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The construction and characterization of layered double hydroxides as delivery vehicles for podophyllotoxins

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Abstract The aim of this study was to construct PPT–LDH nanohybrids and compare their tumor inhibition effects with that of free PPT. Anticancer drug podophyllotoxin (PPT) was encapsulated in the galleries of Mg–Al layered double hydroxides (LDHs) by a two-step approach. Tyrosine (Tyr) was first incorporated into the interlayer space by co-precipitation with LDH, propopening the layers of Mg–Al/LDH and creating an interlayer environment inviting drug molecules. PPT was subsequently intercalated into the resulting material lamella by an ion exchange process. The intermediate and final products, which can be termed drug-inorganic nanocomposites, have been characterized by powder X-ray diffraction (XRD), UV-VIS spectrophotometer, transmission electron microscopy (TEM) and in cell culture. Our results demonstrate that the interlayer spacing distance of the PPT–LDH nanohybrids (34% w/w of drug/material) is 18.2 Å. LDHs do not harm normal cells (293T) based on toxicity tests. Ex-vivo anticancer experiments reveal that the PPT–LDH nanohybrids have higher tumor suppression effects than intercalated PPT. We conclude that the higher tumor inhibition effects of PPT–LDH hybrids result from the inorganic drug delivery vehicle, LDHs.

Introduction

Podophyllotoxins (PPTs) are naturally-occurring lignans, found in plants, particularly in the genus podophyllum. Podophyllotoxins have been used as medications for over 1,000 years [1]. To date, podophyllotoxin and its derivatives represent a group of clinically useful drugs in the armamentarium of cancer chemotherapeutic agents [2]. However, their usefulness has been hindered by several deficiencies such as poor water solubility, fast metabolic inactivation, drug resistance, myelosuppression and poor bioavailability [3–5]. Therefore, efforts for improving their clinical efficiency and expanding the scope of application are unceasing and prove to be challenging.

Layered double hydroxides (LDHs), known as hydrotalcite-like compounds and anionic clays, consist of cationic brucite-like layers and exchangeable interlayer anions [6]. The most common group of LDHs can be represented by the general formula $[M^{2+}_{1-x}\ M^{3+}_{x}\ (OH)_2][A^{n-}]_{x/n} \cdot mH_2O$, where M^{2+} is a divalent metal cation (Mg^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}), M^{3+} is a trivalent cation (Al^{3+}, Cr^{3+}, Fe^{3+}, V^{3+}, Ga^{3+}), and A^{n-} is an exchangeable anion. The metal cations occupy octahedral positions within the host layers of hydroxide sheets; while A^{n-} anion can compensate for the charge on the layers. In addition, LDHs are the only host lattices with positive charge on the brucite-like layers.

The unique anion exchange capability of LDHs meets the requirement of inorganic matrices for encapsulating a great variety of functional molecules (biomolecules, pharmaceuticals etc.) in aqueous media [7]. Such functional molecules can be incorporated between hydroxide layers by a simple reaction to form LDH nanohybrids [8]. For example, various amino acids [9–11], drugs [12–15], organic dyes [16], biomolecular anions such as DNA, mononucleotides, genes, enzymes [7, 8, 17, 18] have been inserted within interlayer structures in the layers of inorganic matrix of LDHs, resulting in the formation of the so-called layered nanocomposite materials [19]. The charge neutralization through hybridization between LDHs

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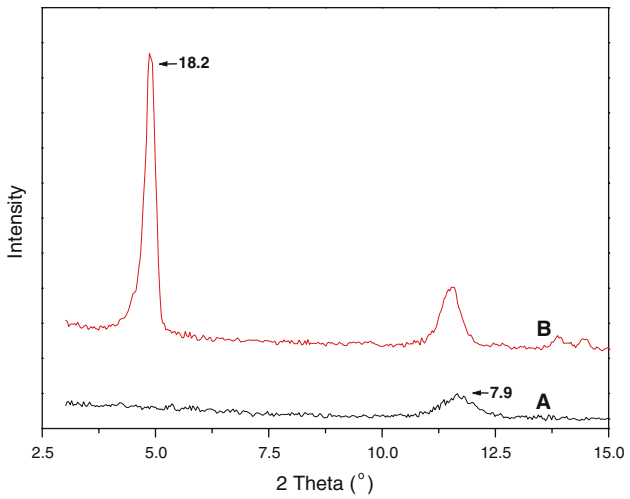

