Regular article

Zepeng Jiao^a, Bin Zhang^a, Chunya Li, Weicong Kuang^a, Jingxian Zhang, Yongqiang Xiong, Shaozao Tan*, Xiang Cai* and Langhuan Huang*

Carboxymethyl cellulose-grafted graphene oxide for efficient antitumor drug delivery

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Abstract: A drug delivery system based on carboxymethyl cellulose-grafted graphene oxide loaded by methotrexate (MTX/CMC-GO) with pH-sensitive and controlled drug-release properties was developed in this work. CMC was grafted on graphene oxide by ethylenediamine through hydrothermal treatment. CMC serves as a pH-sensitive trigger, while CMC-GO serves as a drug-carrying vehicle due to the curved layer and large plain surface. Different amounts of drugs could be loaded into CMC-GO nanocarriers by control of the original amount of drug/carrier ratios. Additionally, low cytotoxicity against NIH-3T3 cells and low *in vivo* toxicity was observed. *In vivo* tumor growth inhibition assays showed that MTX/CMC-GO demonstrated superior antitumor activity than free MTX against HT-29 cells. Moreover, prolonged survival time of mice was observed after MTX/CMC-GO administration. The MTX/CMC-GO drug delivery system has a great potential in colon cancer therapy.

Keywords: cancer therapy; carboxymethyl cellulose; colorectal liver metastasis; drug delivery; graphene oxide.

1 Introduction

Cancer remains one of the leading causes of mortality in the world [1]. Among the various types of cancers, colorectal cancer incidence rates are still rising rapidly in many low-income and middle-income countries [2]. Surgical resection, chemotherapy, radiotherapy, and hormone therapy are the most commonly used anti-tumor therapeutic approaches [3]. Targeted cancer therapies are usually more effective than other conventional treatments [4, 5]. Targeted drug delivery systems hold immense potential to improve the treatment of cancer by the release of the drug at or near the target site, which enhances the efficiency of the drug and lowers the required dosage [6, 7].

Over the past decades, natural polymers have been intensively studied for drug delivery, regarding their biocompatibility and biodegradability, and potential to delivery drugs and genes to specific organs [8–10]. So far, various types of polymers have been used in drug delivery studies [11, 12], including synthetic polymers (polylactic acid, polyglycolide, polylactide-co-glycolide, polycyanoacrylate) and natural polymers (chitosan, gelatin, sodium alginate, etc.). Among the natural polymers, cellulose is much more preferred for modification and use because of its low cost, abundance, and intermolecular hydrogen bond patterns that form mechanically stable fibers [13]. Carboxymethyl cellulose (CMC), a common semisynthetic natural polymer derived from cellulose, shows promising applications for biological and biomedical engineering, including drug delivery [14–16], wound healing [17, 18], bone tissue engineering [19, 20], and biosensing [21]. Crosslinking CMC with polyethylene glycol diglycidyl ether (PEGDE) helps to increase the physical strength of the hydrogels and achieves good adsorption abilities toward lysozyme [22], which would be expected to be a drug carrier for protein drugs.

Recently, an increasing number of graphene-based nanocarriers have been developed for drug delivery [23, 24]. Benefitting from easy modification, high surface

^aZepeng Jiao, Bin Zhang and Weicong Kuang: These authors contributed equally to this work.

*Corresponding authors: Shaozao Tan and Langhuan Huang, Guangdong Engineering and Technology R&D Center of Graphene or Its Like Materials and Products, Department of Chemistry, Jinan University, Guangzhou 510632, P.R. China, e-mail: tsztan@jnu.edu.cn (S. Tan); thuanglh@jnu.edu.cn (L. Huang); and Xiang Cai, Department of Light Chemical Engineering, Guangdong Polytechnic, Foshan 528041, P.R. China, e-mail: cecaixiang@163.com

Zepeng Jiao: Analysis and Test Center, Jinan University, Guangzhou 510632, P.R. China

Bin Zhang, Chunya Li, Weicong Kuang, Jingxian Zhang

and Yongqiang Xiong: Guangdong Engineering and Technology R&D Center of Graphene or Its Like Materials and Products, Department of Chemistry, Jinan University, Guangzhou 510632, P.R. China

area, excellent stability, and outstanding mechanical properties [25–27], graphene and graphene-based materials were widely used as drug delivery vehicles [28–31]. Meanwhile, the six-membered carbon ring structure, which can allow π - π interactions and hydrophobic interactions with drugs having aromatic rings make them quite suitable for delivering aromatic drugs [32]. Kim and coworkers reported functionalized reduced graphene oxide (PEG-BPEI-rGO) as a nano-template for photothermally triggered cytosolic drug delivery, which has the ability to achieve a high concentration of doxorubicin (DOX) and demonstrate great potential in photothermally triggered cytosolic drug delivery via endosomal disruption [33].

