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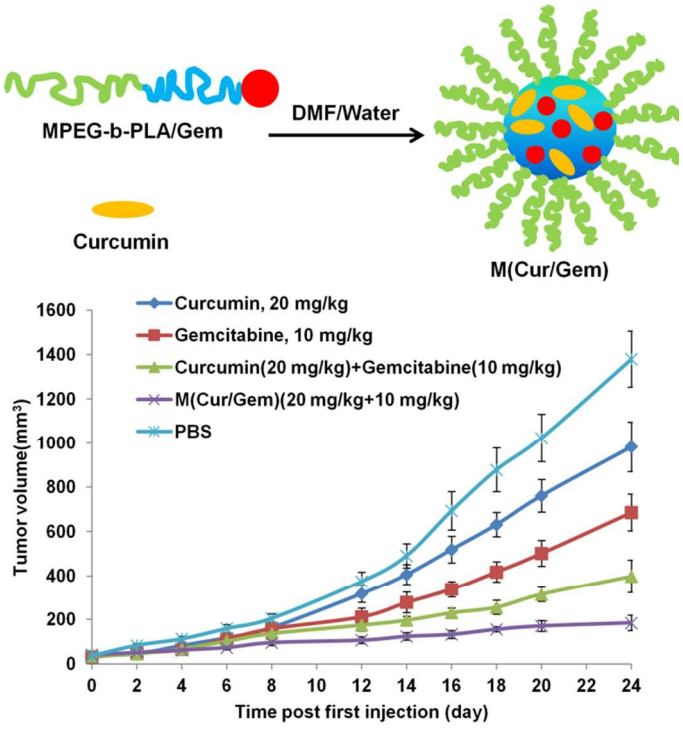

Delivering curcumin and gemcitabine in one nanoparticle platform for colon cancer therapy

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Abstract: As gemcitabine and curcumin show different target in colon cancer cells, combination of them may bring benefits. Here, curcumin and gemcitabine were formulated into a biodegradable polymer platform for combination therapy for treatment of colon cancer. In doing so, a FDA approved biodegradable polymer mPEG-PLA (methoxyl-polyethylen glycol-block-polylactide) was chosen as a drug carrier. At first, a mPEG-PLA/Gem conjugate was designed. Thereafter, simply using this drug conjugate to encapsulate curcumin, polymeric micelles loaded with both curcumin and gemcitabine were obtained. Varying the feed ratio of the two drugs, a series of micelles with different ratios of curcumin and gemcitabine could be prepared. The as-prepared dual drug loaded nanoparticles showed spherical structures with mean diameters ranging from 118 nm to 149 nm by DLS. *In vitro*, M(Cur/Gem) almost showed greater synergy than free combination of curcumin/gemcitabine. *In vivo*, better antitumor effect and lower systemic toxicity of M(Cur/Gem) were observed on murine xenograft model. The present study provides the possibility of combining curcumin and gemcitabine in a nanoparticle formulation, and translation of this combination may bring benefit for future clinic use.

Keywords: curcumin, gemcitabine, combination therapy, drug delivery, colon cancer

1. Introduction

Colon cancer which forms in the tissues of the colon is currently the third most commonly diagnosed cancer [1]. Colon cancer is also the second leading cause of cancer death in men and women combined in USA [2]. Though the great advances in medicine and technology, colon cancer mortality still remains unacceptably high [1,3]. Therefore, there exists an urgent need for alternative drugs or therapeutic strategies for treatment of colon cancer.

Gemcitabine is currently an anticancer drug used in clinic for pancreatic cancer, breast cancer, ovarian cancer, and lung cancer, and may be used for other cancers as well [4]. Gemcitabine is a low molecular weight, deoxycytidine analogue which performs its anticancer activity as an inhibitor of cellular DNA synthesis [3]. Although gemcitabine is very effective against various cancers, it only has an extremely short half-life of 8-17 min [5], which is caused by the degradation of plasma deaminases [5,6]. Therefore, to achieve sufficient efficacy, gemcitabine is used at a very high dose which in turn will cause great side effects in clinic [7]. Researchers around the world have tried various drug delivery systems for

protecting and delivering gemcitabine based on the premise that it could protect gemcitabine from degradation, preferentially transport the drug to the tumor site and reduce the serious side effects [8,9].

