Synthesis and in vitro evaluation of pH-sensitive magnetic nanocomposites as methotrexate delivery system for targeted cancer therapy

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Abstract

This study focuses on the synthesis and in vitro evaluation of magnetic nanocomposites ($\text{Fe}_3\text{O}_4@\text{LDH}$) as methotrexate (MTX) delivery system for targeted anticancer therapy. In which, the Fe_3O_4 nanoparticles acted as magnetically responsive carriers; the coating layer of layered double hydroxide (LDH) was used as a storehouse for MTX. The prepared $\text{Fe}_3\text{O}_4@\text{LDH}$ nanocomposites exhibited a suitable size, good stability and magnetic responsibility (M_s 36.66 emu/g); MTX successfully intercalated into the LDH of $\text{Fe}_3\text{O}_4@\text{LDH}$ at an entrapment rate (%) of 91.78% (the drug loading was 18.36%) by host-guest exchange process. Moreover, the release studies in vitro showed that the drug delivery system ($\text{Fe}_3\text{O}_4@\text{LDH}$ -MTX) had excellent pH-sensitivity, 84.94% of MTX was released within 48 h at pH 3.5 via the co-effect of dissolution of LDH layer and ion-exchange. WST-1 assays in cancer cells (MCF-7 and HepG2) and normal cells (HUVEC) demonstrated that $\text{Fe}_3\text{O}_4@\text{LDH}$ -MTX exhibited high anticancer activity while low toxicity to normal cells, and also the $\text{Fe}_3\text{O}_4@\text{LDH}$ composites were practically non-toxic. Thus, our results revealed that $\text{Fe}_3\text{O}_4@\text{LDH}$ -MTX would be a competitive candidate for targeted delivery and sustained and

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controlled release of MTX.

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1. Introduction

Methotrexate (MTX), a stoichiometric inhibitor of dihydrofolate reductase, is well known for effective treatment of certain human cancers such as, head and neck, breast, skin, lung and other malignant tumors [1-3]. Unfortunately, the very short plasma half-life and high efflux rate of MTX compared to the influx rate have required a high administration dose, and often, MTX administered to an organism does not reach the tumor environment, but is distributed throughout the body, resulting in a variety of toxic effects on normal bone marrow, mucosal, and lymphoid cells, which places many restrictions on its clinical applications [4-6]. Thus, there is a clear need for developing effective delivery systems for MTX to maximize the therapeutic activity presents and that allow for efficient and cell-specific drug delivery with low carrier-induced toxicity.

Many types of novel MTX delivery systems have been reported, such as nanoparticles [7-9], microspheres [10], liposomes [11], polymeric micelles [12] and miscellaneous multiparticulate systems [13]. Among these, nanoparticles, especially the magnetic iron oxide (Fe₃O₄) nanoparticles seem to be a promising tool for site specific delivery [14,15], based on the fact that they will release the anticancer drug on tumor cells owing to the enhanced permeability and retention (EPR) effect [16,17] and passive drug targeting. Furthermore, recently layered double hydroxide (LDH) nanoparticles have been proven effective for site-specific delivery of anticancer drugs to tumor and reduce its side effects, due to their inherent properties such as high biocompatibility [18], low cytotoxicity [19], the ability to accommodate various biologically important molecules including genes and drugs [20-22], and the partial dissolution of LDH layers in endosome that not only stops the passive control release of drugs,

but also buffers the excess protons, which helps the drugs to escape from endosome, improves the viability of drugs in cytoplasm, and enhances the delivery efficacy [23]. MgAl-LDH, specifically, is reported to be more efficient than other LDHs because of its weak alkalinity and the pH-sensitive ability of slow degradation in an acidic environment like endosomes (pH 5.0-6.0) or lysosomes (pH 3.5-4.5) [24,25], and the yielded ions such as Mg^{2+} , Al^{3+} , or NO_3^- may leave the cell through ion-tunnels [26,27]. Therefore, MgAl-LDH maintains the right balance between chemical stability and biodegradability, which precisely meets the requirements for targeted pH-sensitive drug release.

Upon these senses, a Fe_3O_4 @LDH-drug system composed of Fe_3O_4 , MgAl-LDH and MTX is designed in this study. Biocompatible Fe_3O_4 @LDH nanoparticles were fabricated via self-assembly of MgAl-LDH and Fe_3O_4 through electrostatic interaction in hydrothermal process and the stable Fe_3O_4 @LDH-MTX system was prepared by host-guest exchange (shown in Fig. 1). MTX-loaded Fe_3O_4 @LDH nanoparticles were evaluated for physicochemical properties, release kinetics and in vitro cytotoxicity. This study would be helpful to the future design of more effective and safer drug-carrier systems for drug delivery.

2. Materials and methods

2.1 Materials

Ferric chloride ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 98%), magnesium nitrate hexahydrate ($\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$, 98%), aluminum nitrate nonahydrate ($\text{Al}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$, $\geq 98\%$), dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), acridine orange base (AO) and ethidium bromide (EB) were purchased from Sigma-Aldrich. Sodium acetate (NaAc), ethylene glycol (EG) and sodium hydroxide (NaOH) were supplied by Aladdin. 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-1) was purchased from Beyotime Biotechnology Co., Ltd., Shanghai, China. The human umbilical vein

endothelial cells (HUVEC), the human liver hepatocellular carcinoma cells (HepG2) and the model breast cancer cells (MCF-7) were supplied by Chinese Academy of Sciences Cell Bank, Shanghai, China. All chemicals in this experiment were of analytical grade and used as received without further purification.