In this study, we designed and synthesized carboxymethyl cellulose-grafted graphene oxide (CMC-GO), which could serve as drug carrier to encapsulate methotrexate (MTX). The nanoparticles not only achieved high drug loading efficiency but also exhibited pH-sensitive and sustained drug-release behavior. Here, the preparation of CMC-GO carrier and MTX-loaded nanoparticles, cytotoxicity against NIH-3T3 cells and HT-29 cells, *in vivo* toxicity, and *in vivo* antitumor activity of MTX/CMC-GO were investigated.

2 Experimental section

2.1 Materials

Graphite powder (325 mesh) was purchased from Xiya Chemical Industry Co., Ltd. (Shandong, China). Potassium persulfate, phosphorus pentoxide, concentrated sulfuric acid, potassium permanganate, 30% hydrogen peroxide, sodium periodate, ethylenediamine, glycol, and ethyl alcohol were purchased from Guangzhou Chemical Reagents Co., Ltd (Guangdong, China). All of the above reagents were of analytical grade and were used without further purification. Methotrexate and sodium CMC were provided by Dongkangyuan Biotechnology Co., Ltd (Wuhan, Hubei, China). Thiazolyl blue tetrazolium bromide (MTT) substance was purchased from Sigma-Aldrich (Shanghai, P.R. China). Mouse fibroblast cell line (NIH-3T3) and human colon cancer cells (HT-29) were supplied by the General Hospital of Guangzhou Military Command (Guangdong, China).

2.2 Animals

Balb/c female mice (4–6 weeks old, 12–17 g) and male nude mice (4–6 weeks old) were purchased from Guangdong Medical Laboratory Animal Center (Guangzhou,

Guangdong, China). Mice were maintained in the experimental animal facilities at the Southern Medical University under controlled temperature and specific pathogen-free conditions. The Institutional Administration Panel for Laboratory Animal Care approved the experimental design. The university guidelines for care and use of laboratory animals were strictly followed. All animals were housed and fed in the Experimental Animal Center and were specific pathogen free.

2.3 Synthesis of carboxymethyl cellulose-grafted graphene oxide

First, graphene oxide was synthesized using a modified Hummers' method as we introduced before [34]. Then, sodium CMC was oxidized by sodium periodate. Typically, 100 ml of Na_5IO_6 (1%) and 100 ml of sodium CMC (1%) were added to a flask. After stirring for 24 h at room temperature and dark condition, 5 ml of glycol was added to terminate the reaction. Afterward, 200 ml of ethyl alcohol was added to the solution. Oxidized sodium CMC was then deposited from the solution. After filtration and freeze drying, oxidized sodium CMC was obtained.

CMC-GO was synthesized as follows: Graphene oxide (0.2 g) was dispersed in 50 ml of distilled water and sonicated for 20 min. Then, CMC (0.2 g) was added. Ethylene diamine (5 ml) was added dropwise. The suspension was magnetically stirred for 2 h to obtain a homogeneous dispersion. The solution was put into 25-ml glass bottles and maintained at 90°C for 10 h until a colloidal precipitate was formed. After rinsing and freeze drying, CMC-GO was obtained.

2.4 Methotrexate loading

Accurately weighed MTX was dissolved in 0.1 M sodium hydroxide solution. The weighed quantity of CMC-GO was added afterward. The suspension was sonicated for 40 min. The amount ratio of MTX and CMC-GO was adjusted to 1:1, 2:1, 3:1, and 4:1 to obtain various drug-loading capacities. MTX-loaded CMC-GO were collected by centrifuge (10,000 rpm for 10 min, 10,615×g) and freeze dried. The MTX concentrations after drug loading were measured using a UV spectrophotometer at 303 nm. The drug-loading efficiency (LE) and entrapment efficiency (EE) were determined as follows:

$$\text{LE (\%)} = \frac{W}{W_c + W} \times 100\%$$

$$\text{EE (\%)} = \frac{W}{W_0} \times 100\%$$

where W , W_c , and W_0 , respectively, represent the entrapped drug amount, the weight of carrier, and the amount of drug initially added. Tests were carried out in triplicate, and the average values were reported.

