Fabrication of folate-phytosterol-carboxymethyl cellulose nanoparticles derived from plant material as carrier of anticancer drug

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In this work, folate-phytosterol-carboxymethyl cellulose nanoparticles (FPCMC NPs) derived from plant material were fabricated and investigated as carrier of hydrophobic anticancer drugs. Firstly, hydrophobic phytosterol was grafted onto the framework of water-soluble carboxymethyl cellulose. Then folate, as tumor-targeting ligand, was coupled to the phytosterol-carboxymethyl cellulose to become self-assembled FPCMC NPs. The physicochemical properties of the fabricated FPCMC NPs were characterized. Doxorubicin (DOX) was selected as model anticancer drug that was entrapped in prepared FPCMC NPs with satisfactory loading content (7%) and loading efficiency (71.2%). The in vitro drug release test showed that the release amount of DOX from drug-loaded FPCMC NPs at pH 5.3 was much higher than that at pH 6.5 or pH 7.4. The research results indicated that the fabricated FPCMC NPs had the potential as nanocarrier of hydrophobic anticancer drugs for further experimental study.

1. Introduction: The chemotherapy is one main method of cancer treatments, which uses anticancer drugs to prohibit the proliferation and migration of cancer cells. Although chemotherapy is an effective way to treat a variety of cancer, it can also damage normal cells and cause serious side effects. The cancer cells exhibit the resistance mechanism to chemotherapeutic drugs for several reasons. The detoxification of the drug is improved by increasing metabolism and reducing drug uptake. Therefore, the development of the unique carrier system entrapping a large amount of chemotherapeutic drugs and targeting tumor cells is essential for successful cancer chemotherapy.

Nanotechnology has been vastly used in the field of drug delivery and cancer treatment, which does improve the therapeutic effects on cancer treatment [1, 2]. The polymeric nanoparticles (NPs) have been developed in recent years as anticancer drug carriers to improve the therapeutic efficacy of anticancer drugs by facilitating local drug uptake and increasing drug bioavailability due to passive targeting capability through enhanced permeability and retention effect [3]. Among an assortment of polymeric NPs for targeting delivery of anticancer drugs, the NPs based on natural polysaccharides, such as heparin [4, 5], dextran [6], curdlan [7, 8], xyloglucan [9, 10], arabinogalactan [11, 12], hyaluronic acid [13–15], alginate [16–19], pullulan [20–22], and chitosan [23–26] had attracted great attention from scientists.

Carboxymethyl cellulose (CMC) is a water-soluble polysaccharide derivative derived from plant with carboxyl groups and hydroxyl groups on its backbone [27]. CMC has many distinctive advantages over other biopolymers, such as the low cost, non-toxicity, biodegradability, and hypo-allergic reaction [28, 29]. CMC has been used in the pharmaceutical field for controlling the release of drugs, prolonging contact time of drugs with target tissues, improving bioavailability of drug, increasing the anticancer effect and reducing side effects [30, 31]. Phytosterol occurring in plants is a cholesterol-like steroid compound that can lower serum cholesterol concentration and reduce the risk of cardiovascular disease. Preliminary studies have suggested that phytosterol may help prevent ovarian cancer, breast cancer, prostate cancer, colon cancer, stomach cancer, and lung cancer [32]. Folate, also known as vitamin B_{11}, is a convenient targeting ligand for a wide variety of tumors, since folate exhibits very high affinity for its corresponding receptors, which are often over-expressed on the membrane surface of various human cancer cells such as breast, ovary, lung, kidney, colon, brain cancer, and so on [33–35].

In this study, amphipathic self-assembled phytosterol-CMC (PCMC) NPs were fabricated with phytosterol as hydrophobic moieties. Folate, mainly derived from green leafy vegetables, was then coupled to PCMC NPs as a target ligand for delivery of anticancer drugs. The physicochemical properties of folate-PCMC (FPCMC) NPs were fully studied using nuclear magnetic resonance (NMR), transmission electron microscopy (TEM), dynamic light scattering (DLS) and fluorescence emission spectroscopy (FES). Doxorubicin (DOX), a first-line anticancer drug used to treat various cancers, was encapsulated in self-assembled FPCMC NPs by physical dialysis procedure and the release profiles of drugs were studied under different pH conditions.