2.2 Synthesis of layered double hydroxide (MgAl-LDH)

MgAl-LDH was synthesized by co-precipitation method according to the previously reports [4]. Briefly, 11.54 g $\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ and 5.63 g $\text{Al}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ were dissolved in about 50 mL of deionized water, then the resulted solution was transferred to a 150 mL 3-necked reaction flask and titrated with 2.9 M NaOH solution at room temperature. The final pH of the solution was maintained at 10.5. The resulting white precipitate was collected by centrifugation, and washed with deionized water thoroughly. We will use LDH to represent MgAl-LDH in the following text.

2.3 Preparation of Fe_3O_4 @LDH

The biocompatible Fe_3O_4 @LDH nanoparticles were synthesized using a hydrothermal method. First, 1 g LDH was disperse in 40 mL of ethylene glycol (EG) by ultrasound, then 0.5 g $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and 3.0 g NaAc were added and the mixture was sonicated and stirred for 30 min to ensure all materials dissolve completely, finally the uniform mixed solution was transferred to a teflonlined stainless-steel autoclave. The autoclave was heated to 200 °C and maintained at this temperature for 10 h, then cooled to room temperature. The black products were collected by magnetic decantation and washed several times with water.

2.4 Drug loading into Fe_3O_4 @LDH (Fe_3O_4 @LDH-MTX)

25 mg dry Fe_3O_4 @LDH nanoparticles were dispersed in 20 ml of deionized water. 10 mg MTX was added and allowed to react with the nanoparticles for 3 days in the dark at 60 °C, the MTX is stable

at this temperature [28,29]. The products were collected by magnetic decantation and washed twice with deionized water. The final brown Fe₃O₄@LDH-MTX nanoparticles were freeze-dried and stored in the dark at 5°C. The drug loading (%) and entrapment rate (%) were calculated by the following equations [30].

$$\text{Drug loading} = \frac{\text{the amount of MTX in nanoparticles}}{\text{the amount of MTX loaded nanoparticles}} \times 100\%$$

$$\text{Entrapment rate} = \frac{\text{the amount of MTX in nanoparticles}}{\text{the amount of MTX feeding}} \times 100\%$$

2.5 Characterizations

The morphology of the samples was observed by transmission electron microscopy (TEM, Model Tecnai 12, Philips Co. Ltd., Holland), high-resolution electron microscopy (HRTEM, Tecnai G2 F30 S-TWIN, FEI Co. Ltd., USA) and field-emission scanning electron microscopy (FE-SEM, Hitachi S-4800, Hitachi, Japan). The mean particle size, size distribution and polydispersity index were determined by dynamic light scattering (DLS, BI-90Plus, Brookhaven Co. Ltd., USA). Surface charge was evaluated by the zeta potential analyzer (BI-90Plus, Brookhaven Co. Ltd., USA). The crystalline phases were carried out by powder X-ray diffraction (XRD) performed on a Bruker D8 (Bruker Co. Ltd., Germany) advance system using Cu K α radiation ($\lambda=1.5418 \text{ \AA}$). The thermal stability of the dry samples were measured by thermogravimetric analysis (TG-DTA, Model TA2100, TA Instruments, USA) under N₂ at a heating rate of 10°C/min over the range of 50~700°C. Fourier-transform infrared spectroscopy (FT-IR) spectra were recorded on Vector 22 spectrometer (Bruker Co. Ltd., Germany) using the KBr pellet technique. The UV-vis absorbance spectra were recorded by UV-vis spectrophotometry (Agilent Co. Ltd., USA). Magnetic properties of the particles were detected at room temperature using vibrating sample magnetometer (VSM, Model 7410, Lake Shore Co. Ltd., USA).

2.6 In vitro release of MTX

The in vitro release studies were similar to our previous reported [31]. Briefly, 10 mg Fe₃O₄@LDH-MTX was dispersed in 3 mL PBS with different pH of 3.5, 5.0 and 7.4, and then transferred to a dialysis bag (3500 MWCO, 25 mm DIA) which was immersed in 6 mL of the same medium and kept in the oscillator at 37°C. At selected time intervals, PBS (3 mL) outside the dialysis bag was removed for UV-vis analysis at 301 nm and replaced by the fresh with the same volume. To determine the amount of released MTX, calibration curves were run with MTX in corresponding PBS at pH 3.5, 5.0 and 7.4, respectively. The release experiments were conducted in triplicate. The results presented are the average data with standard deviations.

2.7 WST-1 assays

HUVEC, MCF-7 and HepG2 cells were seeded in 96-well plates (5×10^3 cells/well) for 24 h at 37°C in a humidified 5% CO₂-containing atmosphere. Then, 20 µL of free MTX, Fe₃O₄@LDH, or Fe₃O₄@LDH-MTX at various concentrations was added and the cells were incubated for 24 h. After that, 20 µL WST-1 solution (5 mg/mL) was added. The cells were incubated for 2 h, and the absorbance at a wavelength of 450 nm of each well was measured using a multifunctional microplate reader (TECAN Infinite 200 Pro, Austria). The relative cell viability (%) was calculated using equation as follows:

$$\text{Relative cell viability} = \frac{\text{absorbance of treated cells}}{\text{absorbance of control cells}} \times 100\%$$

Cytotoxicity was expressed as percentage of control cells. The inhibition concentration 50% (IC₅₀) is defined as the dose of compound that reaches inhibited cell growth of 50%, was interpolated in the growth curves thus obtained. The fluorescence images of HUVEC, MCF-7 and HepG2 cells were observed by RFP filter under microscope (Olympus, IX81, Japan).