Fig. 1 Powder X-ray diffraction data. (A) Tyr-LDH; (B) PPT–LDH

and functional molecules would facilitate the penetration of hybrids into cells through endocytosis [20, 21], since it greatly reduces the electrostatic repulsive interaction between the negatively charged cell membranes and anionic molecules during the process. Once the hybrids are introduced into cells, the hydroxide layer in the hybrids would be removed slowly in the lysosome where the pH is moderately acidic (pH 4–5); while the intercalated functional molecules would be partially replaced by other anions in the cell electrolyte and released into cytoplasm, enabling these molecules to function in the cells. To explore the drug delivery system of insoluble anticancer agents and to expand the application range of layered double hydroxides nanocomposites, we have constructed, from Mg–Al/LDH and podophyllotoxin (PPT), and characterized the intercalated nanocomposites. Here, we report that the anticancer effects of PPT–LDH hybrids, in cell culture, are higher than that of using naked PPT alone.

Experiment

Materials

Podophyllotoxin was a kind gift from University of Science and Technology of China. Other reagents were purchased from China National Medicines Group Shanghai Chemical Reagents Company and used without further purification. Deionized water was decarbonated by boiling before using in all applications.

Characterization

X-ray diffraction (XRD) patterns of powdered samples were recorded on Rigaku Diffractomer Model Miniflex using CuKα radiation (λ = 1.54060 Å, 40 kV, 40 mA, step of 0.0330°). Transmission Electron Micrographs (TEM) were obtained using JEOL 1230 transmission electron microscope. UV/VIS absorption spectra of all the samples were measured on a CARY 50 spectrophotometer.

Synthesis of the layered inorganic host and its nanohybrids with PPT

The tyrosine-containing Mg–Al/LDH (Tyr-LDH) was prepared, following a conventional procedure [22], by co-precipitation (variable pH method) of tyrosine and Mg–Al/LDH in a nitrogen-filled hood to avoid, or at least to minimize, the contamination from atmospheric CO_2.

The mixed solution of 1 M $Mg(NO_3)_2{\cdot}6H_2O$ and $Al(NO_3)_3{\cdot}9H_2O$ (Mg:Al ratio of 3:1) was added drop-wise to an aqueous solution of 1 M tyrosine with vigorous stirring in a nitrogen-filled hood at room temperature. The pH of final solution was adjusted to 10.0 ± 0.2 by drop-wise adding of 1 M NaOH solution, and stirred vigorously at 80 °C for 5 hrs in nitrogen-filled environment with occasional adjustment of pH. The resulted precipitates were filtered and washed thoroughly with decarbonated water and vacuum-dried at 60 °C.

PPT–LDH nanohybrids were prepared by an ion-exchange procedure. The freshly prepared 0.1 M Tyr-LDH suspension was added to a 0.1 M podophyllotoxin solution (pH previously adjusted to 12 with NaOH). The solid was kept in suspension with stirring at 80 °C for 5 hrs in a nitrogen-filled hood. Then it was filtered, washed and dried as above.