Curcumin, which is the major active ingredient of turmeric (*curcuma longa*) shows no discernable toxicity [10]. Curcumin is an antioxidant in the polyphenol family and it has shown anti-inflammatory, anti-viral, anti-bacterial, and anticancer properties [10,11]. Research on curcumin have found that free curcumin induces cell cycle arrest and/or apoptosis and blocks nuclear factor kappa B (NF-κB) activation in various cancer cell lines [12]. Recent research have shown that curcumin could prevent the development of intestinal adenomas in Min+/− mice, a model of human familial adenomatous polyposis [13].Moreover, it was reported that curcumin inhibits EGFR activation in colon cancer cells [14]. However, curcumin has a low bioavailability due to its poor solubility in water [15]. Therefore formulation and delivery of curcumin via drug carrier must be carefully considered.

Combination therapy where multiple drugs would be used in one regimen is becoming increasingly important in most malignancies therapy. This is because in each cancer, multiple pathways become dysfunctional [16,17]. Therefore, therapeutic benefit against tumor development could be achieved within drug combinations that will affect several targets [18,19]. Gemcitabine and curcumin have different targets in cancer cells as mentioned in the previous part. Moreover, various study showed that Curcumin has a potent antiproliferative activity and can also potentiate the antitumor effect of gemcitabine both *in vitro* and *in vivo* [18]. Combination of curcumin and gemcitabine has shown to bring benefit to pancreatic cancer in a previous study [20]. Combination of the two drugs may also bring synergistic effect on colon cancer. However there is almost no report on delivering these two drugs together directly to the cancer cells to achieve the synergistic effect till far. To combine two anticancer drugs in one single nanoparticle shows great advantages as following: 1) to trap two drugs at a desirable ratio that results in synergistic effect *in vitro*, which therefore can best translate into *in vivo* synergistic effect and clinic benefits; 2) to maximize the two drugs at desirable ratio at the tumor site via so-called enhanced permeation effect (EPR)[21]. Here, based on this assumption, we try to formulate curcumin and gemcitabine in a single nanoparticle for combinational use and drug delivery. Biodegradable polymer mPEG-PLA was chosen as a drug carrier for the delivery of curcumin and gemcitabine (Scheme 1). At first, a mPEG-PLA/Gem conjugate was designed, then by using this drug conjugate to encapsulate curcumin, polymeric micelles loaded with both curcumin and gemcitabine can be obtained (Scheme 1). Just by simply changing the feed ratio of the two drugs, a series of micelles with different ratios of curcumin and gemcitabine could be obtained with ease. The *in vitro* and *in vivo* evaluation of these composite micelles was studied systematically.

2. Materials and Methods

2.1. Materials

Monomethoxy poly (ethylene glycol)-polylactide (mPEG-PLA) was synthesized as previously described [22] (molecular weight is 8000, mPEG is 5000, ca. 21 lactide units). The two drugs gemcitabine

hydrogen chloride (Gem) and curcumin (Cur) were purchased from Sigma-Aldrich and was dissolved in DMSO and polyoxyethylenated castor oil for in vitro and in vivo study respectively. Rhodamine B (RhB), N,N'-dicyclohexylcarbodiimide(DCC) and 4-dimethyl amino pyridine (DMAP) were purchased from Aladdin (Shanghai, China). All other chemicals were used as received.