2.8 Data analysis and statistics

The results were presented as mean $\pm$ standard deviation (SD). Statistical analysis of the data was performed via one-way analysis of variance (ANOVA) using Data and Statistics software package (DAS, Shanghai, China). Statistical difference between data was defined as $P < 0.05$.

3. Results and discussion

3.1 Characterization of sample

The size and morphology of the products in different stages were characterized by TEM and FE-SEM. Fig. 2a shows that the naked Fe_3O_4 nanoparticles are spherical and approximately monodispersed. After coating the LDH layer (Fig. 2b, S1), the $\text{Fe}_3\text{O}_4@\text{LDH}$ spheres had a mean diameter of ~ 230 nm with low polydispersity index (0.086) and positive zeta potential (21.5 ± 0.28 mV). The corresponding FE-SEM images (Fig. 2c, d) conform again that the Fe_3O_4 nanoparticles are spherical and coated well with LDH. Moreover, the peaks of the O, Fe, Mg and Al elements are presented in the energy dispersive spectroscopy (EDS) spectrum (Fig. 2e), conforming the existence of Fe_3O_4 nanoparticles and LDH. What's more, the $\text{Fe}_3\text{O}_4@\text{LDH}$ nanocomposites have good stability in dispersion (Fig. S2), after being dispersed in PBS (pH 7.4) for 10 days, there is almost no discernible changes to the average particle diameter.

The detail of the core-shell structure was further characterized by HRTEM. As shown in Fig. 3a, a clear interface is present between the Fe_3O_4 core and the LDH shell, indicating that LDH has coated onto the surface of Fe_3O_4 core. The lattice planes of the core region are measured to be 0.291 and 0.253 nm, corresponding to the spacing of (220) and (311) planes of Fe_3O_4 , respectively. Furthermore, the lattice planes at the edge are 0.353 and 0.289 nm, corresponding to the (003) and (006) planes of LDH (Fig. 3b). Fig. 3c shows the elemental mapping of Fe, O, Al and Mg corresponding to the $\text{Fe}_3\text{O}_4@\text{LDH}$ sphere. The images labeled Fe, O, Al and Mg are uniform along the shape of the sphere, revealing that

the Fe_3O_4 particles are homogeneously coated by LDH. The above results reveal that $\text{Fe}_3\text{O}_4@\text{LDH}$ composites are successfully fabricated by the present method.

The composition and crystallinity of the products were investigated by XRD. In Fig. 4a, the diffraction peaks with 2θ values of 30.06° , 35.44° , 44.54° , 57.37° and 62.88° are correspond to the (220), (311), (400), (511) and (440) reflection planes of magnetite Fe_3O_4 respectively (JCPDS no.19-629). Meanwhile, the series of characteristic peaks with 2θ values of 11.43° , 22.88° , 34.75° , 39.03° , 45.64° , 60.54° and 62.06° correspond to the (003), (006), (009)+(012), (015), (018), (110), and (113) planes typically found in LDH structures [32]. It can also be observed that the XRD spectrum of the core-shell $\text{Fe}_3\text{O}_4@\text{LDH}$ microspheres contains the peaks of both LDH and Fe_3O_4 , substantiating the fact that both phases are present in its structure. In addition, in Fig. 4b, LDH shows the typical XRD pattern of $\text{Fe}_3\text{O}_4@\text{LDH}$ with the basal spacing of $8.4 \text{ \AA}$. After intercalation of MTX, the interlayer space leads to a significant increase ($19.9 \text{ \AA}$). Since the sheet thickness of LDH is $\sim 4.8 \text{ \AA}$, the gallery heights can be estimated to be $15.1 \text{ \AA}$ for LDH-MTX of $\text{Fe}_3\text{O}_4@\text{LDH-MTX}$. This value is smaller than the longitudinal molecular length of MTX ($\sim 21.2 \text{ \AA}$), but much longer than its lateral length. It is suggested from the gallery heights that the intercalated molecules are arranged in tilted longitudinal monolayer with the tilting angle of $\sim 46^\circ$ for MTX to the hydroxide layer, as illustrated in Fig. 4b (the insect). Thus, the MTX is intercalated by host-guest exchange reaction with nitrate ions of LDH and preserved safely in the hydroxide interlayer space by electrostatic interaction.

TG-DTA curves of $\text{Fe}_3\text{O}_4@\text{LDH}$ and $\text{Fe}_3\text{O}_4@\text{LDH-MTX}$ are shown in Fig. 5. The thermal decomposition of $\text{Fe}_3\text{O}_4@\text{LDH}$ is represented by two stages (Fig. 5a). The first stage (up to about 200°C) was the removal of water physisorbed on the external surface of $\text{Fe}_3\text{O}_4@\text{LDH}$ as well as water intercalated in the interlayer galleries. The second stage was the destruction of the layered structure,

involving the dehydroxylation of the OH^- intralayer anions and loss of volatile species (NO_x) which were formed by thermal decomposition of the interlayer NO_3^- anions. For the $\text{Fe}_3\text{O}_4@\text{LDH-MTX}$ (Fig. 5b), the first step was also attributed to the removal of surface adsorbed water molecules and the loss of interlamellar water. The second exothermic step arose from the decomposition of intercalated MTX around 400°C . These temperature was somewhat higher than the decomposition temperatures of MTX ($185\text{-}204^\circ\text{C}$). The enhanced thermal stability supports its stable electrostatic interaction with LDH.

