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ScienceDirect
International Journal of Pharmaceutics 326 (2006) 148–152
INTERNATIONAL JOURNAL OF PHARMACEUTICS
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Pharmaceutical Nanotechnology

Synthesis and properties of cordycepin intercalates of Mg–Al–nitrate layered double hydroxides

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Received 3 February 2006; received in revised form 8 June 2006; accepted 27 June 2006
Available online 1 July 2006

Abstract

Layered double hydroxides (LDHs) were investigated as cordycepin delivery nanocarriers for the first time in this study. Negatively charged biomolecule-cordycepin was intercalated in the gallery spaces of [Mg–Al–NO_3] as the charge-compensating species, which was confirmed by the results of XRD, FT-IR, TEM, CZE and electrophoretic mobility. Cell experiment suggested that the new bio-LDH nanohybrid could prevent cordycepin decomposition by adenosine deaminase. This new formulation could possibly be used as a novel form cordycepin intravenous injection.

Keywords: Layered double hydroxide; Cordycepin; Nanohybrid; Intercalate

1. Introduction

The incorporation of organic guests into layered double hydroxides (LDHs) has received much attention recently because of the potential uses of the resulting inorganic–organic nanohybrid materials in catalysis, adsorption, optics, nanocomposite engineering materials and medical science (You et al., 2002; Ambrogi et al., 2001; Whilton et al., 1997; Tyner et al., 2004; Khan et al., 2001; Li et al., 2004; Darder et al., 2003; Hussein and Seng, 2001; Hibino, 2004). LDHs can be represented by the general formula: $[M_{1-x}^{II}M_x^{III}(OH)_2]^{x+}X_{x/m}{}^{m-}\cdot nH_2O$, abbreviated as: $[M^{II}–M^{III}–X]$ where M^{II} is a divalent metal ion such as Mg^{2+}, Ni^{2+}, Cu^{2+} or Zn^{2+}, M^{III} is a trivalent metal ion such as Al^{3+}, Cr^{3+}, Fe^{3+} or Ga^{3+} and X^{m-} is an anion charged m such as $CO_3{}^{2-}$, Cl^-, $NO_3{}^-$ or other inorganic and organic anions (Cavani et al., 1991; Rives and Ulibarri, 1999; Vaccari, 1999; Khan and O'Hare, 2002). The net positive charge, due to substitution of trivalent by divalent metal ions, is compensated by an equal negative charge of the interlayer solvated anions (Meyn et al., 1990). LDHs are layered materials with ion-exchange ability and biocompatibility (Choy et al., 1999; Choy et al., 2000; Tamura et al., 2004; Kwak et al., 2004; Nakayama et al., 2004), *in vivo* testing in animals indicated that they are noncytotoxic to animals (Kwak et al., 2004). These findings suggested that LDHs could act as new inorganic carriers for encapsulating functional biomolecules between hydroxide layers through the electrostatic interaction between cationic layers and negatively charged biomolecules to form steady bio-LDHs nanohybrid.

Cordycepin (3′-deoxyadenosine), a nucleoside derivative has been shown to inhibit the growth of various tumor cells (Sun et al., 2003; Rodman et al., 1997; Ahn et al., 2000; Huang et al., 2003), making it a potential therapeutic agent for cancer and microbial diseases. However, cordycepin is quickly deaminated by adenosine deaminase (ADA), an enzyme present in many tissues. Coadministration of 2′-deoxycoformycin, a potent inhibitor of ADA, markedly increases the toxicity of cordycepin. Increasing the dose, consequence of high cost level and drug resistance. How to protect cordycepin from deamination by ADA and retain its activity in the biological system is still an unsolved problem. There is a high possibility that the cordycepin-LDHs [Mg–Al–cordycepin] nanohybrid can resolve this problem. Intercalation of cordycepin into LDHs may prevent biomolecules from degradation of enzyme and other substances during transportation, whereas anion exchange along with acid dissolution may result in the release of the cordycepin.

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doi:10.1016/j.ijpharm.2006.06.037

In the present study, we prepared the [Mg–Al–cordycepin] by intercalation of cordycepin into [Mg–Al–NO_3] which was prepared by a non-steady coprecipitation method, and briefly discussed the characterization and properties of the [Mg–Al–cordycepin]. Application efficiency of [Mg–Al–cordycepin] was also investigated through *in vitro* bioassay with a tumor cell U937.