2. Materials and methods

2.1. Materials and reagents: Sodium CMC, folate, dicyclohexyl carbodiimide (DCC), 4-dimethylaminopyridine (DMAP) and N,N-dimethyl acetamide (DMAc) were bought from Sigma Chemical Co., USA. Phytosterol was acquired from Xian Lantian Bioengineering Co. Ltd., China. DOX hydrochloride (DOX·HCl) was purchased from Zhejiang Hisun Pharmaceutical Co. Ltd., China. Other chemical compounds used in the study were analytical grade and obtained from commercial sources.

2.2. Synthesis of PCMC and FPCMC: Phytosterol was conjugated to CMC by DCC/DMAP-mediated esterification reaction. Briefly, 3 g of sodium CMC was dissolved in 150 mL of dimethyl sulfoxide (DMSO), and then 500 mg of DCC and 250 mg of DMAP were added with stirring for 1 h at ambient temperature to activate carboxyl groups of carboxymethyl cellulose. Thereafter 1.0 g of phytosterol was added with stirring to the above mixture and allowed to react for 24 h. After the grafting reaction, the mixed reactant was filtered and then dialysed against distilled water for

48 h with a dialysis bag (molecular cut-off: 12 kDa).Distilled water used in dialysis operation was replaced with fresh distilled water at intervals of 4 h. Amphipathic grafting product was collected by filtration and then washed with diethyl ether and distilled water, followed by lyophilisation.

FPCMC was fabricated with similar method. Briefly, 600 mg of folate was dissolved in 20 mL of DMSO and then 400 mg of DCC and 200 mg of DMAP were added with stirring at ambient temperature for 4 h to activate carboxyl groups of folate. Subsequently, the activated folate mixture was added into 130 mL of DMSO solution including above lyophilised PCMC and reacted for 24 h at ambient temperature with constant stirring. Then, the reaction product was filtered, dialysed, washed, and eventually freeze-dried to get dried FPCMC. The fabrication route of FPCMC was outlined in Fig. 1.

The structures of the CMC and synthesised FPCMC were analysed by ^{1}H NMR spectroscopy and Fourier transforms infrared (FTIR) for affirming the grafting of phytosterol and folate on CMC.

2.3. Preparation of self-assembled NPs: Amphipathic self-assembled NPs were created by probe sonication in the aqueous medium. PCMC or FPCMC was dispersed in distilled water with gentle shaking at 37°C for 48 h, followed by sonication with a probe-type sonifier at 100 W for 2 min. The above step was carried out three times until the size of self-assembled NPs met the requirement. The pulse program (pulse on 2.0 s, pulse off 2.0 s) was used to prohibit heat built-up in the sample solution during the process. The solution of self-assembled NPs was then filtrated through a Millipore filter (1.0 μm) to remove any precipitation and impurity. The morphological observation of self-assembled FPCMC NPs was executed by TEM. The sizes, polydispersity index (PDI) and zeta potential of prepared NPs were determined by DLS and ELS.

2.4. Fluorescence spectroscopy of FPCMC: The critical aggregation concentration (CAC) and self aggregation behaviour of FPCMC were examined by FES with pyrene as a hydrophobic fluorescent probe [19].The pyrene solution (1.0×10^{-4} M) in acetone was placed into a series of test tubes and the acetone solvent was removed by evaporation under the stream of nitrogen gas. Then, different concentrations of FPCMC solutions were placed into each test tube to make the 6.0×10^{-7} M of final pyrene concentration, which was equal to the solubility of pyrene in water at 22°C. The above solutions were sonicated for 30 min in an ultrasonic bath and the pyrene emission spectra in each test tube were measured by fluorescence spectrophotometry. The probe was excited at 343 nm and its emission spectra were collected in the range of 360–420 nm. The two straight lines were plotted according to the intensity (peak height) ratio of the third band (386 nm, I3) to the first band (374 nm, I1) against the logarithmic value of the FPCMC concentration. The CAC value was obtained from the intersection of two straight lines.

2.5. Preparation of DOX-loaded FPCMC NPs: DOX-loaded FPCMC NPs were fabricated by ultrasonic treatment and dialysis method [19, 36]. Briefly, the FPCMC (20–100 mg) of different weights were dispersed in predetermined amount of phosphate buffered saline (PBS) solution (1/15 M, pH 7.4), respectively and stirred for 12 h. DOX·HCl (5 mg) was blended with three