Fig. 6 shows the FT-IR spectra of the materials used in this study. For Fe_3O_4 (Fig. 6a), the peak at about 580 cm^{-1} is caused by the Fe-O symmetric stretch, while the peaks at 3430 cm^{-1} is the stretching vibration of the water molecules adsorbed on the surface of the Fe_3O_4 particles [33]. The LDH (Fig. 6b) displays the characteristic O-H bending at about 3450 cm^{-1} , of both the hydroxyl groups in the brucite layers as well as the hydroxyl groups in the interlayer water molecules. The stretching of the interlayer nitrate (NO_3^-) is responsible for the peak at about 1383 cm^{-1} , while the peak at 603 cm^{-1} is caused by metal-oxygen-metal stretching [34]. The peaks present in Fe_3O_4 and LDH can also be found in the spectra for $\text{Fe}_3\text{O}_4@\text{LDH}$ and $\text{Fe}_3\text{O}_4@\text{LDH-MTX}$ (Fig. 6c, e), suggesting that both materials are present. In Fig. 6d, the absorption band at 1645 cm^{-1} corresponds to C=O stretching, whereas the bands at 1605 , 1500 and 1403 cm^{-1} correspond to the skeleton vibration of benzene ring of MTX [28]. The peaks present in MTX can also be found in the spectrum for $\text{Fe}_3\text{O}_4@\text{LDH-MTX}$, suggesting that the intercalation reactions are successfully completed without any denaturation of MTX molecules.

To further verify the successful synthesis of $\text{Fe}_3\text{O}_4@\text{LDH-MTX}$, UV-vis absorption spectra were also collected (Fig. S3). In Fig. S3a, the MTX exhibits characteristic absorption peaks at 220 , 250 and 303 nm because of the $\pi\rightarrow\pi^*$ (enone), $\pi\rightarrow\pi^*$ (carboxylate) and $n\rightarrow\pi^*$ (enone) transitions, respectively [35]. For the $\text{Fe}_3\text{O}_4@\text{LDH-MTX}$ (Fig. S3e), the peak at 220 nm is not identified due to the scattering

effect of LDH particles. The distinct peaks show the small red shift by about 2-5 nm, suggesting that positively charged layers of LDH withdraw the electrons of carbonyl groups of MTX. These results confirm that MTX molecules are stabilized by electrostatic interaction with the positively charged LDH. In addition, the stability of Fe₃O₄@LDH-MTX has also been investigated and the results are shown in Fig. S4. As we can see that the average particle size do not change significantly even after being dispersed in PBS (pH 7.4) for 10 days, revealing that the Fe₃O₄@LDH-MTX has excellent stability, which is important for the application in biological systems.

In Fig. 7, we can see that the magnetization of Fe₃O₄ (72.73 emu/g) is reducing successively along with the LDH coating (36.66 emu/g) and MTX intercalation reaction (29.89 emu/g). These reveal that the LDH has coated onto Fe₃O₄ and MTX molecules have effectively intercalated into the interlayer galleries. The inset (I) of Fig. 7 shows an enlarged section of the magnetization plot near the zero magnetic fields. Saturation remanence (M_{rs}) and coercivity (H_c) can be determined from the intersection of the hysteresis loop with two axes at 1.45 emu/g and 3.64 Oe, respectively. These two values indicate that a rather low residual magnetization is present when the external magnetic field is removed and that low-intensity magnetic field is required to reduce the magnetization to zero [36]. Moreover, the inset (II) of Fig. 7 shows a typical magnetic separation of Fe₃O₄@LDH-MTX dispersed in PBS solution (pH 7.4), when a magnet was placed outside the tube, the Fe₃O₄@LDH-MTX was concentrated at the magnet site, whereas the bulk solution became colorless. Therefore, it is expected that Fe₃O₄@LDH-MTX can be easily controlled by an external magnetic field, and then the drug can be easily delivered to the target area.

3.2 *In vitro* drug release

Here, the *in vitro* release profiles of MTX from Fe₃O₄@LDH-MTX at different pH have been

investigated (the drug loading (%) and entrapment rate (%) were 18.36% and 91.78%, respectively). As can be seen in Fig. 8, the release rate of MTX from Fe₃O₄@LDH-MTX was found to increase with decreasing pH, such that 27.59, 62.01 and 84.94% of MTX were released within 48 h when stored in PBS of pH 7.4, 5.0 and 3.5, respectively. In addition, a rapid release of MTX occurs at the initial stage, which followed a relatively slower release then. Thus, the pH-sensitive drug release could occur once the Fe₃O₄@LDH-MTX were internalized into the cancer cells and the two-step and prolonged release behavior could benefit the therapeutic treatment, as the initial fast release rapidly provides a therapeutic dose, and the subsequent sustained release allows maintenance of this dose over a long period of time [29]. Consequently, the pH-triggered MTX release will decrease the drug loss during blood circulation and improve intracellular drug release at cancer cells.