2. Experimental

All the chemicals used in this work were of analytical grade and used without any further purification. Cordycepin (Sigma), RPMI-1640 (Gibco), HEPES (Sigma), fetal bovine serum (FBS, Gibco), penicillin (Gibco), streptomycin (Gibco), cell counting kit-8 (Dojindo Laboratories).

The [Mg–Al–NO_3] was synthesized by the non-steady coprecipitation (Yang et al., 2005).

[Mg–Al–cordycepin] was prepared by ion-exchanged route with modifications. The 0.058 mL dense suspensions of [Mg–Al–NO_3] (10.0 wt.% solid content) were mixed with 1.3 mL 0.6% aqueous solutions of cordycepin, shaken by hand for 10 min and filtrated through 0.22 μm filter film for disinfection. The filtrate was then stored at 4 °C for 7 days followed by separated by centrifugation. The precipitate was washed thoroughly with deionized water and freeze-dried to afford [Mg–Al–cordycepin] nanohybrid 7.82 mg. We calculated the quantity of intercalated cordycepin by comparing the change of the weights of LDHs. For example, after reacting with cordycepin, the quantity of LDHs changed from 5.8 to 7.82 mg. Since the respective formula weight of the cordycepin and NO_3^- is 251 and 62, the quantity of intercalated cordycepin can be calculated by the formula: $(7.82 - 5.8)/(251 - 62) \times 251 = 2.68$ mg.

Powder X-ray diffraction (XRD) used was a D/max-γ B diffractometer with Cu Kα radiation. Data were collected over the 2θ range from 2° to 40° in the increments of $0.02^\circ\ s^{-1}$ at room temperature. Transmission electron microscopy (TEM) analysis was performed using a JEM-100CX^{II} electron microscope. TEM samples were prepared by dipping amorphous carbon coated copper TEM grids into dilute aqueous suspensions of the samples powder, which was washed only by deionized water. Capillary zone electrophoresis (CZE) separations were carried out with a Beckman P/ACE^{TM} System MDQ (Fullerton, CA, USA) utilizing System Software Version 2.2 for control and data collection. FT-IR spectra were recorded in the regions 4000–400 cm^{-1} on a Nicolet 50X infrared spectrophotometer. The electrophoretic mobility (μ) of samples was measured using a DXD-I microelectrophoresis instrument with a flow through sample cell. Samples were prepared by dispersing 0.05 vol.% of each samples powder.

2.1. In vitro bioassay

Human leukemia cell (U937) was used in this study. Cells were routinely cultured at 37 °C in a humidified atmosphere with 5% CO_2 in 75 cm^2 flasks containing 10 mL of RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin (Gibco) and 100 μg/mL streptomycin (Gibco). Cordycepin, LDHs, and nanohybrid were dissolved in aseptic water at different concentrations. The concentration of [Mg–Al–cordycepin] nanohybrid was the concentration of the intercalated cordycepin. The U937 cells were collected by centrifugation and resuspended in culture medium at 1.5×10^5 cells/mL. About 100 μL of the testing samples at different concentrations were placed in the wells of a 96-well plate followed by adding 100 μL of the U937 cells to each well. It was gently mixed and incubated in a CO_2 incubator at 37 °C for 46 h. At 46 h, 10 μL of a solution of cell counting kit-8 (Dojindo Laboratories) was added to each well according to the manufacture's instructions, and incubated for another 2 h. The number of viable cells in culture was assessed using the cell counting kit-8, which colorimetrically measured intracellular NADH.

3. Results and discussion

3.1. Characterization of the intercalation compound

The XRD patterns for the [Mg–Al–NO_3] and the [Mg–Al–cordycepin] nanohybrid are shown in Fig. 1. It shows that the basal spacing (d_{003}) of the [Mg–Al–NO_3] is 0.84 nm corresponding to the sum of the gallery height (0.36 nm) and the thickness of the brucite layer itself (0.48 nm). The basal spacing of [Mg–Al–cordycepin] nanohybrid is 1.62 nm (Fig. 1b), indicating that the basal spacing of LDHs has been expanded because of the intercalation of cordycepin into the LDHs gal-