2.2. Methods

^{1}HNMR spectra were recorded at room temperature on a Bruker 400 NMR spectrometer. The micelles were characterized by Transmission electron microscopy (JEOL JEM-1011) and dynamic light scattering (DLS) with a vertically polarized He-Ne laser (DAWN EOS, Wyatt Technology, USA). The zeta potential of the micelles prepared in this paper was conducted on a Malvern Zetasizer Nano ZS.

2.3. Synthesis of mPEG-PLA/Gem conjugates

mPEG-PLA and its carboxyl end-capped polymer mPEG-PLA-COOH were synthesized as previously described [22]. To prepare the mPEG-PLA/Gem conjugate, mPEG-PLA-COOH (100 mg) was dissolved in 50 ml anhydrous CH_2Cl_2 in a flask and then gemcitabine hydrochloride (18.5 mg), DCC (12.7 mg) and DMAP (5 mg) were added into the flask. The reaction mixture was left at room temperature and kept stirring for 24 h. After that, it was subjected to flirtation and precipitated by CH_3OH/ ether (30%:70% v/v). The precipitates were collected via filtration and dried under vacuum. Then it was dissolved in 10 mL DMF and subjected to dialysis against 2 L water by changing the dialysis water for 3 times and lyophilized to powder for further use.

2.4. Preparation of single drug loaded micelles

Gemcitabine and curcumin single drug loaded micelles were prepared using a nano-precipitation method. For gemcitabine loaded micelles M(Gem),10 mg mPEG-PLA/Gem conjugate was dissolved in 2 mL THF in a bottle flask under vigorous stirring, then 6 mL de-ionized water was added drop-wise into this flask. After this process ended, THF was removed using vacuum evaporation. Then the micellar solution was subjected to ultra-filtration and the nanoparticles were re-suspended in 5 ml of water and lyophilized as a powder for further use. For curcumin single drug loaded micelles, mPEG-PLA was used as drug carrier to encapsulate it. Briefly, 0.5 mg curcumin and 10 mg mPEG-PLA were dissolved in 2 mL DMF in a bottle flask under vigorous stirring, then 6 mL de-ionized water was added drop-wise into this bottle. After this process ended, the DMF was removed using dialysis. Thereafter, curcumin loaded micelles were lyophilized as powder for further use.

2.5. Preparation of double drug loaded micelles at different curcumin/gemcitabine ratios and Rhodamine B labeled micelles.

For the potential combination of curcumin with gemcitabine at desirable ratios, the double drug loaded micelles were prepared. Here, we use mPEG-PLA/Gem to encapsulate curcumin. To make

nanoparticles with double drugs at different ratios, we first set the amount of mPEG-PLA/Gem unchanged and increase the amount of curcumin to vary the ratio of curcumin/gemcitabine in the nanoparticles. Here, we set four ratios of curcumin/gemcitabine during preparation of the nanoparticles, namely, Cur/Gem=0.1:1, Cur/Gem=0.5:1, Cur/Gem=1:1 and Cur/Gem=2:1.Here, 0.1:1, 0.5:1, 1:1 and 2:1 stand for the aimed ratio of curcumin and gemcitabine in the micelles respectively. The obtained micelles with both curcumin and gemcitabine can be called M(Cur_x/Gem), where x stands for the ratio of curcumin/gemcitabine. Take the preparation of aimed M(Cur_1/Gem) as an example, curcumin (0.5 mg) and mPEG-PLA/Gem(14 mg) were weighed at a drug molar ratio of 1:1 and then they were dissolved in 2 mL THF. 8 mL de-ionized water was added drop-wise under vigorous stirring to make the nanoparticles containing both curcumin and gemcitabine. Thereafter, THF was removed via vacuum evaporation. The micelles solutions were purified by ultra-filtration and re-suspended in 5 mL water and lyophilized to powder for further use. The drug loading of curcumin and gemcitabine in the nanoparticles were quantified by HPLC as previously described [23, 24]. For Rhodamine B(RhB) labeled micelles preparation, 0.2 mg RhB, 0.5 mg curcumin and 14 mg mPEG-PLA was dissolved in 2 mL THF. 8 mL de-ionized water was added drop-wise under vigorous stirring to make the nanoparticles containing RhB, curcumin and gemcitabine. Thereafter, THF was removed by rotator evaporation. The micelles solutions were purified by ultra-filtration and re-suspended with 5 mL water and lyophilized to powder for further use.

