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Cellular uptake behavior of [γ-^{32}P] labeled ATP–LDH nanohybrids†

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The inorganic layered double hydroxide support, $Mg_4Al_2(OH)_{12}(NO_3)_2{\cdot}mH_2O$, could be obtained by coprecipitation in aqueous solution; the interlayered NO_3^- anions can be replaced by biomolecules such as cytidine 5′-monophosphate, adenosine 5′-monophosphate, guanosine 5′-monophosphate and adenosine 5′-triphosphate to form new biomolecule–LDH hybrids. Upon intercalating these biomolecules into hydroxide layers, the interlayer distance increases from 8.7 Å (for NO_3^-) to 14.5 Å, 16.9 Å, 18.4 Å, and 19.4 Å, respectively. According to spectroscopic analysis, it is also found that the intercalated biomolecules maintain their chemical properties unchanged in the interlayer space of the layered double hydroxide. Furthermore, the isotope dilution test for the transfer efficiency of [γ-^{32}P] ATP–LDH hybrid into eucaryotic cells verifies reproducibly much higher transfer efficiency of the hybrid than for ATP molecules alone due to the charge neutralization of anionic phosphate molecules by cationic hydroxide layers in the hybrid. These experimental data suggest the potential usefulness of LDHs as innovative inorganic reservoirs and delivery carriers for genes, drugs, and other biomolecules.

Introduction

Over the past few years there has been considerable interest in nanoscale materials which often exhibit unique physical and chemical properties that are dramatically different from their bulk counterparts.[1–3] More recently, nanohybrids, defined as composites consisting of two disparate nanoscale materials with two different functions, *e.g.* a mixed conductor consisting of an electronic conducting phase and ionic conducting one,[4] which are chemically bound in an organized way. Such materials often exhibit extraordinarily high synergetic and complementary behavior.

In particular, the combination of two-dimensional layered materials and the intercalation technique offers new areas for developing new hybrids with desired functionality.[5–14] Since most biomolecules, such as nucleoside monophosphates and ATP are negatively charged, they can be incorporated between the hydroxide layers as the charge compensating anions through ion exchange.[15,16] Layered double hydroxides (LDHs), also called 'anionic clays', have received considerable attention due to their technological importance in catalysis, separation technology, optics, medical science, and nanocomposite materials engineering. LDHs consist of positively charged metal hydroxide layers, in which the anions (along with water) are stabilized in order to compensate the positive layer charges. The composition can be generally represented as $[M^{2+}_{1-x}M^{3+}_x(OH)_2][A^{n-}]_{x/n}{\cdot}mH_2O$, where M^{2+} is a divalent cation (Mg^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+}), M^{3+} is a trivalent one (Al^{3+}, Cr^{3+}, Fe^{3+}, V^{3+}, and Ga^{3+}), and $A_{x/n}{}^{n-}$ is an anion with charge n. Various kinds of inorganic or organic anions (A^{n-}) have been introduced between the hydroxide layers by simple ion-exchange reaction or coprecipitation.[17–20] Especially, LDHs consisting of magnesium and aluminium have already been used as antiacid and antipepsin agents, therefore, we believe that the present LDH is quite bio-compatible. In the present study, novel bio-hybrids of layered double hydroxide (LDH) and biomolecules (ATP or nucleoside monophosphates) are designed and organized artificially on the nanometer scale to provide opportunities for reservoir and delivery carriers of functional biomolecules in gene therapy and drug delivery. Therefore, the purpose of this study is not only to prepare new bio-inorganic nanohybrids but also to provide basic data for developing new inorganic carriers.

Experimental

Preparation of pristine LDH

Firstly, the pristine LDH was prepared by coprecipitation under N_2 atmosphere following the conventional route.[17–20] In a typical coprecipitation, a mixed aqueous solution containing Mg^{2+} (0.024 M, from $Mg(NO_3)_2$) and Al^{3+} (0.012 M, from $Al(NO_3)_3$) was titrated into a NaOH solution dropwise with vigorous stirring. During the titration, the solution pH was adjusted to 10 ± 0.2 and the temperature was controlled to 25 °C. The resulting white precipitates were collected by centrifugation and washed with deionized water thoroughly.

