Applied Clay Science 121-122 (2016) 103–110

Contents lists available at ScienceDirect

Applied Clay Science

journal homepage: www.elsevier.com/locate/clay

Research paper

Synthesis of methotrexatum intercalated zinc–aluminum-layered double hydroxides and the corresponding cell studies

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ARTICLE INFO

Article history:
Received 6 October 2015
Received in revised form 26 November 2015
Accepted 30 November 2015
Available online 11 December 2015

Keywords:
Layered double hydroxide
Morphology control
Bioassay explore

ABSTRACT

To study the influence of morphology on release property, drug efficacy and other properties, a series of methotrexate intercalated zinc–aluminum-layered double hydroxides (MTX/ZnAl-LDHs) hybrids were synthesized through the traditional coprecipitation method, by the way of changing pH value and using different solvents. X-ray diffraction (XRD) patterns and fourier transform infrared spectroscopy (FTIR) investigations manifested the successful intercalation of MTX anions for all hybrids. TEM photographs indicated that low pH value was in favor of improving the morphologies, and addition of polyethylene glycol (PEG) in solvent would lead to the formation of regular particles. Lastly, in-vitro release and the bioassay tests showed that regular particles had much longer release duration and were more efficient in the suppression of the tumor cells.

1. Introduction

Recently nanocarrier mediated drug delivery system, particularly to treat cancers, has been a highly attractive research area. Especially, inorganic nanoparticles are alternatively obtaining more attention nowadays because of their high drug loading, pronounced stability and biocompatibility. And then various inorganic nanocarriers including magnetite, calcium phosphate, carbon, gold, silica oxide and layered double hydroxide (LDHs) have been evaluated for delivering cytotoxic drugs (Peter et al., 2005; Cheng et al., 2008; Liu et al., 2008a; Zhang et al., 2009b, 2013a; Wu et al., 2013). In such efforts to develop new drug delivery systems, LDHs are less toxic than other conventional drug carriers, thus making them a promising alternative for drug delivery. The general formula of LDHs is represented as $[M^{2+}_{1-x}M^{3+}_{x}(OH)_2]^{x+}[A^{n-}]_{x/n}\cdot mH_2O$, where M^{3+} and M^{2+} are tri- and divalent metal ions, and A^{n-} is the exchangeable anion (Das et al., 2004; Gao et al., 2009). From the formula, the positive charge of LDHs is balanced by a variety of anions (A^{n-}) locating in the interlayers. As a result, anionic drugs, which can replace the interlayer anions laying between the two positive metal hydroxide sheets of LDHs material, enable a controllable ion-exchange mechanism. Further, the abilities of controlled-release and pH dependency are additional advantages of using LDHs as drug delivery system (Kura et al., 2013). More importantly, the positively charged outer layer of the delivery system, which can attract a negatively charged cell membrane, enables easy penetration of LDHs into cells.

Methotrexate (MTX), one of the antifolate drugs, is well known for the effective treatment of certain human cancers such as osteosarcoma (bone cancer), leukemia, and other malignant tumors (the chemical structure is shown in Scheme 1). Unfortunately, the very short plasma half-life and high efflux rate of MTX compared to the influx rate have required a high administration dose, which restricts its clinical applications (Choy et al., 2004). Therefore, considerable attention had been paid to improve the efficacy of MTX. The matrices, frequently used for the sustained release of MTX, mainly aims to decrease the taken frequency, and then to keep a steady level of the drug in the bloodstream (Carriazo et al., 2010). This fact is of paramount importance in some cases since it would help to avoid unnecessary doses that can produce collateral effects. Fortunately, given that the intercalation of drugs into LDHs not only can increase their solubility or improve their therapeutic profile, but also can help to decrease its pristine harmful capacity, LDHs have been usually used for this purpose (Oh et al., 2006; Choi et al., 2008, 2010; Chakraborty et al., 2011; Rives et al., 2013, 2014).