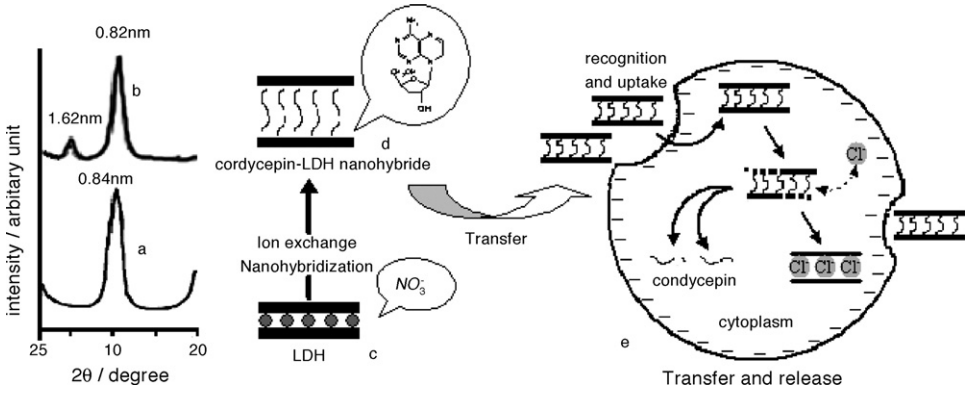

Fig. 1. XRD patterns of the samples and the route of nanohybrid transfer into cell.

leries. The observed gallery height of 1.14 nm (1.62–0.48 nm) in the nanohybrid is well consistent with the thickness of the cordycepin molecule (1.03 nm), so the orientations of cordycepin in the gallery of LDHs may be vertical monolayer (Fig. 1d).

The electrophoretic mobility of [Mg–Al–NO_3] particle was $+2.80 \times 10^{-8}\ m^2\ V^{-1}\ s^{-1}$. After interaction with cordycepin, the electrophoretic mobility of the nanohybrids particle decreased dramatically to $+0.02 \times 10^{-8}\ m^2\ V^{-1}\ s^{-1}$. The [Mg–Al–cordycepin] nanohybrid particle is about 300 nm in length (Fig. 3b), and exhibits a nearly neutral μ-value. Since cells have the ability to engulf neutralized nanoparticles through phagocytosis or endocytosis, it is most likely that the [Mg–Al–cordycepin] nanohybrid particles were taken by cells through endocytosis. The charge neutralization would facilitate the penetration of nanohybrid into cells through endocytosis, since it greatly reduces the electrostatic repulsive interaction between negatively charged cell membranes and negatively charged cordycepin molecule during this process. It is conceivable that once nanohybrids are introduced into cells, such as U937 cancer cells, the LDHs layers would then be dissolved slowly in the cytoplasm, which has a lower pH (pH 4–5). At the same time, interlayer cordycepin molecules would be partially replaced by other anions (such as Cl^-). Consequently the encapsulated cordycepin would be released inside of the U937 cell from the bio-LDHs nanohybrids (Fig. 1e).

Fig. 2 shows the FT-IR spectra of [Mg–Al–NO_3], cordycepin and [Mg–Al–cordycepin] nanohybrid in the 4000–400 cm^{-1} wave number ranges. A broad absorption band at around 3462 cm^{-1} is attributed to OH stretching due to the presence of hydroxyl group of LDHs. A strong absorption band at 1383 cm^{-1} is due to the presence of nitrates. Vibration band corresponding to OH stretching of water is observed at 1640 cm^{-1} (Fig. 2a). The presence of cordycepin in intercalation compound is evidenced by the characteristic C–H stretching bands of methylene groups over the 2850–2930 cm^{-1}. The presence of C–H stretching vibration and C–H sector mutation vibration of glucoside can be implied by the observation of bands at 1300–1500 cm^{-1}. The bands corresponding to aromatic C=C and C=N are also clearly observed at about 1700–1570 cm^{-1}. However, there is an absorption band around 1361 cm^{-1}, which indicates that a few nitrates still in the gallery of LDHs (Fig. 2c). Because our nanohybrid products were formed by ion-exchange