Preparation of biomolecule–LDH hybrids

The biomolecule–LDH hybrids were then prepared by ion-exchanging the interlayer nitrate ions in the pristine LDH with nucleotides such as adenosine 5′-monophosphate (AMP), guanosine 5′-monophosphate (GMP), cytidine 5′-monophosphate (CMP), and adenosine 5′-triphosphate (ATP, containing 40 μCi of [γ-^{32}P] ATP, DuPont) at pH = 7. The pristine LDH was dispersed in a deaerated aqueous solution containing an excess of dissolved AMP, GMP, CMP or ATP, and reacted for 48 hours with constant stirring. The reaction products were then isolated and washed as described above.

†Electronic supplementary information (ESI) available: histogram for Mg solubility of Mg_2Al–LDH as a function of pH; FITC–LDH exchange rate according to NaCl concentration (FITC = fluorescein 5-isothiocyanate); cytotoxicity test of Mg_2Al–NO_3^-–LDH. See http://www.rsc.org/suppdata/jm/b0/b008680k/

Table 1 Compositional data for the pristine LDH and biomolecule–LDH hybrids[a]

Sample	Chemical composition
Pristine LDH	$Mg_{0.68}Al_{0.32}(OH)_2(NO_3)_{0.32}\cdot 1.2H_2O$
AMP–LDH	$Mg_{0.66}Al_{0.34}(OH)_2(AMP)_{0.14}(OH)_{0.20}\cdot 0.9H_2O$
CMP–LDH	$Mg_{0.66}Al_{0.34}(OH)_2(CMP)_{0.19}(OH)_{0.14}\cdot 0.7H_2O$
GMP–LDH	$Mg_{0.66}Al_{0.34}(OH)_2(GMP)_{0.14}(OH)_{0.20}\cdot 0.9H_2O$
ATP–LDH	$Mg_{0.67}Al_{0.33}(OH)_2(ATP)_{0.08}(CO_3)_{0.05}\cdot 1.0H_2O$

[a]AMP, CMP, GMP: monoanion, ATP: trianion.

Sample characterization

The stoichiometry of each biomolecule–LDH hybrid was determined by elemental analysis (CHN), thermogravimetry (TG), and inductively coupled plasma spectrometry (ICP) as shown in Table 1. The synthesis of each hybrid was confirmed by XRD measurement using Ni-filtered Cu-Kα radiation with a graphite diffracted beam monochromator. Infrared spectra were obtained with a Bruker IFS-88 FT-IR spectrometer by the standard KBr disk method.

Cellular uptake of radioactive isotope labeled LDH hybrid

The isotope labeled ATP–LDH hybrid and [γ-^{32}P] ATP only were added to 4×10^6 HL-60 cells in 20 ml of RPMI-1640 with 10% heat-inactivated fetal bovine serum, and then incubated in a 5% CO_2 incubator at 37 °C for 1, 2, 4, 6, 20 or 24 hours.[21] For each reaction time, 1 ml of sample was taken, centrifuged, then the separated supernatant was collected and the cell pellet washed once with 1 ml of phosphate buffer (10 mM Na_2HPO_4, pH 7.4, 150 ml NaCl) followed by sedimentation. The supernatant was again separated and collected, and the cell pellet was lysed in 200 µl of lysis buffer (10 mM Tris/Cl, pH 7.4, 150 ml NaCl, 1% sodium dodecyl sulfate) and then extracted with 200 µl of phenol. After separating the aqueous phase, the phenol phase was extracted again with 200 µl of water. Aliquots of the combined aqueous extracts, cell walls, and culture-medium supernatant were analyzed by liquid scintillation counting. The percentage of hybrid and [γ-^{32}P] ATP taken up by the cells was calculated by dividing the counts in the combined aqueous phases of the cell pellet extract by the total counts in the cell pellet, cell wash, and culture-medium supernatant. All the procedures were repeated three times to check the reproducibility.