2.6. Drug release study from M(Cur/Gem) micelles

In vitro release of curcumin and gemcitabine from the nanoparticles was studied by the dialysis bag method in phosphate-buffered saline (PBS pH 7.4) and acetate buffered solution (pH 5.0) containing 1% v/v Tween 80 as previously described. Here, Tween 80 was added to maintain perfect sink conditions since curcumin has limited solubility in PBS.50 mg M($Cur_{0.89}$/Gem) and dialyzed against 20 mL of the release medium maintained at 37 °C. At predetermined time intervals, the whole medium was removed and replaced by fresh medium to maintain perfect sink conditions. The release experiments were carried out in the dark and 1 mg/ml ascorbic acid was used in the release medium to protect curcumin from degradation. Release of curcumin and gemcitabine were measured by HPLC as previously described [23, 24].

2.7. Cell use and culture conditions

HCT-116(human colorectal cancer cells) were purchase from ATCC and passaged in McCoy's 5A medium in the presence of 10% FBS, 0.03% L-glutamine and 1% penicillin/streptomycin in 5% CO_2 at 37 °C in a 95% humidified atmosphere.

2.8. In vitro intracellular uptake of M(Cur/Gem) by confocal laser scanning and flow cytometry

The cancer cells HCT-116 were plated onto a glass bottom petri dish at 100,000 cells per plate in 1 mL of culture medium for 12 h before treatment of M($Cur_{0.095}$/Gem/RhB) both at 37 °C and 4 °C and examination with confocal laser scanning microscopy (CLSM, Nikon A1R). 4',6-diamidino-2-phenylindole(DAPI) was used to stain the nuclei. Cells were imaged at 1 h post-treatment of micelles with excitation of 550 nm for RhB and 340 nm for DAPI.

2.9. In vitro evaluation of drug pairs of gemcitabine and M(Gem) as well as curcumin and M(Cur) via MTT assay

HCT-116 cells were seeded in 96-well plate at a density of 5000 cells/well overnight. The cells were then treated with gemcitabine and M(Gem) as well as curcumin and M(Cur) at a concentration ranging from 0.0125 μM to 100 μM. After 72 h treatment of drugs, the culture media was removed and the cells were thoroughly washed by cold PBS to remove possible remaining curcumin. Then, 100 μL of fresh culture media was added to each well and the cells were put into the incubator for warm-up. Thereafter, 10 μL of MTT solution at 5 mg/mL was added to each well and further incubation of 4 h was preceded. After that, the absorbance of each plate was measured by a microplate reader. The cell viability was calculated by comparing the drug treated groups with the non-treated groups as controls.

2.10. In vitro evaluation of free drug combinations of curcumin/gemcitabine

HCT-116 cells were seeded in 96-well plate at a density of 5000 cells/well overnight. The cells were then treated with free combinations of curcumin and gemcitabine at a molar ratio of of curcumin/gemcitabine at 0.095:1, 0.46:1, 0.89:1 and 1.75:1.The concentration range of gemcitabine was set from 0.0125 μM to 25 μM. After 72 h treatment of drugs, the culture media was removed and the cells were thoroughly washed by cold PBS to remove possible remaining curcumin. Then, 100 μL of fresh culture media was added to each well and the cells were put into the incubator for warm-up. Thereafter, 10 μL of MTT solution at 5 mg/mL was added to each well and further incubation of 4 h was preceded. After that, the absorbance of each plate was measured by a microplate reader. The cell viability was calculated by comparing the drug treated groups with the non-treated groups as controls.

