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Methotrexate intercalated calcium carbonate nanostructures: Synthesis, phase transformation and bioassay study

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ABSTRACT

The formation and stabilization of amorphous calcium carbonate (ACC) is an active area of research owing to the presence of stable ACC in various biogenic minerals. In this paper, the synthesis of calcium carbonate ($CaCO_3$) under the participation of methotrexate (MTX) via a facile gas diffusion route was reported. The results indicated that the addition of MTX can result in the phase transformation of $CaCO_3$, and then two kinds of hybrids, i.e., MTX-vaterite and stable MTX-ACC came into being. Interestingly, the functional agent MTX served as both the target anticancer drug loaded and effective complexation agents to modify and control the morphology of final samples. The examination of MTX-ACC biodegradation process revealed that the collapse of MTX-ACC nanoparticles was due to the synergistic effect of drug release and the phase transformation. Finally, our study also proved that MTX-ACC exhibited the most excellent suppressing function on the viability of cancer cells, especially after long-time duration.

1. Introduction

In the last decades, nanocarrier system for the drug delivery against cancers has been a highly attractive research area [1–3]. Especially, inorganic nanoparticles are obtaining more attention nowadays because of their high drug loading, pronounced stability and biocompatibility. Thus, various inorganic nanocarriers including magnetite, calcium phosphate, carbon, gold, silica oxide and layered double hydroxide etc. have been evaluated for delivering cytotoxic drugs [4–9]. Among numerous inorganic particles, calcium carbonate has been of interest due to its excellent biocompatibility, good affinity, simple chemical composition, as well as low cost and ease of large-scale production [10]. To date, $CaCO_3$ has been widely used as a successful carrier for the delivery of drugs, genes, and proteins [11]. Usually, $CaCO_3$ exists as three polymorphs (calcite, aragonite, and vaterite) and an unstable amorphous form. Among these, the formation and stabilization of ACC is an active area of research owing to the presence of stable ACC in various biogenic minerals [6,9,10]. It has been proved that the presence of magnesium is essential for the formation of the stable ACC phase [11,12]. However, the mixture of magnesium ions with ACC led to the inconvenience of synthesis process and further application. (See Scheme 1.)

Here, methotrexate (MTX) was used as complex agents to regulate the crystal growth of ACC structure by interacting with the nucleation sites and crystal faces, thus giving rise to the formation of stable architectures. More importantly, MTX is also a kind of chemotherapeutic agent for treating lots of cancers [9]. As a result, MTX was served as both the target anticancer drug loaded and effective complex agents to modify and control the structure and morphologies of the samples, which has not been reported before [13]. Moreover, the drug-release and anticancer effect of MTX-ACC nanostructure were explored emphatically, for further clinical application.

2. Experimental section

2.1. Chemicals

Calcium chloride ($CaCl_2 \cdot 2H_2O$), ammonium hydrogen carbonate (NH_4HCO_3), methotrexate (MTX), absolute ethanol (C_2H_5OH), ammonia solution ($NH_3 \cdot H_2O$), were all of analytical purity. MTX was purchased from Huzhou Prospect Pharmaceutical Co. (Zhejiang, CN). Human lung adenocarcinoma cells (A549) were purchased from Shanghai cell bank (Shanghai, CN).

2.2. Synthesis of pristine $CaCO_3$ and MTX-$CaCO_3$ compounds

Pristine $CaCO_3$ and MTX-$CaCO_3$ were prepared by the typical gas diffusion method [14,15]. $CaCl_2 \cdot 2H_2O$ (200 mg) was dissolved in absolute ethanol (100 mL), and then $NH_3 \cdot H_2O$ (25%) was used to regulate the pH

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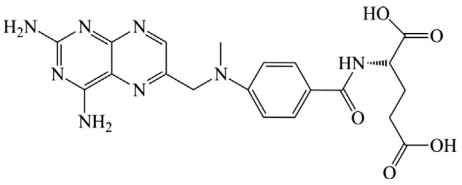

Scheme 1. The chemical structure of MTX.

value to 4.5, 7.2 and 8.5. After that, the mixture was transferred into a glass bottle and covered by parafilm with several pores. Then, the bottle was left in a desiccator along with two glass bottles of ammonia bicarbonate (NH_4HCO_3) at 35 °C. After reaction for 2–3 days, the white products were centrifuged at 5000 rpm and redispersed in absolute ethanol. At last, the corresponding samples were named as *a*, *b* and *c* according to the pH value of 4.5, 7.2 and 8.5. A typical synthesis of MTX-$CaCO_3$ was also described as follows: 5 mg of MTX was added into the solution of calcium chloride, the rest of experimental section was the same with that of synthesizing pristine $CaCO_3$. At last, the final samples were named as *d*, *e* and *f* according to the pH value of 4.5, 7.2 and 8.5, respectively. The detailed experimental conditions were listed in Table 1.