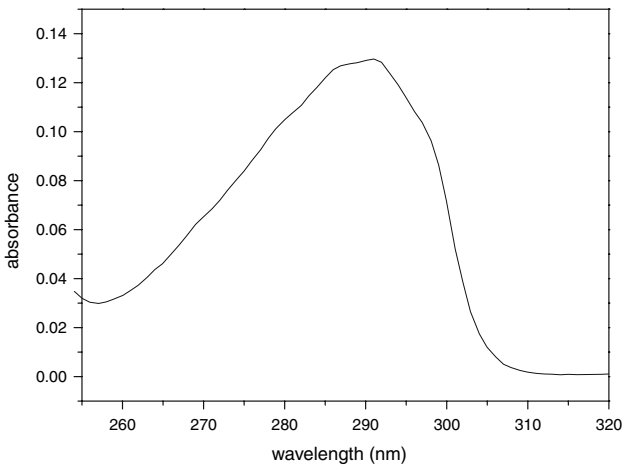

Fig. 2 UV-VIS spectrum of PPT–LDH. Peak at 290 nm indicates successful intercalation of PPT in the layers of LDHs

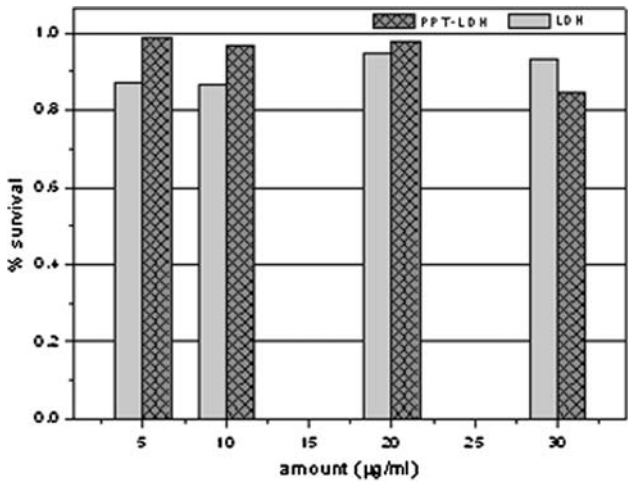

Fig. 3 Cytotoxicity tests of LDH and PPT–LDH with different volume in vitro

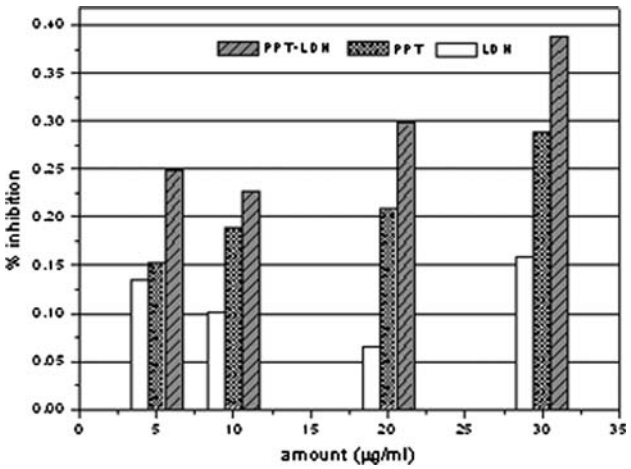

Fig. 4 Anticancer efficiency test for EJ cell treated with LDH, PPT and PPT–LDH

Determination of podophyllotoxin loading

A known volume of the nanohybrids suspension was placed in a flask. 5 mL of 1 M HCl was added, and then the flask was filled with 100% ethanol until the total mixture volume reached 10 mL. The solution was analyzed by UV-VIS spectrophotometer against a series of standards made with the same method. Absorbance value at 290 nm was measured, and the concentrations were calculated by calibration graph (A = 0.01160 C-0.00500, r = 0.99859).

Cell studies

293T and EJ cells were routinely cultured in flasks, incubated at 37 °C in a humidified hood filled with 5% CO_2, containing 10 mL per flask of media of DMEM or RPMI-1640, supplemented with 10% fetal bovine serum, for 293T cells and EJ cells, respectively. At 70–80% confluence, cells were trypsinized and plated at a density of 2.0×10^4 cells per well in a 96 well plate and then 100 μL of medium was added per well. The cells were then