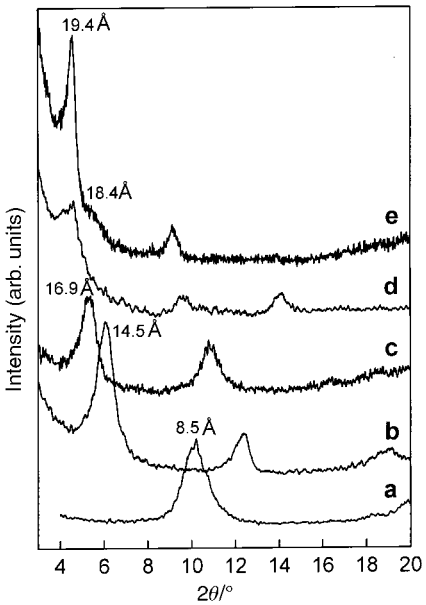

Fig. 1 Powder X-ray diffraction patterns: (a) the pristine LDH, (b) CMP–LDH, (c) AMP–LDH, (d) GMP–LDH, and (e) ATP–LDH.

Results and discussion

Crystal structure of biomolecule–LDH hybrids

The X-ray diffraction patterns for the pristine LDH and its biomolecule–LDH hybrids are shown in Fig. 1. The diffraction peak at 8.5 Å for the pristine sample corresponds to the basal spacing of NO_3^- incorporated in hydroxide layers. Upon substituting NO_3^- ions with biomolecules, the (00*l*) reflections shift to lower angles, indicating that the hydroxide layers are further expanded upon intercalation of bulky anionic biomolecules. In addition, well ordered (00*l*) series imply that the anion exchange reaction occurs topotactically without any reconstruction of the lamellae structure of the pristine hydroxide layers. Taking into account the brucite-like LDH sheets (4.8 Å), the gallery heights of biomolecule–LDH hybrids were estimated to be 9.7 Å (for CMP), 12.1 Å (for AMP),

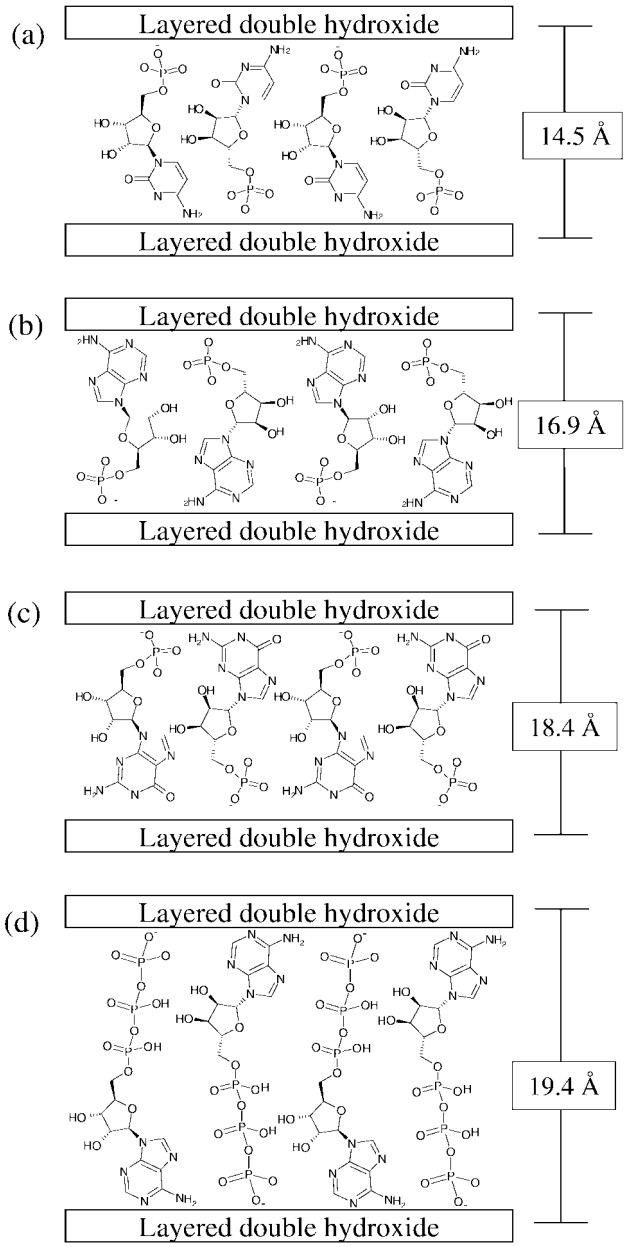

Fig. 2 Schematic illustration of each nucleotide structure intercalated in the LDH inorganic layer: (a) CMP–LDH, (b) AMP–LDH, (c) GMP–LDH, and (d) ATP–LDH.