2.5 Material characterization

The morphology of the samples was evaluated by JEOL-2100F electron microscope (Japan) and Zeiss Ultra 55 field scanning electron microscope (Germany). UV-visible diffuse reflectance spectra were taken by Shimadzu UV-2501PC UV-Vis spectrophotometer (Japan). Hydrogen-1 nuclear magnetic resonance (^1H NMR) spectra were analyzed on a ^1H NMR Spectrometer (Bruker 500 ultrashield, Germany). The FT-IR spectra were recorded on a VERTEX-70 spectrometer (Bruker, Germany). Particle size distribution and ζ potential were measured using Zetasizer NANO ZS particle size and ζ potential equipment (Malvern, UK).

2.6 Cytotoxicity assay

The cytotoxicity of MTX/CMC-GO was determined using MTT assay. The NIH-3T3 cells and HT-29 cancer cells were cultured in Dulbecco's modified Eagle's medium (DMEM) medium plus 10% fetal bovine serum (FBS) at 37°C under 5% CO_2 . The cells were seeded in 96-well plates ($1.5\text{--}2 \times 10^4$ cells/well) for 24 h, and then, different concentrations of MTX, CMC-GO, MTX/CMC-GO (0.1, 1, 10, 100, 500, and 1000 $\mu\text{g/ml}$, diluted in DMEM) were added to the wells. After incubation for 24 h, the MTT reagent [5 mg/ml of MTT in phosphate-buffered saline (PBS)] was added to each well with further incubation for 4 h. After removing the medium, the cells were lysed in dimethyl sulfoxide (150 $\mu\text{l/well}$) and incubated to dissolve the purple formazan crystals. The absorbance in each well was measured at 490 nm using a Model-550 Enzyme-linked immunosorbent microplate (Bio-Rad, USA). Cellular viability was calculated in triplicate, and the average values were reported.

In vivo toxicity test was performed to further investigate the biosafety of MTX/CMC-GO. Twenty-four healthy female Balb/c mice (4–6 weeks) were randomly divided into four different experimental groups, including the (a) blank control group, (b) mice treated with MTX/CMC-GO by i.p. injection, (c) mice treated with CMC-GO by i.p. injection, and (d) mice treated with MTX by i.p. injection. Mice were fed over 21 days and finally sacrificed for further tests. The major organs (liver, heart, lung, spleen, and kidney) from those mice were harvested and fixed in 4% formaldehyde for hematoxylin and eosin (H&E) examinations.

2.7 *In vitro* and *in vivo* drug-release studies

An *in vitro* release study of MTX from synthesized nanoparticles was investigated by the dialysis method. Briefly, 0.1 g of MTX/CMC-GO was sealed into a dialysis bag (MWCO 1000) and extensively dialyzed against 400 ml of release medium at 37°C under mechanical shaking at 100 r/min (release medium: simulated gastric fluid, prepared by diluted HCl, adjusted pH to 1.0; simulated intestinal fluid and simulated colon fluid, prepared by 0.2 mol/l of monopotassium phosphate solution and 0.2 mol/l of sodium hydroxide solution, adjusted pH to 6.8 and 7.4). At predetermined intervals of time, a 4-ml sample of the released medium was taken and replaced with the same amount of fresh medium. The drug-released content was diluted with distilled water and analyzed at 488 nm using a UV-visible spectrophotometer.

An *in vivo* release study of MTX was also carried out. Male Sprague-Dawley rats were fasted for 12 h prior to drug administration. MTX/CMC-GO (equivalent to 3 mg/kg of MTX) was dissolved in saline and orally administered to rats. Blood samples were collected at predetermined intervals of time within 48 h after oral administration. The concentrations of MTX were analyzed by high-performance liquid chromatography (HPLC) using Shimadzu Prominence LC-20AT HPLC instrumentation with the following conditions: Ultimate® AQ-C18 Column, 5 μm , 4.6*250 mm; mobile phase, methanol: pH 3.5 sodium acetate solution (10:90, v/v), at a flow rate of 1.0 ml/min; SPD-M20A UV-visible detector (302 nm).