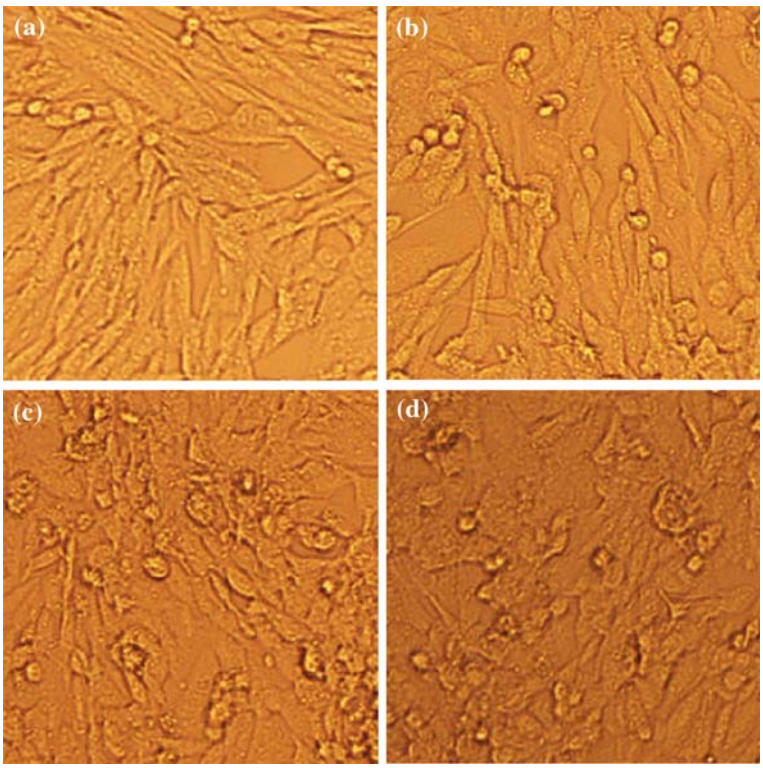

Fig. 5 Pictures of (**a**) blank EJ cell, (**b**) EJ cell with LDH, (**c**) EJ cell with PPT, (**d**) EJ cell with PPT–LDH

incubated at 37 °C in a 5% CO_2 humid environment for 24 h. The number of viable cells was determined by MTT assay with 3-(4, 5-dimethylthiazole-2-yl)-2, 5-phenyltetrazolium bromide.

PPT–LDH nanohybrids, free PPT and LDH were added to the wells in increasing concentrations corresponding to 5, 10, 20, 30 μg/mL. The cells were incubated as above for 48 h. 20 μL of MTT was then added to all the wells, and the plate was further incubated for 4 h to deoxidize MTT. After incubation, the supernatant of the solution was removed and 150 μL of DMSO was added into all wells. The absorbance was measured at 490 nm in an ELX 800 reader.

Evaluation of sustained- or controlled-releasing properties

The sustained- or controlled-releasing capabilities of LDH and PPT–LDH nanohybrids were evaluated by monitoring the change of pH value in respective suspensions with addition of 0.1 M HCl aqueous solution [23]. A typical experiment was performed by using 100 mg of the sample in 10 mL of deionized-distilled water and the flask was kept stirring at 37 °C. Then, HCl aqueous solution was added to the suspensions until pH reached the value of ca. 1.

Results and discussions

Characterization of nanohybrids

Successful synthesis of the Tyr-LDH was verified from powder X-ray diffraction data (Fig. 1). A d-spacing of 7.9 Å corresponding to the tyrosine ion between the layers was obtained for the pristine complex. This space increases to 18.2 Å for PPT–LDH nanohybrids which indicates expansion of the interlayer spacing that may be ascribed to the podophyllotoxin intercalation. The successful intercalation was verified by UV-VIS spectrum (Fig. 2), an absorption peak at 290 nm indicates the intercalation of podophyllotoxin.

The amount of PPT loaded into the nanohybrids was calculated by UV-VIS spectrophotometer. PPT–LDH nanohybrids prepared by using the Tyr-LDH as a precursor have a w/w loading of 34%.