13.6 Å (for GMP), 14.6 Å (for ATP), respectively, which means that the nucleotides tend to have a monolayer arrangement. It is thought that anionic substituents (phosphate groups) are oriented towards the LDH layers to maximize the electrostatic attraction. Taking into account the charge density of the layers, about 25.0 $Å^2/e^-$,[22] intercalants are perpendicularly arranged to hydroxide layer. The schematic molecular arrangements in the interlayer of LDH are represented in Fig. 2, based on the basal spacings and the molecular size of the corresponding intercalants.

Chemical and biological integrity of biomolecule–LDH hybrids

Fig. 3 shows the infrared spectra for biomolecule–LDH hybrids. All the characteristic bands, corresponding to biomolecules, are superimposed on those of LDH. The absorption band at 1360 cm^{-1} due to the stretching vibration of NO_3^- in the pristine LDH completely disappears after ion exchange reaction, suggesting that the interlayer NO_3^- ions are completely replaced by biomolecules. The absorption bands at 1000–1100 cm^{-1} correspond to the ν_1,ν_3 (P–O) modes of PO_3^{2-} groups in the biomolecules.[23] Comparing them with those of the native biomolecules, however, the bands are relatively broad owing to the interaction between the biomolecules and the hydroxide sheets. The other characteristic bands due to various functional groups such as the aromatic C=C or C=N, as well as conjugates C=O, –N–C=O in the biomolecules are also clearly observed as shown in Fig. 3. Here, the absorption maxima are almost identical with those of native biomolecules, which strongly suggests that the biomolecules are intercalated in the hydroxide layers with their integrity preserved.

The transfer efficiency of [γ^{32}-P] ATP–LDH hybrid

As discussed above, the biomolecules are well stabilized in the LDH lattice, but they can be, if necessary, deintercalated by ion-exchange reaction with other anions or atmospheric CO_2. These features will allow LDHs to be applied as new drug or gene carriers if the transfer efficiency of the biohybrids to target organs or cells is proved. To elucidate the transfer efficiency, isotope-labeled [γ-^{32}P] ATP–LDH hybrid was prepared by ion exchange and the uptake of such hybrids by eucaryotic cells was monitored with respect to incubation time.[21] Fig. 4 clearly demonstrates that the exogeneously introduced ATP–LDH hybrid can enter into HL-60 cells effectively within a relatively

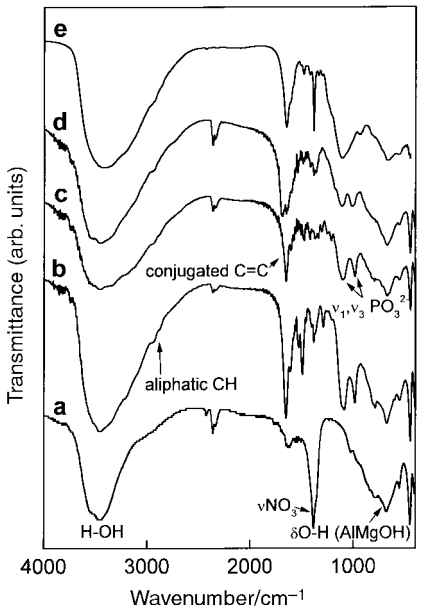

Fig. 3 Infrared spectra for (a) the pristine LDH, (b) CMP–LDH, (c) AMP–LDH, (d) GMP–LDH, and (e) ATP–LDH.