2.8 *In vivo* tumor growth inhibition study

A metastatic tumor model was established by splenic injection of 200 μl of HT-29 cells (1×10^7 cells/ml) to nude mice. Twenty mice were randomly assigned into four groups (five mice per group): (1) PBS control, (2) CMC-GO, (3) MTX, (4) MTX/CMC-GO (3 mg $\cdot$ kg $^{-1}$). Mice were intragastrically administrated for 5 days. At day 40, mice were sacrificed to harvest tumor tissues. Tumor growth was monitored by hematoxylin and eosin examinations.

2.9 Statistical analysis

All data were analyzed using Statistical Product and Service Solutions statistical software (SPSS version 13.0, USA). Statistical significance was determined via Student's t-test with $p < 0.05$ considered to be significant.

Figure 1: FTIR spectral image of GO (A), CMC (B), CMC-GO (C) and MTX/CMC-GO (D); ^1H NMR spectra of GO (E), CMC (F), CMC-GO (G) and MTX/CMC-GO (H).

3 Results and discussion

3.1 Characterization of MTX-loaded CMC-GO

The chemical structure of each sample was confirmed by Fourier-transform infrared spectroscopy (FTIR) and ^1H NMR analysis. The characteristic bands of GO in Figure 1A at 1720 cm^{-1} , 1620 cm^{-1} , 1400 cm^{-1} , 1220 cm^{-1} , and 1048 cm^{-1} were attributed to C=O stretching, C=C stretching, carboxyl O-H bending, C-OH stretching, and C-O stretching, respectively. The characteristic bands of CMC in Figure 1B at 1325 cm^{-1} , 1420 cm^{-1} , and 1630 cm^{-1} were attributed to C-O stretching, O-H bending, and C=C stretching, respectively. After grafting on GO, the FTIR spectrum of CMC-GO

(Figure 1C) showed an absorption peak at $\approx 1580\text{ cm}^{-1}$, which was attributed to N-H bending (amide bond), indicating that CMC was successfully grafted on GO. The broad absorption band at 3490 cm^{-1} was owing to the stretching frequency of the hydroxyl group (-OH). The strong absorption band at 1660 cm^{-1} was attributed to the presence of the carboxyl group (-COOH). The amide group was further confirmed by ^1H NMR spectroscopy. As shown in Figure 1E, the peaks at $\delta \approx 1.34\text{ ppm}$ were attributed to -C=C-, and the peaks at $\delta \approx 3.42\text{ ppm}$ were attributed to -OH. In Figure 1F, the peaks at $\delta \approx 3.58\text{ ppm}$ were attributed to -ROH, and the peaks at $\delta \approx 8.37\text{ ppm}$ were attributed to -COOH. In Figure 1G, the peaks at $\delta \approx 2.8\text{--}3.2\text{ ppm}$ were attributed to the presence of $-\text{CH}_2\text{NH}-$. The characteristic peak at $\delta \approx 7.62$

Figure 2: TEM and FSEM images of CMC-GO (A, B) and MTX/CMC-GO (C, D); particle size distribution (E) and zeta potential of each sample (F).

(d, $J = 8.9$ Hz, 1H), $\delta \approx 6.81$ (d, $J = 8.9$ Hz, 2H) in Figure 1H was attributed to methotrexate [35], confirming that methotrexate was successfully loaded on CMC-GO.

The morphology of CMC-GO and MTX/CMC-GO was observed by transmission electron microscopy (TEM) and field-emission scanning electron microscopy (FSEM). The samples for the TEM observation were prepared by dropping a small amount of the dispersion onto a carbon-coated copper grid and air drying. CMC-GO showed a curved layer morphology (Figure 2B). In the TEM image of MTX/CMC-GO (Figure 2C), we can see monodispersed MTX nanoparticles with a particle size around 200 nm, which was in accordance with particle size distribution results (Figure 2E). Particle size distribution survey indicated that MTX/CMC-GO conjugates took an average particle size of 980 nm (Figure 2E). The ζ potentials of CMC-GO and MTX/CMC-GO were -25.1 mV and -10.8 mV, respectively.

3.2 MTX loading and release behaviors

The antitumor drug MTX was used to evaluate the feasibility of using CMC-GO as a drug carrier. In our approach, MTX was loaded onto CMC-GO by sonication in different ratios. CMC-GO offer abundant aromatic rings to adsorb MTX through π - π stacking and hydrophobic interactions [36]. As shown in Table 1, the drug-loading efficiency and

encapsulation efficiency increases with the increasing MTX concentration, and achieves the highest amount of $39.33 \pm 0.70\%$, $29.50 \pm 0.53\%$, respectively, when the mass ratio of methotrexate to CMC-GO was 3:1. Then, the drug loading decreases with the rising MTX concentration.