Efficacy studies

Cytotoxicity tests were carried out on the 293T cell. As shown in Fig. 3, LDHs do not harm normal cells. When PPT was intercalated in the layers of LDH, its cytotoxicity effects on the normal cell was similar to that of pristine

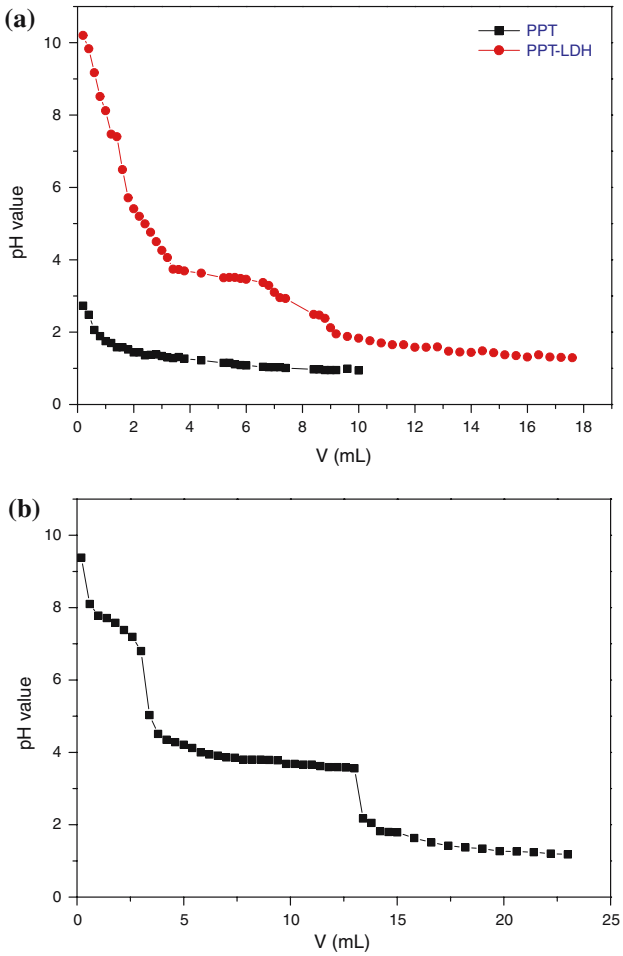

Fig. 6 Titration curves (100 mg/10 mL H_2O) for (**a**) PPT, PPT–LDH and (**b**) LDH (V corresponds to the added volume of a 0.1 mol/L HCl aqueous solution)

LDH. These results prove that LDH is a noncytotoxic, biocompatible and safe delivery vector.

The anticancer efficacy of PPT–LDH nanohybrids compared with free PPT and pristine LDH was evaluated by bioassay using EJ cell. Fig. 4 shows the concentration-dependent inhibition as calculated by EJ cell survival rates. In Fig. 5, the tumor cells with or without LDH have no visible differences in morphology, while the growth of the cells with PPT or PPT–LDH has been significantly inhibited. The overall results demonstrate that both PPT and LDH–PPT nanohybrids gradually suppress the tumor cell growth with increased concentration.

Furthermore, according to anticancer efficiency results, PPT–LDH nanohybrids have higher tumor suppression efficiency compared to free PPT. In Fig. 4, the maximum suppression effects of PPT and PPT–LDH nanohybrids are observed at the concentration of 30 μg/mL respective 29% and 39% inhibition, as calculated by cell survival rates. As shown in Figs. 3 and 4, LDHs do not harm either normal or

tumor cells. The nontoxic effects of LDHs affirm that the high anticancer effects of PPT–LDH nanohybrids surely result from the enhanced permeation of drug delivery vector, LDH. Our results confirm that LDHs are a group of effective drug carriers.