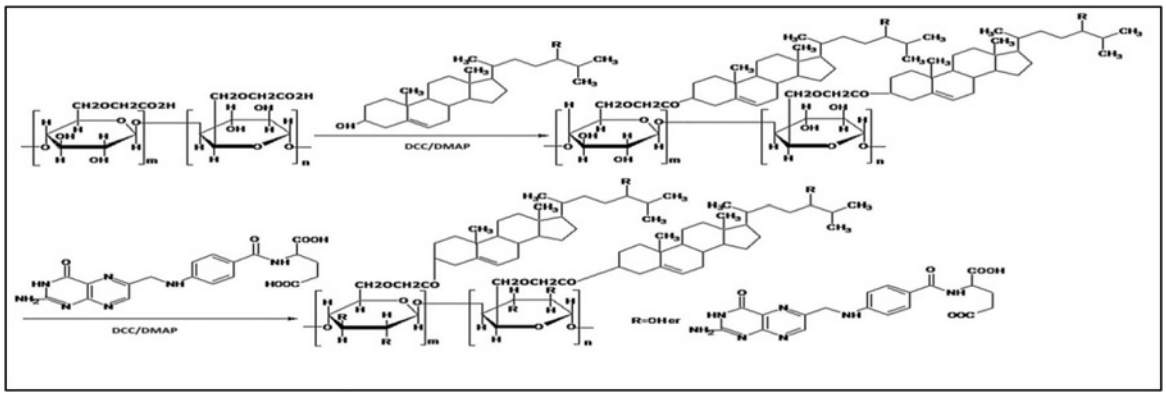

Fig. 1 *Speculated synthetic route of FPCMC*

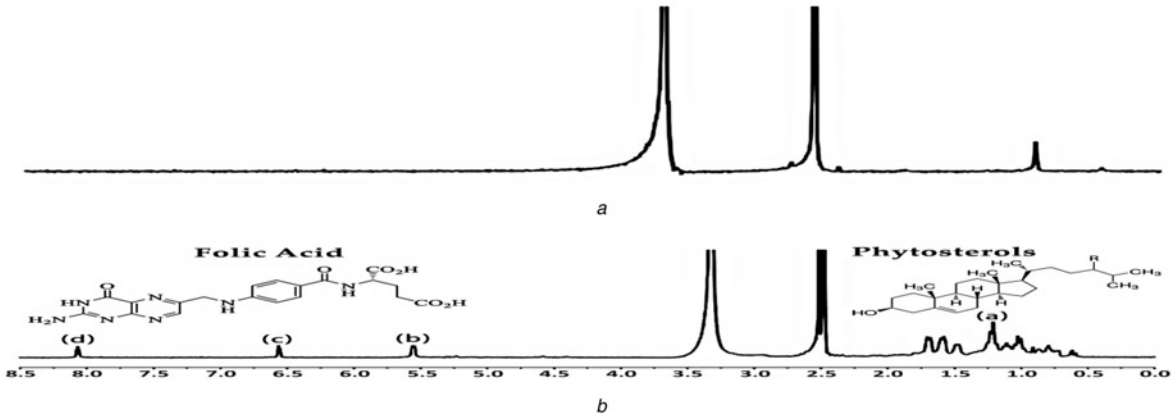

Fig. 2 *^{1}H NMR spectra of*
a CMC
b FPCMC

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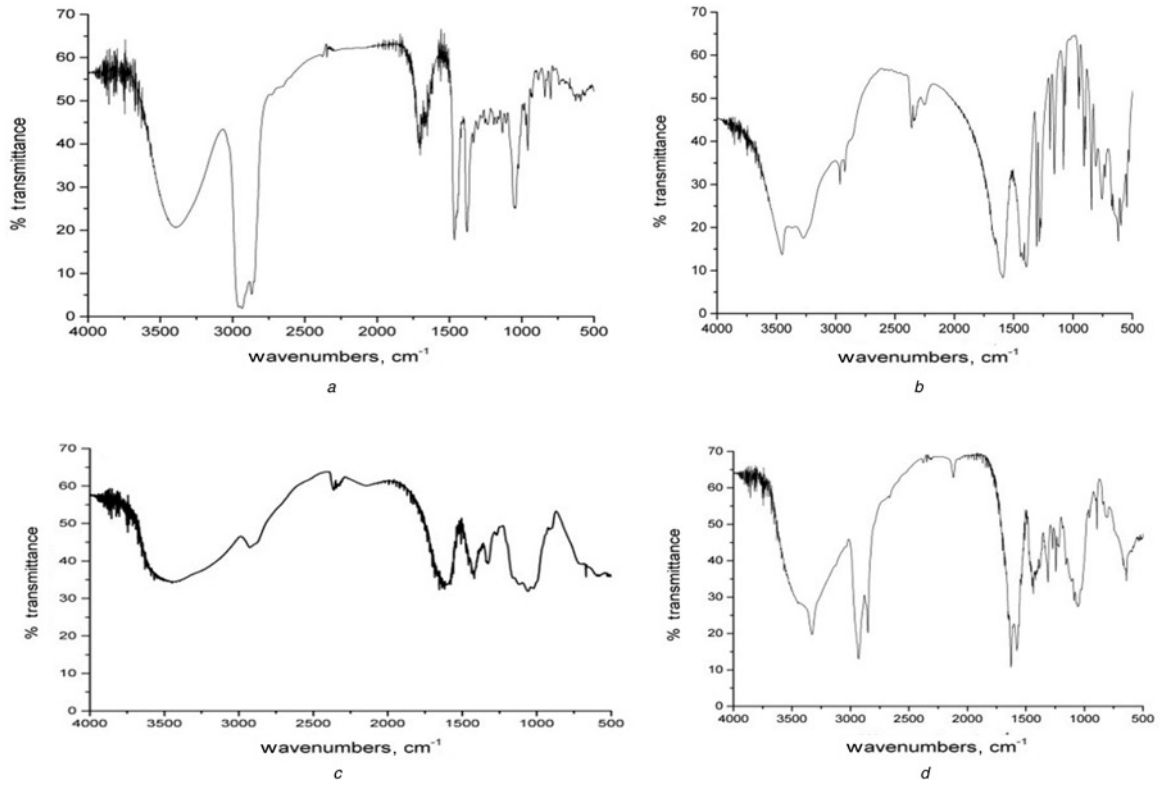

Fig. 3 *FTIR spectra of*
a Phytosteriol
b Folate
c CMC
d FPCMC

equivalents of triethylamine in 2 ml of DMAc to make DOX basic adduct. Then the DOX solution and FPCMC solution was mixed under probe sonication for 3 min. After that, the mixture was stirred overnight at 4°C in the dark and dialysed against above PBS solution for 72 h to remove free DOX and organic solvent. The dialysis medium was changed with fresh PBS solution every 4 h in the first 24 h, then daily. The dialysis product was filtered through a Millipore filter (1.0 μm) and lyophilised to get powder of DOX/FPCMC NPs.

The sample (weight ratio of FPCMC NPs to DOX = 12) was used to perform subsequent drug release experiments.

2.6. Determining loading content (LC) and loading efficiency (LE): On the basis of the drug loading method mentioned above, the drug loading content (LC, %) and loading efficiency (LE, %) were determined. Briefly, the above several DOX/FPCMC NPs were respectively dissolved in DMAc with vigorous stirring for 2 h and then sonicated for 3 min. The supernatant of samples was separated by centrifuging at 20,000 × g for 30 min and the drug concentration in liquid