Figure 3A shows the MTX release profiles *in vitro* at different pH environments. The specified pH 1.0, pH 6.8, and pH 7.4 buffer solution were corresponded to simulated gastric, intestinal, and colon environments [37], respectively. The results showed that drug-release rate was intensively affected by the pH value of the environment. The cumulative release of MTX was only 4.76% after 6 h in pH 1.0-simulated buffer solution, while it was 10.90% after 3 h in pH 6.8-simulated buffer solution. However, at a buffer solution of pH 7.4, the release rate of MTX increased sharply to 67.4% and 82.2% within 6 h and 48 h, respectively. It was reported that CMC exhibits a lower swelling ratio at pH 1.2 but a higher swelling ratio at pH 7.4 due to the protonation of $-\text{COO}^-$ groups [38]. In brief, the release results proved the potential of CMC-GO as a pH-responsive drug carrier delivering MTX into the colon with a controlled drug-release behavior.

The *in vivo* drug-release profile is shown in Figure 3B, and the corresponding pharmacokinetic parameters are listed in Table 2. There were significant differences in the pharmacokinetic parameters for MTX and MTX/CMC-GO. The $C_{\max}$ and $T_{\max}$ of the MTX/CMC-GO in the portal vein blood were $10.38 \mu\text{g/ml}$ and 9 h, respectively; the $C_{\max}$ and $T_{\max}$ of the free drug MTX in the portal vein blood were $34.01 \mu\text{g/ml}$ and 2.5 h, respectively. MTX/CMC-GO can maintain a stable blood drug concentration than the free drug MTX, thus, enhancing the effectiveness and safety of medication. Also, we noticed a hysteresis effect for MTX/CMC-GO ($t_0 = 3.5$ h). The half-life ($t_{1/2}$) of MTX/CMC-GO was 32.36 h, much longer than that of free MTX (12.29 h). These results demonstrated

Table 1: Drug-loading efficiency and encapsulation efficiency.

w(MTX)/w(CMC-GO)	EE (%)	LE (%)
1/1	8.08 ± 0.14	16.16 ± 0.29
2/1	17.47 ± 0.31	28.18 ± 0.47
3/1	29.50 ± 0.53	39.33 ± 0.70
4/1	20.62 ± 0.37	25.77 ± 0.46

EE, Encapsulation efficiency; LE, loading efficiency.

Figure 3: (A) *In vitro* cumulative drug release at pH 1.0, pH 6.8 and pH 7.4; (B) *In vivo* mean plasma concentration versus time curve of free MTX and MTX/CMC-GO.

Table 2: Pharmacokinetic parameters for MTX and MTX/CMC-GO.

Pharmacokinetic parameters	MTX in portal vein blood	MTX in blood	MTX/CMC-GO in portal vein blood	MTX/CMC-GO in blood
T_{max} (h)	2.5	3	9	9
C_{max} (μg/ml)	34.01	30.21	10.38	6.96
t_0 (h)	0	0	3.5	3.5
$t_{1/2}$ (h)	12.29	10.98	32.36	31.94
$AUC_{0 \rightarrow 48}$ (mg/l)	457.22	400.98	324.81	296.99

that MTX/CMC-GO was able to achieve a long time and sustained drug release.

3.3 Toxicity assay

The *in vitro* cytotoxic effects of MTX, CMC-GO, and MTX/CMC-GO were screened against NIH-3T3 cells (normal cells) and HT-29 cells (cancer cells) by MTT assay. The results of the MTT assay showed a dose-dependent

reduction in the viability percentage of NIH-3T3 cells and HT-29 cells after 24 h. As shown in Figure 4, there is no significant cytotoxicity effect for CMC-GO (cell viability >90%, Figure 4A and C), indicating that CMC-GO shows low toxicity. MTX showed significant cytotoxicity to the NIH-3T3 cells and HT-29 cells. Serious cell injury and membrane disruption were observed after interaction for 24 h (Figure 4B). The cell viability of the NIH-3T3 cells for the MTX/CMC-GO group at all concentrations was higher than that of the free MTX group,