2.3. Characterization

The X-ray diffraction (XRD) patterns were obtained with a D/max-2500PC rotating anode X-ray powder diffractometer (Rigaku Co.), using Cu Kα radiation (λ = 1.5406 Å) from 2° to 90° at a scanning rate of 1 °/min. Fourier transform infrared spectroscopy (FTIR) spectra were recorded on a Bruker Tensor 27 spectrometer in the wavenumber region of 400 to 4000 cm^{-1} using KBr pellets (with a weight ratio of sample to KBr being 1:100), and the resolution of the instrument is 4 cm^{-1}. Transmission electron microscope (TEM) images were obtained using a H-7650-HITACHI (Hitachi Medical Co.) machine operating at 200 kV. Samples for TEM were prepared by depositing a drop of sample solution onto a carbon-coated copper grid and the grid was dried in air. TG analyses of the powders were carried out with a heating rate of 10 °C/min in N_2 atmosphere with the flow of 80 mL/min from ambient to 1200 °C in Al_2O_3 crucible by STA-449C (Netzsch Co.) machine. A Cary 50 UV–vis (Varian Co. USA) spectrophotometer was used to determine the drug-loading capacity and release property. The particle size distribution was characterized by Sirion 200 (FEI Co. Holland) Scanning Electron Microscopy (SEM) machine. Dynamic light scattering (DLS) measurements were performed by using Zetaplus/90plus Dynamic Light Scattering instrument (Brookhaven Instrument Co., USA). The DLS instrument was operated under the following conditions: temperature 25 °C, detector angle 90 °C, incident laser wavelength 683 nm and laser power 100 mW.

2.4. Drug-loading capacity

To determine the amount of MTX loaded into $CaCO_3$ nanostructures, 0.01 g of MTX-$CaCO_3$ was dissolved in HCl solution (pH = 1.2) and then diluted to 500 mL in volumetric flask. The concentration of MTX was measured by UV–vis spectroscopy at 306 nm, and then the loading capacity can be determined. The data were collected in triplicate and presented in Table 1.

2.5. In vitro drug release

To measure the amount of MTX released from MTX-$CaCO_3$ nanostructures, in vitro drug release was performed as follows: 0.02 g of MTX-$CaCO_3$ was added into 500 mL of phosphate buffer solution (pH = 7.4) in a closed glass bottle at a constant temperature of 37 °C and the bottle was magnetically stirred. 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. Dissolution medium was maintained at constant volume by replacing the samples with a fresh dissolution medium. 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. The data were collected in triplicate and presented in Fig. 3D.

2.6. In vitro bioassay

Human lung adenocarcinoma cells (A549) and mouse adrenal pheochromocytoma cells (PC-12) were used here. 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% of fetal bovine serum (FBS) solution, and 100 U/mL of penicillin and 100 mg/mL of 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% of 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-$CaCO_3$ samples, and further incubated for 24 h. The effect of MTX-$CaCO_3$ nanoparticles on cell proliferation was determined using MTT (a yellow tetrazole) assay [16]. Briefly, after the supernatant was removed, 10 μL of MTT (5 mg·mL^{-1} in PBS, pH 7.4) stock solution and 90 μL of 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 incubation time. The data are collected in triplicate and presented in Fig. 4. As for the phase contrast images, after the cell attachment of 24 h, the medium in the wells was replaced by sample *f* with the incubation of 0.5, 1 and 2 h, respectively. Then the cells were observed using a light microscope with a 40× objective lens at different incubation time.

Table 1
Detailed experimental parameters for the synthesis of some typical samples by gas diffusion method and their drug loadings (Three independent experiments (n = 3) were carried out, and data are means ± SD.)

Samples	pH value	Reaction system	abbreviation	A_{ln} (%)
a	4.5	$CaCl_2·2H_2O + NH_4HCO_3$	Calcite	0
b	7.2	$CaCl_2·2H_2O + NH_4HCO_3$	/	0
c	8.5	$CaCl_2·2H_2O + NH_4HCO_3$	Vaterite	0
d	4.5	$CaCl_2·2H_2O$ + MTX + NH_4HCO_3	HVN-MTX	26.33 ± 0.46
e	7.2	$CaCl_2·2H_2O$ + MTX + NH_4HCO_3	/	29.62 ± 0.38
f	8.5	$CaCl_2·2H_2O$ + MTX + NH_4HCO_3	ACC-MTX	30.63 ± 0.26

Data points represent mean ± SD (n = 3).
A_{ln} represents the drug-loading capacity.