was measured by spectrophotometry at 490 nm. The supernatant from the blank FPCMC NPs was performed as correction. All samples were evaluated in triplicate and the LC and LE values were calculated according to the following equations:

$$\text{LC \%} = \text{mass of DOX in NPs} \times 100/\text{mass of used NPs}$$
$$\text{LE \%} = \text{mass of DOX in NPs} \times 100/\text{mass of used DOX}$$

2.7. Release profiles of DOX from drug-loaded NPs: The DOX-loaded FPCMC NPs (6 mg) were dispersed in PBS solution (5 mL) of pH 5.3, 6.5, or 7.4, respectively. The suspension was placed into a dialysis tube (molecular cut-off: 12 kDa) and the sample was immersed in 25 mL of the PBS solution listed above at 37°C with gentle shaking. At desired time intervals, the solution containing DOX was withdrawn and replaced with fresh PBS solution and then DOX concentration was measured by spectrophotometry at 490 nm.

3. Results and discussion

3.1. Characterisation of structure of FPCMC

3.1.1. ^{1}H NMR study: The structure of grafting FPCMC was confirmed by comparing its ^{1}H NMR spectrum with that of original CMC. In the ^{1}H NMR spectrum of FPCMC (Fig. 2*b*), the resonance peaks (a) from 0.6 to 1.7 ppm represented methyl protons at an aliphatic side chain of phytosterol moiety, which indicated that hydroxyl groups of phytosterol were successfully conjugated to carboxyl groups of CMC. The resonance signals (a, b) at 5.5 and