Figure 4: (A) The viability of NIH-3T3 cells after treatment with no stimulus (blank control), CMC-GO, MTX/CMC-GO and MTX for 24 h. (B) Photo of microscopy depict NIH-3T3 cells incubated for 24 h with no stimulus (blank control), CMC-GO, MTX/CMC-GO and MTX (100 μg/ml). (C) The viability of HT-29 cells after treatment with no stimulus (blank control), CMC-GO, MTX/CMC-GO and MTX for 24 h. (D) Photo of microscopy depict HT-29 cells incubated for 24 h with no stimulus (blank control), CMC-GO, MTX/CMC-GO and MTX (100 μg/ml).

indicating a lower toxicity of MTX/CMC-GO than free drug. The cytotoxicity effect for MTX/CMC-GO to HT-29 cells showed no significant difference compared to that of MTX: both presented a higher toxicity against tumor cells (25.9% and 24.4% cell viability after 24-h incubation, Figure 4C).

To further investigate the *in vivo* cytotoxicity of MTX/CMC-GO, the organs were sliced and stained

with H&E. From the H&E staining results (Figure 5), no severe lesions were observed among the samples from the groups treated with free MTX, CMC-GO, and MTX/CMC-GO. In addition, no difference was observed between the blank control group and treated groups. These evidences proved that MTX/CMC-GO does not induce damage to the organs of mice; MTX/CMC-GO shows good biocompatibility.

Figure 5: Microscopy images of H&E stained tissue slices (heart, liver, spleen, kidney, and lung) of (A) MTX/CMC-GO, (B) CMC-GO, (C) MTX injected mice and (D) blank control group. (Magnification = 400×).

3.4 In vivo antitumor activity

The *in vivo* antitumor efficacies are demonstrated in Figure 6. As shown in Figure 6A, CMC-GO possessed no *in vivo* antitumor activity, which was in agreement with the cytotoxic results of HT-29 cells. Free MTX exhibited relatively tight tumor-inhibiting capacity, while enhanced

antitumor efficacy was observed in MTX/CMC-GO, implying that the inhibition of tumor growth could be enhanced by the delivery of CMC-GO carriers. Histological examination showed a lower proliferation of tumor cells in the MTX- and MTX/CMC-GO-treated mice. In addition, we counted the number of liver metastases, and the results are shown in Table 3. The liver metastasis of colon cancer

Figure 6: (A) Representative photos of tumors treated with (a) no stimulus (blank control), (b) CMC-GO, (c) MTX and (d) MTX/CMC-GO. (B) H&E staining of tumors treated with (a) no stimulus (blank control), (b) CMC-GO, (c) MTX and (d) MTX/CMC-GO; (C) Kaplan-Meier survival curve between groups (a) blank control, (b) CMC-GO, (c) MTX and (d) MTX/CMC-GO.

Table 3: Inhibition of colon cancer liver metastasis.

Group	Number of mice	Liver metastatic rate (%)	Inhibition rate (%)	0	I	II	III*	Number of liver metastases >2 mm	Inhibition rate of liver metastases >2 mm (%)
Blank control	18	100%	–	0	3	4	11	6.27 ± 2.73	–
CMC-GO	18	100%	–	0	4	4	10	6.11 ± 2.22	–
MTX	18	27.8%	72.2%	13	1	2	2	1.06 ± 0.39 ^a	83.1%
MTX/CMC-GO	18	16.7%	83.3%	15	1	2	0	0.56 ± 0.11 ^b	90.6%

*Compared with the blank control group, ^a*p* < 0.05, ^b*p* < 0.01. *Number of metastatic tumors: grade 0 (0), grade I (1–5), grade II (6–10), and grade III (>10).

can be effectively inhibited after administration. The inhibition rate of liver metastasis in the MTX-treated group was 72.2%, while the inhibition rate in the MTX/CMC-GO-treated group was 83.3%. The Kaplan-Meier survival curve (Figure 6B) suggested that enhanced survival of the animals was achieved through MTX/CMC-GO administration.

4 Conclusion

In summary, CMC-GO was developed for efficient delivery of MTX. As expected, MTX/CMC-GO drug delivery system exhibited several advantages: high drug-loading efficiency (39.33%) and encapsulation efficiency (29.50%); good pH-dependent drug-release properties; reduced cytotoxicity against NIH-3T3 normal cells but high cytotoxicity against HT-29 cancer cells; higher plasma drug concentration and longer action time; superior tumor inhibition activities and liver metastasis-inhibition activities. The MTX/CMC-GO drug delivery system provides a promising option for colon cancer therapy.

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