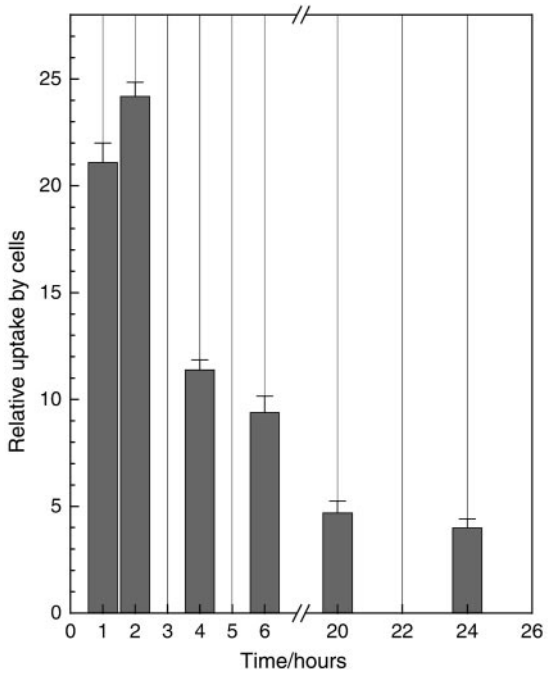

Fig. 4 Histogram for uptake efficiency of [γ-^{32}P] radioactive isotope labeled ATP–LDH hybrids into HL-60 cells. The uptake efficiency of ATP–LDH hybrids was normalized to that of ATP only. The error bars represent the standard deviation for three measurements.

short time. The transfer efficiency was found to be much higher, up to about 25-fold after 2 hours incubation, than that of ATP only, whereas after 4 hours incubation, the uptake amount of the hybrids becomes lower, below 12-fold. The triphosphate group of [γ-^{32}P] ATP has a negative charge, which inhibits [γ-^{32}P] ATP from being internalized in the cell through the negatively charged cell walls. In contrast, the hybridization between ATP and LDH neutralizes the surface charge of anionic phosphate groups in ATP due to the cationic charge of LDH, which leads to favorable endocytosis of cells, and eventually results in enhanced transfer efficiency. The longer the incubation time is in a CO_2 atmosphere, the more the ATP will be released from the interlayer space of the hydroxide lattice. In spite of this, the transfer efficiency of the hybrid remains higher than that of ATP only, up to about 4-fold after 24 hours incubation. This result reflects that the hybridization between cationic layers and anionic biomolecules greatly enhances the transfer efficiency of biomolecules to mammalian cells or organs, as illustrated in Fig. 5. The charge neutralization through hybridization between LDH and ATP facilitates the penetration of hybrids into cells, *via* so-called endocyto-

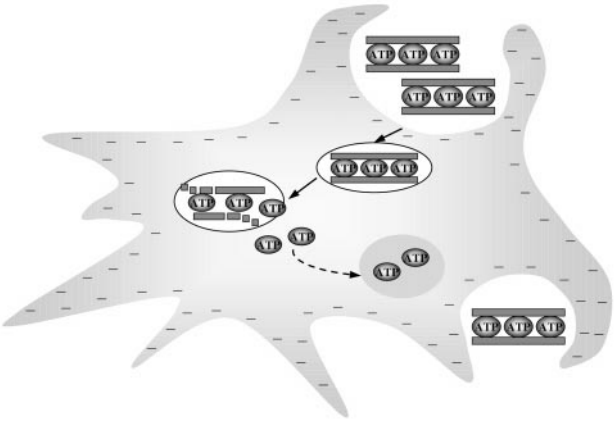

Fig. 5 Schematic illustration for the transfer mechanism of the ATP–LDH hybrid.

sis,[24,25] because it greatly reduces the electrostatic repulsive interaction between negatively charged cell membranes and anionic biomolecules during endocytosis. Once bio–LDH hybrids are introduced into cells, then the hydroxide layer in the bio–LDH hybrids will be removed slowly in the lysosome where the pH is slightly acidic (pH = 4–5), since the hydroxide layers of Mg and Al dissolve in an acidic environment. At the same time, interlayer biomolecules would partially be replaced by other anions in the cell electrolyte in such a way that the encapsulated biomolecules could be released in the inside of a cell from an LDH hybrid.