2.11. In vitro evaluation of micellar nanoparticle based drugs M(Cur/Gem) and mixed solutions of double micelles (M(Cur)+ M(Gem))

HCT-116 cells were seeded in 96-well plate at a density of 5000 cells/well overnight. The cells were then treated with micelles M(Cur/Gem) loaded with both curcumin and gemcitabine at a molar ratio of curcumin/gemcitabine at 0.095:1, 0.46:1,0.89:1 and 1.75:1.Namely, the micelles of M(Cur/Gem)=0.095:1, M(Cur/Gem)=0.46:1, M(Cur/Gem)=0.89:1 and M(Cur/Gem)=1.75:1 were used. The concentration range of gemcitabine in the micelles was set from 0.0125 μM to 25 μM. For comparisons, the two separate micelles M(Cur) and M(Gem) were dissolved and mixed together at a the same molar ratio of 0.095:1, 0.46:1,0.89:1 and 1.75:1 were used to treat the cells. After 72 h treatment of drugs, the

culture media was removed and the cells were thoroughly washed by cold PBS to remove possible remaining curcumin. Then, 100 μL of fresh culture media was added to each well and the cells were put into the incubator for warm-up. Thereafter, 10 μL of MTT solution at 5 mg/mL was added to each well and further incubation of 4 h was preceded. After that, the absorbance of each plate was measured by a microplate reader. The cell viability was calculated by comparing the drug treated groups with the non-treated groups as controls.

2.12. In vivo study

2.12.1. Animal use

Female nude mice (6-8 week old, 20 g) were obtained from SLRC Laboratory animal center (Shanghai, China). All animal studies were conducted under animal project license in Capital University of Medical Sciences (Beijing, China) and all the animal study was performed in compliance with the laws and guidelines on animal use of Capital University of Medical Sciences (Beijing, China).

2.12.2. Animal tumor model

Mice were inoculated with 5×10^6 HCT-116 cancer cells suspended in PBS subcutaneously into the right flanks.

2.12.3. In vivo evaluation of antitumor efficacy of drugs

The tumor was established as described in the former section. Female nude mice (6–8 weeks old) were divided into 5 groups with 6 animals per group. When the tumors reached 40-50 mm^3, the treatment was started. The drug groups were set as follows: 1) curcumin, 20 mg/kg; 2) gemcitabine, 10 mg/kg; 3) curcumin (20 mg/kg)+ gemcitabine (10 mg/kg); 4)M(Cur/Gem) (20 mg curcumin/kg plus 10 mg gemcitabine/kg);5) PBS treated control group. Drugs (100 μL) and PBS (100 μL) were given via tail vein at day 0 and day 6.The day that the mice received the first injection of drugs was set as day 0. The tumor volume and body weight were monitored from day 0 for a period of ca. 24 days. The tumor volumes were calculated by using the following formula, $V = 0.5 \times a \times b^2$, where a and b are the length and width of tumor, respectively. The tumor volume and relative body weight data were shown as mean value ± standard deviation of 6 mice at each time points. The tumor weight was measured at day 24 by sacrificing three random mice in each group.

3. Results and discussions

3.1. Synthesis of mPEG-PLA/Gem conjugate

Polylactide is a biodegradable polymer approved by the Food and Drug Administration (FDA, USA) for a variety of clinical applications [25]. Poly(ethylene glycol) (PEG) is also FDA approved for drug and protein conjugation[26]. PEG is non-toxic and has a non-immunogenic nature which provides a great advantage for medical purposes [27]. Moreover, its high hydration capacity is favorable for regulation of the hydrophilicity of other compounds. mPEG-PLA which is an amphiphilic polymer with both hydrophilic PEG and hydrophobic polylactive block can be synthesized by ring-opening polymerization. mPEG-PLA itself can self-assemble in water to micelles with the hydrophilic PEG block as the shell and the hydrophobic PLA as the core [25]. Further transforming the end hydroxyl group (-OH) to carboxyl group (-COOH) was achieved by treating mPEG-PLA with succinic anhydride as previously described [22]. Then, conjugation gemcitabine with the carboxyl group end capped mPEG-PLA polymer results in the gemcitabine conjugates -PLA/Gem.