The evaluation of sustained- or controlled-releasing properties

The sustained- or controlled-releasing properties of LDH and LDH–PPT nanohybrids were evaluated by monitoring the changes of pH values caused by the addition of 0.1 M HCl. The corresponding graphs of pH versus volume of added HCl aqueous solution to the suspensions are shown in Fig. 6. The curve for the host LDH shows the major buffering effects keeping the pH constant at about 4. The neutralizing and buffering capabilities of the host LDH are owing to, obviously, the hydroxyl groups in the layers. It is reported that the LDHs have been effectively used as antacid reagents [24, 25]. In relation to the intercalated materials, the pH curve of PPT–LDH nanohybrids is similar to that of the LDH, with a buffering plateau when pH around 3.5, while the pH curve for free PPT does not show buffering effects and the pH drop sharply along with the addition of HCl. TEM data confirm that the layered inorganic host LDH was biodegradable. When exposed to pH 4.8 and pH 7.4 environments, PPT–LDH crystals showed different morphologies. The crystals degraded in 60 min (Fig. 7) at pH 4.8 but could maintain normal structure in the pH 7.4 environment. The results above suggest the potential applications of LDHs in sustained or controlled release systems.

Conclusion

In this work, we have constructed new drug-inorganic nanohybrids by the hybridization of PPT (podophyllotoxin) with layered inorganic host Mg–Al/LDH, using tyrosine (Tyr) as pre-intercalation agent to prop open LDH layers, creating an environment that accommodates PPT. We show that the layered inorganic host LDH is biocompatible and biodegradable and non-toxic to human cells, and the PPT–LDH nanohybrids carrying significant amount of immobilized podophyllotoxin posess good sustained- or controlled-releasing properties. Furthermore, our results demonstrate, in cell culture, the enhanced anticancer efficacy of PPT encapsulated in the galleries of LDHs compared to free PPT. Considering all the results, we conclude that the layered inorganic host LDH can serve as an excellent host for encapsulating different kinds of drugs and play an important role in sustained- or controlled-releasing drug delivery system. Our study illustrates the utility of Mg–Al/LDH for enhancing drug effects with possible applications in cancer chemotherapy, and validation of novel drug delivery system.

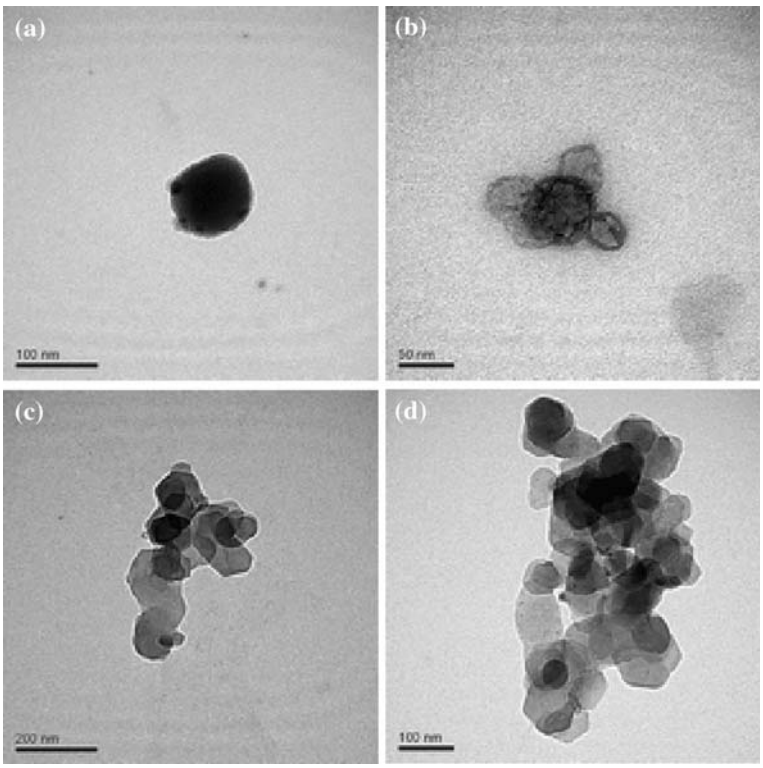

Fig. 7 TEM pictures of PPT–LDH exposed to pH 4.8 and pH 7.4 environments with different time duration. (**a**) pH 4.8, 10 min (**b**) pH 4.8, 60 min (**c**) pH 7.4, 10 min (**d**) pH 7.4, 60 min

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