Usually, magnesium–aluminum-LDHs (MgAl-LDHs for short) have been widely applied in drug delivery systems due to their fabulous features, such as biocompatibility, easy excretion from the body, the tendency to accommodate different kinds of biomolecules, ease of preparation and so on (Qi et al., 2012, 2013). The reports have shown that MTX/MgAl-LDHs hybrids exhibited excellent controlled-release and good anticancer effect (Qi et al., 2012, 2013; Liu et al., 2014; Tian et al., 2014; Zhang et al., 2014). While compared with the extensive study of MgAl-LDHs, the research on ZnAl-LDHs as drug carrier has been relatively less because of the difficulty on morphology and size control (Jin et al., 2010; Pang et al., 2013;

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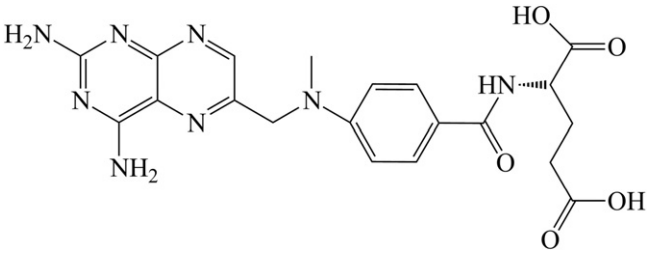
http://dx.doi.org/10.1016/j.clay.2015.11.025
0169-1317/



Scheme 1. The chemical structure of MTX ($C_{20}H_{22}N_8O_5$).

Table 1
Characteristic data of MTX/ZnAl-LDHs hybrids at different synthesis conditions.

Sample	pH value	Composition of solvents	Basal spacing (nm)	Interlayer spacing (nm)	A_{In} (%)	a(nm)
a	8.0	H_2O	2.05	1.57	49.50 ± 0.14	0.15
b	8.5	H_2O	2.17	1.69	55.12 ± 0.25	0.15
c	9.0	H_2O	2.11	1.63	45.63 ± 0.86	0.15
d	9.5	H_2O	2.31	1.83	42.33 ± 0.45	0.15
e	8.0	PEG/H_2O	2.24	1.76	48.35 ± 0.78	0.15
f	8.0	EtOH/H_2O	2.10	1.62	51.98 ± 0.23	0.15

Zeng et al., 2014; Barahuie et al., 2015; Eili et al., 2015). However, Zn is a very important element involved in the life activities, like protecting DNA from damage, beneficial for the treatment of cancers, or controlling metabolic activities of 300 enzymes. It is also critical to the tissue growth and the immune system function. Zinc deficiency may result in birth defects, growth retardation, cancers and depression (Scheplyagina, 2005; Maret and Sandstead, 2006). Hence, the choice of ZnAl-LDHs as pristine for MTX carriage is in urgent need. Here, we aimed at improving the morphologies of MTX/ZnAl-LDHs, and exploring their suppression effect on the tumor cells emphatically, and then helping us develop a new dosage form in the future. This paper is a follow-up of our series studies (Tian et al., 2014; Zhang et al., 2014; Liu et al., 2015a, 2015b; Tian et al., 2015). In our previous studies, different preparation routes to obtain MTX/LDH hybrids were emphatically explored, concluding that coprecipitation had the best anticancer effects. In this work, different MTX/LDH samples were synthesized and detailed procedure was refined as well.

2. Experimental sections

2.1. Experimental material

Zinc nitrate ($Zn(NO_3)_2 \cdot 6H_2O$), aluminum nitrate ($Al(NO_3)_3 \cdot 9H_2O$), ammonia ($NH_3 \cdot H_2O$), methotrexate (MTX) are all of analytical purity. MTX and human lung adenocarcinoma cells (A549) were purchased from Zhejiang Province Huzhou Prospect Pharmaceutical Co. and Chinese Academy of Sciences (Shanghai, CN), respectively.