Figure 1a and Figure 1b showed the ^{1}HNMR spectra of gemcitabine and mPEG-PLA/Gem. It can be found that apart from the typical chemical shift of lactide proton (C**H** at 5.17ppm, C$\mathbf{H}_3$ at 1.57ppm), and protons of PEG(-C$\mathbf{H}_2$-C$\mathbf{H}_2$-O-) in MPEG-PLA-Gem conjugates, there are characteristic chemical shifts of gemicitabine(3.8 to 4.3ppm) (Figure 1b), denoting the successful synthesis of mPEG-PLA/Gem conjugates. The drug content in mPEG-PLA/Gem was 2.58 w/w% via UV/vis spectra at an absorbance wavelength of 269 nm. This means 85% of the end capped carboxyl groups in mPEG-PLA-COOH was conjugated with gemcitabine.

3.2. Preparation of curcumin and gemcitabine single drug loaded micelles

mPEG-PLA which is an amphiphilic polymer and can self-assemble into aqueous solution as nanoparticles itself with the hydrophilic PEG block as shell and hydrophobic PLA block as core. This amphiphilic nature provides the possibility of encapsulating the hydrophobic curcumin into the core and protecting it [28]. In a similar way, mPEG-PLA/Gem conjugate can also form nanoparticles itself. The as-prepared curcumin and gemcitabine loaded micelles were defined as M(Cur) and M(Gem) respectively. The size and size distribution of M(Cur) and M(Gem) were systematically characterized via DLS and TEM and the results were collected in Table 1. Both M(Cur) and M(Gem) had spherical structures as shown in TEM images(data not shown), suggesting the successful formation of nanoparticles. Moreover, the mean diameter for M(Cur) and M(Gem) were 77 nm and 61 nm by TEM respectively. It seems M(Cur) is slightly larger than M(Gem). DLS were used to monitor the mean diameter of M(Cur) and M(Gem) in aqueous solution to get a better insight of their behavior in solution as the nanoparticles could be further used in aqueous solution. As shown in Table 1, the mean diameter of M(Cur) and M(Gem) in aqueous solution were 93 nm and 78 nm. The diameter by DLS was larger than those detected by TEM for both M(Cur) and M(Gem), due to the fact that the nanoparticles are stretched in aqueous solution while they are condensed after drying during sample preparation for TEM. At last, M(Cur) and M(Gem) showed a zeta potential of -8.6 mV and -3.5 mV receptively (Table 1).

3.3. Preparation of curcumin and gemcitabine co-loaded micelles M(Cur/Gem) at different drug ratios

For drug combinations, different ratio of drugs may be used at a certain ratio, the drug efficacy may be maximized [29]. Therefore, we aimed to prepare a single nanoparticle platform with both curcumin and gemcitabine. As conjugation of gemctabine with the drug carrier mPEG-PLA will not change the amphiphilic nature, encapsulation of curcumin via this conjugate is possible. Thus mPEG-PLA/Gem was used to encapsulate curcumin. In this way, nanoparticles with both curcumin and gemcitabine could be obtained with ease. Four aimed ratios of curcumin/gemcitabine were set (curcumin/gemcitabine: 0.1:1, 0.5:1, 1:1 and 2:1). After mixing them and removing the organic solvent, the micelles with dual drug were prepared. Then curcumin content in the micelles was determined by UV at 420 nm. Compared to the feed ratio of curcumin/gemcitabine at 0.1:1, 0.5:1, 1:1 and 2:1, four kinds of micelles were obtained as shown in Table 1.

