 for 10 min at 37°C for the delivery tests that followed.

Light-microscopic imaging of HEK 293T cells showed very bright 6FAM-positive puncta within the cytoplasm after exposure to dsDNA-6FAM-sLDH complexes (1.0 μg/mL dsDNA) in comparison with the control (dsDNA-6FAM) and dsDNA-6FAM-large LDH (Figure 8A), confirming that sLDH nanoparticles are able to deliver dsDNA much more efficiently into HEK 293T cells than large LDH particles. These puncta are likely to be early endosomes and multi-vesicular bodies, which we have previously shown to be the internalization route for large LDH (Wong et al. 2010; Xu et al. 2008).

The quantitative efficiency of sLDH- and large LDH-mediated dsDNA uptake into HEK 239T cells was further examined. In these experiments, cells were exposed to 0.1, 1.0 or 10 μg/mL of dsDNA-6FAM associated with sLDH or large LDH (mass ratio 1:5) for 4 h, after which the percentage of 6FAM-positive cells was assessed by flow cytometry (FACS). The
cytoplasmic fluorescence of cells exposed to dsDNA alone was equivalent to the intrinsic background fluorescence of the untreated cells (data not shown). When exposed to either dsDNA-6FAM-sLDH or dsDNA-6FAM-large LDH at a dsDNA concentration of 10 μg/mL, almost 100% of cells were 6FAM-positive (Figure 8B, Table 2). However, the extent of internalization in individual cells was markedly greater where sLDH nanoparticles were used as the delivery system. The median fluorescence intensity (FI) for these cells was 127 FI units while dsDNA-6FAM-large LDH complexes results the observed median FI of only 21 units (Table 2). Therefore, sLDH-mediated dsDNA internalization was calculated to be 6-fold more efficient than large LDH-mediated internalization.

Such a difference in sLDH and large LDH uptake efficiency was also observed at a dsDNA concentration of 1.0 μg/mL, albeit the level of intracellular 6FAM fluorescence was lower than observed with 10 μg/mL dsDNA (Figure 8B, Table 2). At 1.0 μg/mL, 72% of cells were 6FAM-positive after exposure to the dsDNA-6FAM-sLDH complexes and the median FI for the population was 15.7, whereas only 50% of cells internalized the dsDNA-6FAM-large LDH complexes and the median FI was 4.5, indicating a 5-fold reduction in uptake efficiency. As previously observed (Wong et al. 2010; Xu et al. 2008), very few cells were found to be 6FAM-positive when using a lower dsDNA concentration of 0.1 μg/mL with either sLDH or large LDH, however, again the sLDH particles were more effective. Consistently, when dsDNA-6FAM was associated with sLDH or large LDH at the mass ratio of 1:1, the internalization efficiency using sLDH was found a few times higher than using large LDH, as reported previously (Chen et al. 2013). In sharp contrast, the dsDNA:LDH mass ratio of 5:1 led to a much higher amount of dsDNA internalized using both sLDH and large LDH than in the case of 1:1.
Taken together, the above results clearly demonstrate that the smaller LDH (sLDH) nanoparticles are able to more effectively transport short double-stranded nucleic acids into HEK 293T cells. The higher transfection effectiveness of sLDH could be attributed to two fundamental aspects: cellular uptake rate and loading capacity. As Choy et al. found, 50-nm LDH particles are more effectively taken up by cells than 100-350 nm LDH (Oh et al. 2009b; Choi and Choy 2011), so sLDHs would be more readily internalized than large LDHs in our tests. Moreover, we also noted that at the mass ratio of 1:5 (dsDNA:LDH), sLDH loaded almost all dsDNA while large LDH carried probably ~50% dsDNA (Figure 7B). The loading capacity may explain that why smaller CO$_3$-LDH (20 nm) exhibited less efficient delivery of pEGFP-N1 DNA than the larger NO$_3$-LDH (180 nm) (Li et al. 2013). Although the small CO$_3$-LDH can be internalized more rapidly, the loading of DNA onto the CO$_3$-LDH surface is much more difficult than that onto NO$_3$-LDH due to the higher affinity of CO$_3^{2-}$ for LDH than NO$_3^-$, which finally reduces the transfection efficiency.

4. Conclusions

sLDH particles engineered by this approach possess a Z-average diameter size of 35 to 50 nm, albeit with heat treatment temperatures ranging from 60 to 100°C and treatment duration up to 144 h, yielding a final sLDH suspension concentration of 6.5-29 mg/mL. Removing salt impurities prior to natural dispersion proves crucial for homogeneous particle dispersion with a narrow particle size distribution. Once prepared the physical characteristics of our sLDH nanoparticles remain as are, for at least one month. The cellular delivery efficiency using our sLDH nanoparticles (40 nm) is markedly improved, with a 5-6 fold enhancement in delivery observed in vitro, when compared to the typically large LDH particles (110 nm).
Acknowledgments

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Biomaterials 31, 8770-8779.


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Table 2 Comparison of dsDNA delivery by sLDH and large LDH into HEK 293T cells after 4 h incubation

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[a] mass ratio dsDNA:LDH = 1:5; [b] FI, fluorescence intensity; [c] FI for control cells = 1 unit.
Figure 1 Features of typical sLDH: A). Typical particle size distribution of small LDH suspension; B). TEM image of small LDH suspension (synthesized at 100°C for 18 h); C). XRD pattern of small LDH suspension (thin film on glass slide); D). FT-IR spectrum of small LDH.
**Figure 2** Particle size (A) and Polydispersity index (B) change of sLDH suspension with dispersion time and sLDH concentrations.

**Figure 3** Particle size distribution of sLDH suspension (A) and XRD patterns of sLDH slurry according to methanol wash time(s) for synthesising sLDH; MW0, MW1 and MW2 represent 0, 1, or 2 times methanol-washing.
Figure 4 XRD patterns (A) and FT-IR spectra (B) of LDH powders obtained with water washing 0, 1 and 2 times (WW0, WW1, and WW2).

Figure 5 Particle size (A) and Polydispersity Index (B) change of sLDH suspension with dispersion time and hydrothermal treatment temperatures; Particle size (C) and PdI (D) change of sLDH suspension with dispersion time and hydrothermal treatment durations.
Figure 6 Size distribution of sLDH suspension coprecipitated at at 0, 23 and 50°C with (A) and without (B) hydrothermal treatment at 100°C for 18 h.
Figure 7 Agarose gel assay of dsDNA intercalation into LDH nanoparticles. (A) Loading dsDNA with sLDH at mass ratios of 1:10 and 1:1 (dsDNA:LDH) after incubation for 10 min, 30 min, 1 h, 4 h and 18 h at 37°C. (B) Intercalation of dsDNA into sLDH and large LDH at various mass ratios (dsDNA:LDH) after incubation at 37°C for 10 min.
Figure 8 LDH-facilitated uptake of fluorescently tagged dsDNA into HEK 293T cells: (A) Microscopy imaging of 6FAM (green fluorescent puncta) of cells after 4 h exposure to 1.0 μg/mL dsDNA; (B) uptake of dsDNA-DCC2-6FAM-S-LDH complexes (0.1-10 μg/mL dsDNA) was observed after incubation 1 h in HEK293 cells. Both are at dsDNA:LDH = 1:5.
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