2.2. Synthesis of pristine LDHs and MTX/LDHs nanohybrids

As the reference materials, pristine ZnAl–NO_3-LDHs were prepared by the typical co-precipitation method (Zeng et al., 2014). The MTX/ZnAl-LDHs hybrids were first prepared by changing pH values. The typical process was as follows: the mixed salt solution, containing 0.032 mol/L of Zn^{2+} and 0.016 mol/L of Al^{3+}, was first prepared. MTX was dissolved into 15 mL of 10% $NH_3 \cdot H_2O$ to get a 0.05 mol/L solution. Then the mixed salt solution was dropped into MTX solution at a constant rate of 3 mL/min, and the final solution was adjusted to pH 8.0, 8.5, 9.0 and 9.5 by adding a certain amount of 10% $NH_3 \cdot H_2O$. Followed by vigorously stirring for 1 h at 60 °C, the products were washed with deionized water and ethanol for several times, while N_2 gas was bubbled into the solutions throughout the coprecipitation operation. At last, they transferred into a Teflon-lined stainless steel autoclave hydrothermally treated at 100 °C for 24 h and the samples were named as *a*, *b*, *c* and *d* with the increased pH values. Further, in order to improve the morphologies, mixed solvents, such as polyethylene glycol (PEG)-400/water and ethanol/water (with a volume ratio of 1:3), were also used at the pH of 8.0 and the final samples were named as *e* and *f*, respectively. The detailed experimental conditions were listed in Table 1.

2.3. Drug-loading capacity

To determine the amount of MTX loaded into the LDHs, 0.01 g of MTX/ZnAl-LDHs were dissolved by HCl solution (pH = 1.2) completely and diluted to 500 mL in volumetric flask. Under this circumstance, it can be assumed that 100% of MTX is released from the hybrid. Then the concentration of MTX was determined by monitoring the absorbance at λ_{max} = 306 nm with UV–vis spectroscopy. It must be mentioned that the concentration was calculated by regression analysis according to the standard curve obtained from a series of standard solution of MTX in HCl solution. At last, the intercalated amount of MTX into the nanohybrids was calculated. The data were collected in triplicate and presented in Table 1.

2.4. In vitro drug release

To measure the amount of MTX released from MTX/ZnAl-LDHs hybrids, the in -vitro release was performed as follows: 0.02 g of MTX/ZnAl-LDHs were added into 500 mL of phosphate buffer solution (pH = 7.4) in a closed glass bottle at a constant temperature of 37 °C. At selected time after addition of the hybrids, 4 mL of solution was withdrawn and centrifuged. Part of the supernatant was used for the measurement, and then the concentration of MTX was measured by UV–vis spectroscopy at λ_{max} = 306 nm. At last, the release profiles were plotted as the relative release percentages of MTX against time. Dissolution medium was maintained at constant volume by replacing the samples with a fresh dissolution medium. The data were collected in triplicate and presented in Fig. 4A.

2.5. In vitro bioassay

Human lung adenocarcinoma cells (A549) purchased from Chinese Academy of Sciences (Shanghai, CN) 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 Dubecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and 100 U/mL penicillin and 100 mg/mL streptomycin. When the cells were grown up to 80–90% of cellular confluence, the fault culture cells were differentiated with trypsin-EDTA and then washed twice with PBS (pH = 7.4) which was prior prepared. Then the cells were diluted with a volume of DMEM containing 10% FBS. For cell proliferation and viability study, cells were seeded onto 96-well plates. Then the cells were incubated overnight at 37 °C under a 5% CO_2 atmosphere. After that the medium in the wells was replaced with fresh medium containing MTX/LDHs nanohybrids, and further incubated for 24 h. The effect of MTX/LDHs nanohybrids on cell proliferation was determined using MTT (a yellow tetrazole) assay (Rives et al., 2014). Briefly, after the supernatant was removed, 10 μL of MTT (5 mg mL^{-1} in PBS, pH = 7.4) stock solution and 90 μL DMEM with no FBS were added into each well and further incubated for 4 h at 37 °C. During the incubation, MTT was reduced to insoluble purple formazan by mitochondrial reductase in living cells. Afterwards, the product was dissolved with 100 μL of dimethylsulfoxide (DMSO). Absorbance was recorded at 570 nm on a microplate reader (Thermo MK3, USA). The MTT assays were also performed with the cells being cultured with different MTX/LDHs concentrations. The data were collected in triplicate and presented in Fig. 5B.