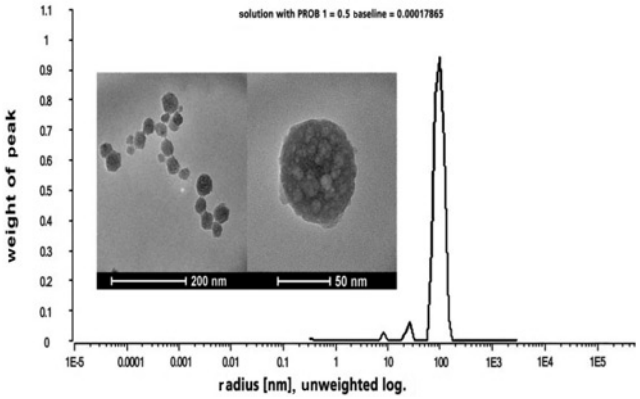

Fig. 4 *TEM images and size distribution of self-assembled FPCMC NPs*

Table 1 Characterisation of prepared NPs

Sample	Diameter (nm)	Zeta potential (mV)	PDI	LC %	LE %
PCMC	91.6	−42.4 ±1.4	0.131	—	—
FPCMC	107.4	−40.2 ±2.6	0.214	—	—
DOX/FPCMC	137.0	−38.5 ±1.7	0.275	7 ± 0.20	71.2 ± 1.85

6.6 ppm were attributed to aromatic protons of folate moiety and the characteristic peak (d) at 8.1 ppm was assigned to methylene protons in the pteridine ring of folate moiety, which indicated that folate was successfully coupled with CMC.

3.1.2. FTIR analysis: As shown in (Fig. 3) the appearance of characteristic IR peaks at about 1580, 1440, and 905 cm^{-1} found in both folate and FPCMC were attributed to the (C = O) stretching vibration of carboxyl group, (C = C) stretching vibration, and (C–H) out-of-plane bending vibration in the aromatic ring of folate, respectively, indicating the covalent coupling of folate moieties on CMC. The C-H stretching vibration from phytosterol ligand at 2870 and 2850 cm^{-1} appeared simultaneously in the FTIR spectra of phytosterol and FPCMC, which indicated that phytosterol ligands had been successfully coupled to CMC molecules.

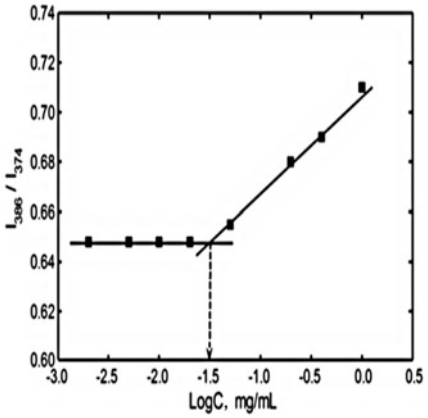

Fig. 5 *Intensity ratio (I_{386}/I_{374}) from pyrene emission spectra against the logarithm of FPCMC concentration*

3.2. Morphology, size, and zeta potential of self-assembled FPCMC: Morphology and size distribution of the prepared self-assembled FPCMC NPs were exhibited in (Fig. 4). The TEM images and DLS analysis indicated that the FPCMC NPs were spherical in shape with an average diameter of 107 nm. The diameter of PCMC NPs was slightly smaller than that of FPCMC NPs and the larger diameter was found in drug-loaded FPCMCNPs than in non-loaded FPCMC NPs (Table 1). For evaluating the stability of prepared NPs dispersed in water, the zeta potential of the NPs was investigated. The PCMC NPs possessed a negative surface charge, but the negative surface charge decreased slightly after conjugation of folate because of the positively charged pteridine ligand. The negative surface charge declined further after drug loading (Table 1). The same surface charge made the NPs stably dispersed without aggregation. This negative charge also generally did not affect the folate receptor-mediated targeting effect. Thus, it was deduced that FPCMC NPs with negative surface charge could be utilised as a drug delivery carrier for the sustained release of drugs.

3.3. CAC of FPCMC: The CAC value of FPCMC was calculated to be 31 mg/L from (Fig. 5) and it was significantly lower than those of glycyrrhetinic acid-modified alginate (66 mg/L) [16] and DOX-modified alginate (58.34 mg/L) [17]. The low CAC value of FPCMC was an important advantage for being used as a carrier of hydrophobic drugs because the hydrophobic inner core of self-assembled FPCMC NPs could act as a container for holding insoluble drugs.