3.3 Release kinetics

To gain more insights into the mechanism of drug release, according to the literature, three types of kinetics models were exploited [37,38]. The Bhaskas equation (1), the first-order equation (2) and the Higuchi equation (3) with modification were chosen to study the release dynamics of Fe₃O₄@LDH-MTX:

$$X = 1 - e^{-k(t-\alpha)^{0.65}} \quad (1)$$

$$X = 1 - e^{-k(t-\alpha)} \quad (2)$$

$$X = k(t-\alpha)^{1/2} \quad (3)$$

where X, t, k, and α are the release percentage, release time, kinetic constant, and modified parameter, respectively.

The fitting results of drug release data at pH 3.5, 5.0, and 7.4 are given in Fig. 9, S5 and S6, respectively. It can be seen from Fig. 9, compared with that of first-order and the Higuchi model

($r=0.9501, 0.9365$), in the case of the Bhaskar model linearity was obtained ($r=0.9801$), indicating that the pH-sensitive drug release process based on $\text{Fe}_3\text{O}_4\text{@LDH-MTX}$ system could be controlled either by dissolution of LDH layer or ion-exchange process, and the ion-exchange process may be the main effect in the co-effect (Fig. 10, S7) [38]. The same kinetic models applied to release data at pH 5.0 and 7.4 were also considered (Fig. S5, S6), the fitting data primarily follow the experimental results.

3.4 *In vitro* bioassay

To test the anticancer activity of MTX released from the $\text{Fe}_3\text{O}_4\text{@LDH-MTX}$, the relative viability of MCF-7 (Fig. 11a) and HepG2 (Fig. 11b) cells was estimated after being exposed to $\text{Fe}_3\text{O}_4\text{@LDH}$, free MTX and $\text{Fe}_3\text{O}_4\text{@LDH-MTX}$ at different concentrations for 24 h by a WST-1 assay. It was apparent that $\text{Fe}_3\text{O}_4\text{@LDH}$ did not show significant cytotoxicity to MCF-7 or HepG2 cells. In the investigated range of concentrations, the viability of the cancer cells was affected in a concentration-dependent manner, and the IC_{50} values of MCF-7 and HepG2 cells that incubated by $\text{Fe}_3\text{O}_4\text{@LDH-MTX}$ were 102.68 $\mu\text{g/mL}$ and 104.58 $\mu\text{g/mL}$, respectively ($P < 0.05$). While the IC_{50} values of MCF-7 and HepG2 cells that incubated by free MTX were 118.65 $\mu\text{g/mL}$ and 119.72 $\mu\text{g/mL}$, respectively ($P < 0.05$). Moreover, the higher inhibition efficiency of $\text{Fe}_3\text{O}_4\text{@LDH-MTX}$ obviously demonstrates that much more MTX was transferred into the cancer cells, and with the increasing of incubation time, the MTX released continuously and accumulated at the tumor site to kill the cells more efficiently (Fig. 11c), which clearly proving that the activity of $\text{Fe}_3\text{O}_4\text{@LDH-MTX}$ against the cancer cells was greater than that of the free MTX. This is most likely because the magnetism of Fe_3O_4 would help the internalization of $\text{Fe}_3\text{O}_4\text{@LDH-MTX}$ within cancer cell compartments, and also the Fe^{3+} had slightly bigger toxicity over cell than Fe^{2+} due to the tendency of oxygen capture from the cell, this result agreed with what was reported by previous studies and showed that the presence of Fe_3O_4

led to increasing cytotoxic effects [30,39]. It is worth mentioning that although the $\text{Fe}_3\text{O}_4@\text{LDH-MTX}$ has excellent effect of inhibiting cancer cells growth, it is practically non-toxic to normal cells (Fig. 11d). These results of WST-1 assay further confirm that $\text{Fe}_3\text{O}_4@\text{LDH-MTX}$ possess a specific drug delivery.

Fig. 12 shows the fluorescence microscopic images of HUVEC, MCF-7 and HepG2 cells in $\text{Fe}_3\text{O}_4@\text{LDH}$, MTX and $\text{Fe}_3\text{O}_4@\text{LDH-MTX}$. From the fluorescence microscopic images, it is observed that the cancer cells incubated by $\text{Fe}_3\text{O}_4@\text{LDH}$ are nearly viable (green images). When the cells are treated with MTX and $\text{Fe}_3\text{O}_4@\text{LDH-MTX}$, it is observed that $\text{Fe}_3\text{O}_4@\text{LDH-MTX}$ produce more orange even red colour cells compare than that of pure MTX. This clearly indicates $\text{Fe}_3\text{O}_4@\text{LDH-MTX}$ have more cytotoxicity than that of pure MTX. Importantly, the $\text{Fe}_3\text{O}_4@\text{LDH-MTX}$ is practically non-toxic to HUVEC cells, revealing that the $\text{Fe}_3\text{O}_4@\text{LDH-MTX}$ could be a competitive candidate with low toxicity to normal tissues for cancer therapy.