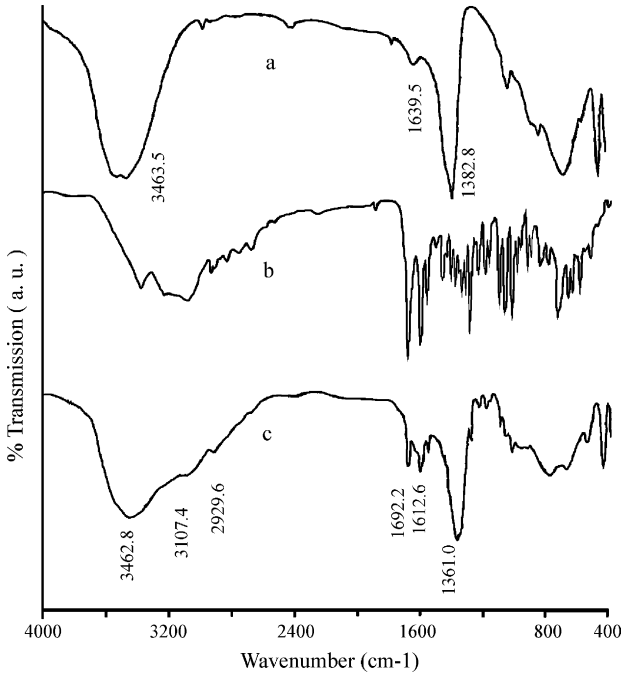

Fig. 2. FT-IR spectra of (a) [Mg–Al–NO_3], (b) standard cordycepin, and (c) [Mg–Al–cordycepin].

reaction at a comparatively low concentration of cordycepin, mixtures of cordycepin and inorganic anions (nitrate) co-occupy the gallery.

TEM micrographs for sample [Mg–Al–NO_3] and [Mg–Al-cordycepin] nanohybrid are shown in Fig. 3. [Mg–Al–NO_3] consist of hexagonal particles with the length about 50–200 nm. Compared to [Mg–Al–NO_3], the samples of [Mg–Al–cordycepin] nanohybrids show some interesting morphological features. Spherical particles can be observed in the suspension when cordycepin is mixed with [Mg–Al–NO_3] for 7 days (Fig. 3b). After the modification of cordycepin, the particles of [Mg–Al–NO_3] turn into spheres due to the effect of solid–liquid interface energy, as the formation of water droplet on the surface of solid. It is generally known that the average diameter of capillaries is around 10 μm and a normal red blood cell is 6–8 μm in diameter. The most common size of small lymphocytes ranges in size from 6 to 10 μm. The diameter of

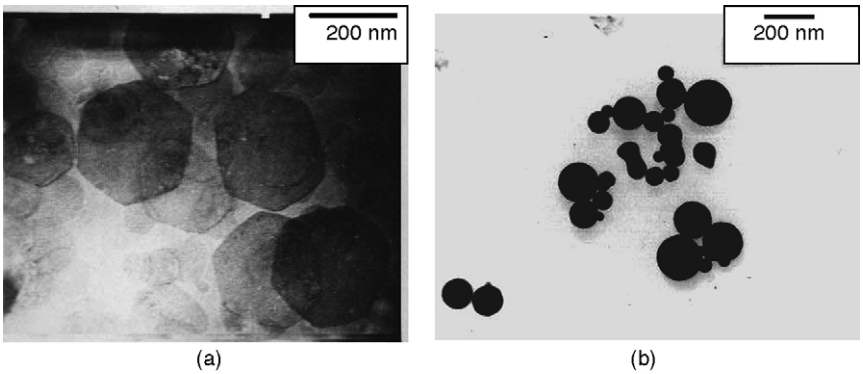

Fig. 3. TEM photographs for LDH and [Mg–Al–cordycepin] nanohybrid samples: (a) [Mg–Al–NO_3]; (b) [Mg–Al–cordycepin] nanohybrid.

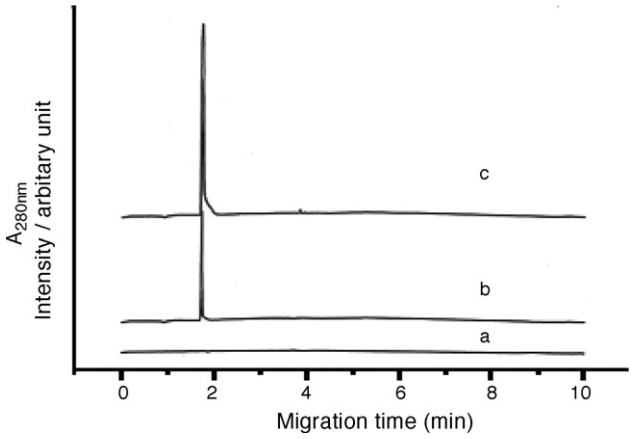

Fig. 4. CZE maps of (a) [Mg–Al–NO_3], (b) [Mg–Al–cordycepin], and (c) [Mg–Al–cordycepin] after adding the standard cordycepin.