Conclusion

It has been clearly demonstrated that nucleoside mono- or triphosphates can be intercalated in inorganic layered double hydroxides, giving rise to biomolecular–inorganic nanohybrids while preserving the physico-chemical and biological integrity of the encapsulated biomolecules. Moreover, the hybridization of ATP with LDH resulted in a remarkable transfer efficiency of ATP into target cells by alleviating the electrical repulsion at the cell walls due to the neutralization of the negative charge of the phosphates with positive hydroxide layers. These unique features of biomolecule–LDH hybrids will open a new opportunity for LDHs to be useful as reservoirs and carriers for genes, drugs, and other functional biomolecules.

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References

1 G. A. Ozin, *Adv Mater.*, 1992, **4**, 612.
2 Y. Lvov, K. Ariga, I. Ichinose and T. Kunitake, *J. Am. Chem. Soc.*, 1995, **117**, 6117.
3 Y. Lvov, K. Ariga, I. Ichinose and T. Kunitake, *Langmuir*, 1996, **12**, 3038.
4 J. H. Choy, Y. I. Kim and S. J. Hwang, *J. Phys. Chem. B*, 1998, **102**(46), 9191.
5 J. H. Choy, S. J. Kwon and G. S. Park, *Science*, 1998, **280**, 1589.
6 J. H. Choy, N. G. Park, Y. I. Kim, S. H. Hwang, J. S. Lee and H. I. Yoo, *J. Phys. Chem.*, 1995, **99**, 7845.
7 J. H. Choy, N. G. Park, S. J. Hwang, D. H. Kim and N. H. Hur, *J. Am. Chem. Soc.*, 1994, **116**, 11564.
8 C. O. Oriakhi, I. V. Farr and M. M. Lerner, *Clays Clay Miner.*, 1997, **45**, 194.
9 V. I. Iliev, A. I. Ileva and L. D. Dimitrov, *Appl. Catal. A: General*, 1995, **126**, 333.
10 G. T. D. Shouldice, P. Y. Choi, B. E. Koene, L. F. Nazar and A. Rudin, *J. Polym. Sci. Part A: Polym. Chem.*, 1995, **33**, 1409.
11 C. O. Oriakhi, I. V. Farr and M. M. Lerner, *J. Mater. Chem.*, 1996, **6**, 103.
12 S. Therias, C. Mousty, C. Forano and J. P. Besse, *Langmuir*, 1996, **12**, 4914.
13 S. G. Starodoubtsev, N. A. Churochkina and A. R. Khokhlov, *Langmuir*, 2000, **16**, 1529.
14 J.-M. Séquaris, *Langmuir*, 1997, **13**, 653.
15 J. O. Rädler, I. Koltover, T. Saldit and C. R. Safinya, *Science*, 1997, **275**, 810.
16 J. H. Choy, S. Y. Kwak, J. S. Park, Y. J. Jeong and J. Portier, *J. Am. Chem. Soc.*, 1999, **121**, 1399.
17 F. Cavani, F. Trifiro and A. Vaccari, *Catal. Today*, 1991, **11**, 173.
18 M. Meyn, K. Beneke and G. Lagaly, *Inorg. Chem.*, 1993, **32**, 1209.
19 V. R. L. Constantino and T. J. Pinnavaia, *Inorg. Chem.*, 1995, **34**, 883.
20 J. H. Choy, Y. M. Kwon, S. W. Song and S. H. Chang, *Bull. Korean Chem. Soc.*, 1997, **18**, 450.
21 E. L. Wickstrom, T. A. Bacon, A. Gonzalez, D. L. Freeman, G. H. Lyman and E. Wickstrom, *Proc. Natl. Acad. Sci. USA*, 1988, **85**, 1028.
22 L. V. Interrante, *Advances in chemistry series 245 – Materials chemistry – An emerging discipline*, American Chemical Society, Washington, DC, 1995.
23 C. J. Pouchert, *The Aldrich library of Infrared spectra*, Aldrich Chemical, Milwaukee, 2nd edn., 1975.
24 S. S. Davis, *Trends Biotechnol.*, 1997, **15**, 217.
25 I. M. Verma and N. Somia, *Nature*, 1997, **389**, 239.