4. Conclusion

In conclusions, biocompatible $\text{Fe}_3\text{O}_4@\text{LDH}$ nanoparticles were successfully synthesized via self-assembly in hydrothermal process and the stable $\text{Fe}_3\text{O}_4@\text{LDH-MTX}$ system was fabricated by host-guest exchange. All the results indicated that $\text{Fe}_3\text{O}_4@\text{LDH}$ had suitable size, good stability and excellent magnetic responsibility. The drug entrapment rate (%) of $\text{Fe}_3\text{O}_4@\text{LDH-MTX}$ is up to 91.78%, and the MTX could be effectively released from the system under acidic condition via the co-effect of dissolution of LDH layer and ion-exchange, 84.94% of MTX was released within 48 h at pH 3.5. Moreover, the $\text{Fe}_3\text{O}_4@\text{LDH-MTX}$ had satisfactory anticancer activity on MCF-7 and HepG2 cells, while it was practically non-toxic to HUVEC cells. To this end, we have enough reasons to believe the application of $\text{Fe}_3\text{O}_4@\text{LDH-MTX}$ in the field of targeted drug delivery for anticancer therapeutics.

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References

- [1] S. Yoon, J.R. Choi, J. Kim, J. Shin, X. Zhang, J. Kang, Treat influence of reduced folate carrier and dihydrofolate reductase genes on methotrexate-induced cytotoxicity, *Cancer Res. Treat.* 42 (2010) 163-171.
- [2] S. Liu, S. Li, X. Li, Intercalation of methotrexatum into layered double hydroxides via exfoliation-reassembly process, *Appl. Surf. Sci.* 330 (2015) 253-261.
- [3] Z. Liu, D. Tian, S. Li, X. Li, T. Lu, MTX/LDHs hybrids synthesized from reverse microemulsions: particle control and bioassay study, *Int. J. Pharm.* 473 (2014) 414-425.
- [4] J.H. Choy, J.S. Jung, J.M. Oh, M. Parka, J.Y. Jeong, Y.K. Kang, O.J. Han, Layered double hydroxide as an efficient drug reservoir for folate derivatives, *Biomaterials* 25 (2004) 3059-3064.
- [5] D. Li, B. Lu, H. Zhang, Z. Huang, P. Xu, H. Zheng, Y. Yin, H. Xu, X. Liu, Y. Lou, X. Zhang, F. Xiong, Synthesis and in vitro evaluation of methotrexate conjugated O,N-carboxymethyl chitosan via peptidyl spacers, *J. Nanopart. Res.* 16 (2014) 2609.
- [6] L. Li, M. Li, D. Li, P. He, H. Xia, Y. Zhang, C. Mao, Chemical functionalization of bone implants with nanoparticle-stabilized chitosan and methotrexate for inhibiting both osteoclastoma formation and bacterial infection, *J. Mater. Chem. B* 2 (2014) 5952-5961.
- [7] Y.H. Chen, C.Y. Tsai, P.Y. Huang, M.Y. Chang, P.C. Cheng, C.H. Chou, D.H. Chen, C.R. Wang, A.L. Shiau, C.L. Wu, Methotrexate conjugated to gold nanoparticles inhibits tumor growth in a syngeneic lung tumor model, *Mol. Pharm.* 4 (2007) 713-722.
- [8] Y. Li, J. Lin, H. Wu, M. Jia, C. Yuan, Y. Chang, Z. Hou, L. Dai, Novel methotrexate prodrug-targeted drug delivery system based on PEG-lipid-PLA hybrid nanoparticles for enhanced anticancer efficacy and reduced toxicity of mitomycin C, *J. Mater. Chem.* 2 (2014) 6534-6548.
- [9] D.R. Nogueira, L. Tavano, M. Mitjans, L. Perez, M.R. Infante, M.P. Vinardell, In vitro antitumor activity of methotrexate via pH sensitive chitosan nanoparticles, *Biomaterials* 34 (2013) 2758-2772.
- [10] L.S. Liang, J. Jackson, W.X. Min, V. Risovic, K.M. Wasan, H.M. Burt, Methotrexate loaded poly(L-lactic acid) microspheres for intra-articular delivery of methotrexate to the joint, *J. Pharm. Sci.* 93 (2004) 943-956.
- [11] N.R. Kuznetsova, C. Sevrin, D. Lespineux, N.V. Bovin, E.L. Vodovozova, T. Meszaros, J. Szebeni, C. Grandfils, Hemocompatibility of liposomes loaded with lipophilic prodrugs of methotrexate and melphalan in the lipid bilayer, *J. Control. Release* 160 (2012) 394-400.
- [12] Y. Sun, Y. Huang, S. Bian, J. Liang, Y. Fan, X. Zhang, Reduction-degradable PEG-b-PAA-b-PEG triblock copolymer micelles incorporated with MTX for cancer chemotherapy, *Colloid. Surface. B* 112 (2013) 197-203.
- [13] H. Kang, J. Kim, S. Han, I. Chang, Self-aggregates of poly (2-hydroxyethyl aspartamide) copolymers loaded with methotrexate by physical and chemical entrapments, *J. Control. Release* 81