3.4. LC and LE of DOX in FPCMC NPs: The LC and LE of drugs were important indexes to evaluate clinic applications of the

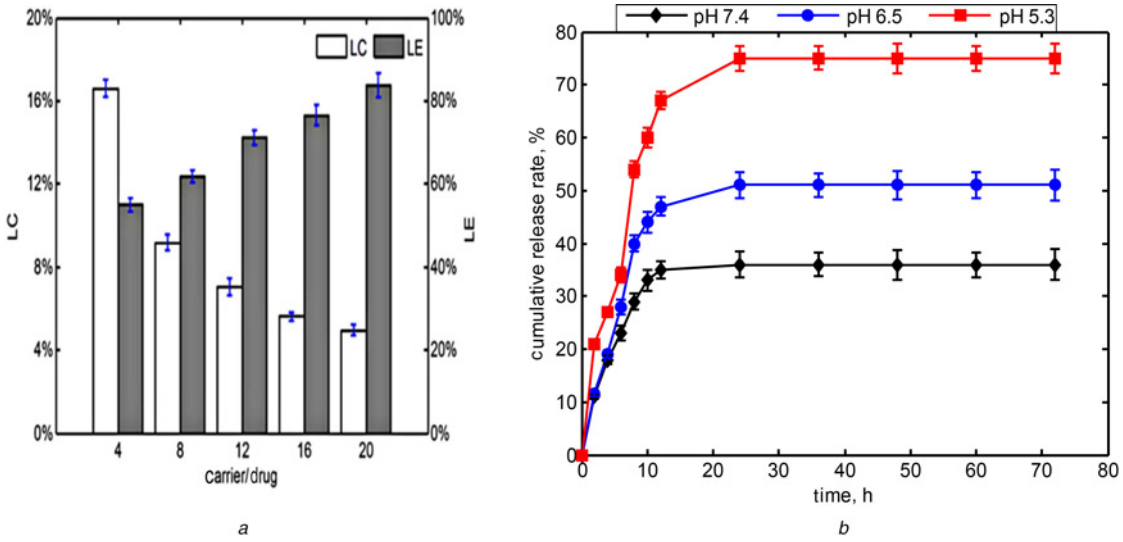

Fig. 6 *DOX loading and release of FPCMC NPs*
a LC and LE of the DOX loaded in FPCMC NPs
b Release profile of DOX from FPCMC

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nanocarrier. The self-assembled FPCMC NPs exhibited relatively excellent LC and LE for DOX. As shown in (Fig. 6*a*), with the increase of carrier/drug weight ratio from 4 to 20, the LE increased from 55.8 to 83.8%, while the LC decreased from 16.5 to 4.9%. The result indicated that FPCMC NPs was an effective carrier for hydrophobic anticancer drugs.

3.5. DOX release behaviour in vitro: The pH value of normal tissues and blood is always maintained at pH 7.4. However, the pH of most tumor tissues is lower than that of normal tissues. For imitating the physiological slightly alkaline environment and pathological acidic environment, three PBS solutions with different pH values (pH: 7.4, 6.5, and 5.3) were used as release medium to study the DOX release profile from DOX/FPCMC NPs. The DOX/FPCMC NPs demonstrated the release characteristic of drugs associated with the pH value of the release medium. As shown in (Fig. 6*b*), the release behaviour of DOX from DOX/FPCMC NPs was clearly pH-sensitive. The release rate of DOX in an acidic environment was faster than that in a near neutral environment, so the cumulative release amount of DOX at acidic pH value was higher than it was in near neutral pH value during the experimental period. The total release amount of DOX at pH 5.3 was 75% in 72 h. However, in the same period, the total release percent of DOX at pH 6.5 and pH 7.4 was approximately 51 and 36%, respectively. The acid-sensitive release mechanism of DOX from DOX/FPCMC NPs may be due to the penetration of proton into the self-assembled FPCMC NPs in acidic condition and the disruption of internal secondary bonds in the self-assembled FPCMC NPs. This drug release profile suggested that FPCMC NPs were suitable as drug delivery vehicles for the sustained release of hydrophobic antitumor drugs in the acidic environment of cancerous tissues

4. Conclusions: The self-assembled FPCMC NPs from plant material was successfully produced and used to encapsulate DOX (hydrophobic anticancer drug) with excellent drug loading effect. The release of DOX from DOX/FPCMC NP relied on environmental pH value and the release of drug was faster in acidic condition than it was in near neutral pH environment. This acid-sensitive release mechanism of drug indicated that the release of anticancer drugs could be induced by the acidic intracellular environment of tumor cells and thus reduced cytotoxicity of DOX/FPCMC NPs to normal tissues. These results confirmed that self-assembled FPCMC NPs had good application prospect as sustained-release carriers of hydrophobic anticancer drugs

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