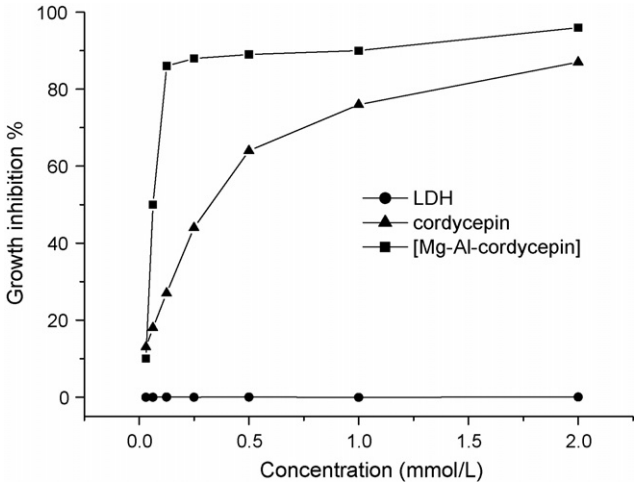

Fig. 5. Effect of the cordycepin, LDH and nanohybrid on the growth inhibition of the U937.

the spherical particles is approximately 50–300 nm. In comparison, we can expect that the particle size of [Mg–Al–cordycepin] nanohybrid is not so large as to block a capillary.

[Mg–Al–cordycepin] nanohybrid was dissolved in deionized water and separated by capillary zone electrophoresis (CZE). The parameters were set as Ling method (Ling et al., 2002) except that the voltage was increased to 25 kV. The pristine LDHs had no UV absorption, it was baseline separated in 10 min shown as Fig. 4a. The intercalated sample exhibited characteristic absorption bands in 258 nm which was attributed to $\pi \rightarrow \pi^*$ or $n \rightarrow \pi^*$ transition in the adenine group (Fig. 4b). The migration time of [Mg–Al–cordycepin] nanohybrid was 1.757 min, which was identified by spiking with the standard of cordycepin (Fig. 4c). This result strongly suggested that cordycepin was intercalated in the LDHs and preserved its integrity.

XRD, FT-IR and CZE results showed that the [Mg–Al–NO_3] could accommodate cordycepin in the interlayer space to get a bigger basal spacing, and the cordycepin retained its integrity and kept a vertical monolayer in the gallery of LDH.

3.2. The effect of the [Mg–Al–cordycepin] nanohybrid on the growth of the U937 cells

The effect of the [Mg–Al–cordycepin] nanohybrid on the growth of the U937 cells was assayed and the results were shown in Fig. 5. The pristine LDH has no effect on the growth of the U937 cells, indicating that it is a biocompatible nanocarrier and is likely not toxic to the cells (Fig. 5). Cordycepin showed strong cell growth inhibition as reported (Sun et al., 2003). However, at the same concentration, [Mg–Al–cordycepin] nanohybrid showed much higher growth inhibition on the U937 cells than cordycepin itself. For instance, at 0.0625×10^{-3} mol/L, the growth inhibition by nanohybrid is 3.185 times higher than that of cordycepin. These results imply that [Mg–Al–cordycepin] nanohybrid was internalized by the cells and eventually inhibit the growth of cancer cells. The much increased activity of nanohybrid is likely due to inhibition of cordycepin decomposition by ADA and therefore more effective to inhibit the growth of U937 cells.

4. Conclusions

In conclusion, [Mg–Al–cordycepin] nanohybrid was prepared and it was shown that cordycepin intercalate into LDHs to get a bio-LDHs nanohybrid. Cell experiments revealed that the nanohybrid had much stronger suppression on the U937 cancer cell growth than cordecypin itself at the same concentration. These results suggested that LDHs through intercalate with pharmaceutically active compounds, could be promising drug delivery nanocarriers.

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