- (2002) 135-144.
- [14] C. Wang, L. Huang, S. Song, B. Saif, Y. Zhou, C. Dong, S. Shuang, Targeted delivery and pH-responsive release of stereoisomeric anti-cancer drugs using β -cyclodextrin assembled Fe_3O_4 nanoparticles, *Appl. Surf. Sci.* 357 (2015) 2077-2086.
 - [15] S. Lv, Y. Song, Y. Song, Z. Zhao, C. Cheng, Beta-cyclodextrins conjugated magnetic Fe_3O_4 colloidal nanoclusters for the loading and release of hydrophobic molecule, *Appl. Surf. Sci.* 305 (2014) 747-752.
 - [16] J. Wu, S. Xu, W. Jiang, Y. Shen, M. Pu, Facile preparation of a pH-sensitive nano-magnetic targeted system to deliver doxorubicin to tumor tissues, *Biotechnol. Lett.* 37 (2015) 585-591.
 - [17] Y. Matsumura, H. Maeda, A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs, *Cancer Res.* 46 (1986) 6387-6392.
 - [18] S.H. Hwang, Y.S. Han, J.H. Choy, Intercalation of functional organic molecules with pharmaceutical cosmeceutical and nutraceutical functions into layered double hydroxides and zinc basicsalts, *B. Kor. Chem. Soc.* 22 (2001) 1019-1122.
 - [19] X. Kong, S. Shi, J. Han, F. Zhu, M. Wei, X. Duan, Preparation of Glycy-L-Tyrosine intercalated layered double hydroxide film and its in vitro release behavior, *Chem. Eng. J.* 157 (2010) 598-604.
 - [20] H. Yang, R.J. van Ee, K. Timmer, E.G.M. Craenmehr, J.H. Huang, F. Cumhuri Öner, W.J.A. Dhert, A.H.M. Kragten, N. Willems, G.C.M. Grinwis, M.A. Tryfonidoud, N.E. Papen-Botterhuis, L.B. Creemers, A novel injectable thermoresponsive and cytocompatible gel of poly(N-isopropylacrylamide) with layered double hydroxides facilitates siRNA delivery into chondrocytes in 3D culture, *Acta Biomater.* 23 (2015) 214-228.
 - [21] J. H. Choy, S. Y. Kwak, J. S. Park, Y.J. Jeong, J. Portier, Intercalative nanohybrids of nucleoside monophosphates and DNA in layered metal hydroxide, *J. Am. Chem. Soc.* 121 (1999) 1399-1400.
 - [22] J. H. Choy, S. Y. Kwak, Y. J. Jeong, J. S. Park, Inorganic layered double hydroxides as nonviral vectors, *Angew. Chem. Int. Edit.* 39 (2000) 4041-4045.
 - [23] J. M. Oh, M. Park, S. T. Kim, J. Y. Jung, Y. G. Kang, J. H. Choy, Efficient delivery of anticancer drug MTX through MTX-LDH nanohybrid system, *J. Phys. Chem. Solids* 67 (2006) 1024-1027.
 - [24] L.E. Gerweck, K. Seetharaman, Cellular pH gradient in tumor versus normal tissue: potential exploitation for the treatment of cancer, *Cancer Res.* 56 (1996) 1194-1198.
 - [25] Q. Wan, S. Chen, W. Shi, L. Li, H. Ma, Lysosomal pH rise during heat shock monitored by a lysosome-targeting near-infrared ratiometric fluorescent probe, *Angew. Chem.* 126 (2014) 11096-11100.
 - [26] Y. Wang, E. Wang, L. Gao, L. Xu, Synthesis and properties of Mg_2Al layered double hydroxides containing 5-fluorouracil, *J. Solid State Chem.* 178 (2005) 736-741.
 - [27] U. Riaz, S. M. Ashraf, Double layered hydroxides as potential anti-cancer drug delivery agents, *Mini-Rev. Med. Chem.* 13 (2013) 522-529.
 - [28] S. Liu, C. Dai, L. Wang, S. Lin, X. Li, Orthogonal test design for optimization of synthesis of MTX/LDHs hybrids by ion-exchange method, *J. Phys. Chem. Solids* 79 (2015) 82-88.
 - [29] L. Lin, W. Xu, H. Liang, L. He, S. Liu, Y. Li, B. Li, Y. Chen, Construction of pH-sensitive lysozyme/pectin nanogel for tumor methotrexate delivery, *Colloid. Surface. B* 126 (2015) 459-466.
 - [30] P. I. Siafaka, A. Titopoulou, E. N. Koukaras, M. Kostoglou, E. Koutris, E. Karavas, D. N. Bikiaris, Chitosan derivatives as effective nanocarriers for ocular release of timolol drug, *Int. J. Pharm.* 495 (2015) 249-264.

- [31] J. Wu, Y. Wang, W. Jiang, S. Xu, R. Tian, Synthesis and characterization of recyclable clusters of magnetic nanoparticles as doxorubicin carriers for cancer therapy, *Appl. Surf. Sci.* 321 (2014) 43-49.
- [32] M. Ramli, M. Z. Hussein, K. Yusoff, Preparation and characterization of an anti-inflammatory agent based on a zinc-layered hydroxide-salicylate nanohybrid and its effect on viability of Vero-3 cells, *Int. J. Nanomed.* 8 (2013) 297-306.
- [33] J. Wu, W. Jiang, S. Xu, Y. Wang, R. Tian, Synthesis of highly selective and sensitive magnetic targetednanoprobe for Cr^{3+} detection in aqueous solution and its application in living cell imaging, *Sensor. Actuat. B-Chem.* 211 (2015) 33-41.
- [34] J. Q. Wang, X. Li, S. P. Li, H. Zhong, Studies on preparation of nanomaterials of methotrexatum/layered double hydroxides compounds by coprecipitation method in ethanol-water medium, *Acta. Chim. Sinica* 69 (2011) 137-144.
- [35] F. M. Rubino, Separation methods for methotrexate, its structural analogues and metabolites, *J. Chromatogr. B* 764 (2001) 217-54.
- [36] S. Sun, H. Zeng, D. B. Robinson, S. Raoux, P. M. Rice, S. X. Wang, G. Li, Monodisperse MFe_2O_4 ($\text{M} = \text{Fe}, \text{Co}, \text{Mn}$) nanoparticles, *J. Am. Chem. Soc.* 126 (2004) 273-279.
- [37] F. Li, L. Jin, J. Han, M. Wei, C. Li, Synthesis and controlled release properties of prednisone intercalated Mg-Al layered double hydroxide composite, *Ind. Eng. Chem. Res.* 48 (2009) 5590-5597.
- [38] H. Zhang, K. Zou, S. Guo, X. Duan, Nanostructural drug-inorganic clay composites: structure, thermal property and in vitro release of captopril-intercalated Mg-Al-layered double hydroxides, *J. Solid State Chem.* 179 (2006) 1792-1801.
- [39] J. Varshosaz, H. Sadeghi-aliabadi, S. Ghasemi, B. Behdadfar, Use of magneticfolate-dextran-retinoic acid micelles for dual targeting of doxorubicin in breastcancer, *Biomed. Res. Int.* 2013 (2013) 1-16.

Figures caption:

Fig. 1. Schematic diagram illustrating the formation of self-assembled $\text{Fe}_3\text{O}_4@\text{LDH}$ and the process of MTX intercalation.

Fig. 2. TEM images of (a) Fe_3O_4 , (b) $\text{Fe}_3\text{O}_4@\text{LDH}$, FE-SEM image of (c) Fe_3O_4 , (d) $\text{Fe}_3\text{O}_4@\text{LDH}$ and (e) EDS spectrum of $\text{Fe}_3\text{O}_4@\text{LDH}$.

Fig. 3. (a and b) HRTEM images, (c) elemental mapping of Fe, O, Al, Mg of the $\text{Fe}_3\text{O}_4@\text{LDH}$ spheres.

Fig. 4. XRD patterns of (a) 2θ from $5-80^\circ$, top to bottom: $\text{Fe}_3\text{O}_4@\text{LDH}$; LDH and Fe_3O_4 , (b) 2θ from $2.5-25^\circ$, top to bottom: $\text{Fe}_3\text{O}_4@\text{LDH-MTX}$ and $\text{Fe}_3\text{O}_4@\text{LDH}$, inset: schematic models for monolayer packing of MTX molecules in the LDH interlayer space.

Fig. 5. TG-DTA curves for (a) $\text{Fe}_3\text{O}_4@\text{LDH}$ and (b) $\text{Fe}_3\text{O}_4@\text{LDH-MTX}$.

Fig. 6. FT-IR spectra of (a) Fe_3O_4 , (b) LDH, (c) Fe_3O_4 @LDH, (d) MTX and (e) Fe_3O_4 @LDH-MTX in KBr pellet.

Fig. 7. VSM measurement results of (a) Fe_3O_4 , (b) Fe_3O_4 @LDH and (c) Fe_3O_4 @LDH-MTX. Insets: (I) the curve of Fe_3O_4 @LDH-MTX with the external magnetic field is near zero, wherein the two markers present the values of saturation remanence [$M_{rs}=1.45 \text{ emu/g}$] and coercivity [$H_c=3.64 \text{ Oe}$] respectively; (II) typical magnetic separation of Fe_3O_4 @LDH-MTX dispersed PBS solution (pH 7.4).

Fig. 8. In vitro cumulative release of MTX from Fe_3O_4 @LDH-MTX at 37°C in PBS buffer at pH 7.4, 5.0 and 3.5. Results are expressed as the mean $\pm$ SD of three independent experiments.

Fig. 9. Plots of different kinetic models for the release of MTX from Fe_3O_4 @LDH-MTX at pH 3.5.

Fig. 10. The expected delivery mechanism of Fe_3O_4 @LDH-MTX into cancer cells.

Fig. 11. The relative viability of MCF-7 (a) and HepG2 cells (b) after being exposed to Fe_3O_4 @LDH, free MTX and Fe_3O_4 @LDH-MTX at different concentrations for 24 h by a WST-1 assay, (c) the relative viability of MCF-7 and HepG2 cells after being exposed to Fe_3O_4 @LDH-MTX at different concentrations for 24 h and 48 h by a WST-1 assay and (d) the relative viability of HUVEC, MCF-7 and HepG2 cells after being exposed to Fe_3O_4 @LDH-MTX at different concentrations for 24 h by a WST-1 assay. Data were expressed as mean $\pm$ SD.

Fig. 12. Fluorescence images of HUVEC, MCF-7 and HepG2 cells incubated with Fe_3O_4 @LDH, MTX and Fe_3O_4 @LDH-MTX at a concentration of $120 \mu\text{g/mL}$. Cells were counterstained with AO (for living cells, the green) and EB (for dead cells, the red).

Graphical Abstract

Synthesis and in vitro evaluation of pH-sensitive magnetic nanocomposites as methotrexate delivery system for targeted cancer therapy

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