Representative TEM images of M($Cur_{0.89}$/Gem) were shown in Figure 2a and the DLS result was shown in Figure 2b. M($Cur_{0.89}$/Gem) had spherical morphology, suggesting the successful preparation of nanoparticles. Similarly, TEM and DLS were used to monitor the mean diameter of other micelles. The data were collected shown in Table 2. Results show that the mean diameter of M(Cur/Gem) increases when the ratio of curcumin/gemcitabine increases. This is possibly due to encapsulation of more hydrophobic curcumin to the core hence increase the size of the core. The zeta potential was also measured for the four micelles and values are listed in Table 2. M($Cur_{0.095}$/Gem), M($Cur_{0.46}$/Gem), M($Cur_{0.89}$/Gem) and M($Cur_{1.75}$/Gem) had a zeta potential of -2.1 mV, -1.6 mV, -3.4 mV and -4.9 mV respectively.

3.4. Study of the drug release

Curcumin and gemcitabine were formulated into one nanoparticle for potential drug delivery and sustained release. To gain some insight into this, the drug release behavior of curcumin and gemcitabine from the micelles could be studied respectively at pH5.0 and pH7.4 by HPLC as previously described [23,24]. Representative drug release data studied on M(Cur) and M(Gem) were shown in Figure 2c. We can found that curcumin was released much faster than gemcitabine both at pH5.0 and pH7.4. This is possibly because gemcitabine is chemically linked to the polymer chain, while curcumin is physically entrapped in the core of the micelles. For both drugs, they have displayed faster drug release in lower acidic pH value than in higher pH value (pH5.0>pH7.4). It is understandable that the polymer chain has PLA block which is acid degradable. Therefore, drug release at pH5.0 is much faster. To be more specific, at 48 h, curcumin were released 89 % and 44% at pH5.0 and pH7.4 respectively, while this was 58% and 24% for gemcitabine at pH5.0 and pH7.4 in the same period of time. The acid dependent drug release makes drug delivery to the cancer cells are meaningful as it is extensively reported that the pH value in the cancer cells are much lower hence promotes the release of drugs.

3.5. In vitro intracellular uptake of M(Cur/Gem) nanoparticles

Curcumin and gemcitabine are loaded into micelles for drug delivery to treat colon cancer. Here, M($Cur_{0.095}$/Gem) micelles were labeled with RhB and the uptake of this labeled micelle M($Cur_{0.095}$/Gem/RhB) by HCT-116 cancer cells was studied at 4 °C and 37 °C for 1 h. 4 °C treatment will inhibit the metabolism of the cancer cells, which will further inhibit the energy dependent process of the cancer cells, ie, endocytosis[30]. In this way, we can prove the nanoparticles are internalized via endocytosis. To give direct insight to this, the cells were treated with M($Cur_{0.095}$/Gem/RhB) at 4 °C and 37 °C at 1 h and then imaged via confocal laser scanning as shown in Figure 3a. It can be clearly found that there was much more red fluorescence in the cells at 37 °C than at 4 °C at 1 h, indicating internalization of more nanoparticles at 37 °C. This is because at 4 °C, the metabolism of the cells were inhibited and less ATP was produced, which would inhibit the energy dependent endocytosis of M($Cur_{0.095}$/Gem/RhB). To further quantify this process, HCT-116 cancer cells treated with M($Cur_{0.095}$/Gem/RhB) at 4 °C and 37 °C at 1 h and 4 h were monitored by flow cytometry. The cells treated with M($Cur_{0.095}$/Gem/RhB) at 37 °C for 4 h were set as controls (100%). The relative uptake efficiency of M($Cur_{0.095}$/Gem/RhB) at other conditions was listed in Figure 3b. One can readily found that the uptake of M($Cur_{0.095}$/Gem/RhB) demonstrated a time and temperature dependent manner. From 1 h to 4 h, uptake was enhanced by more than 2 fold both at 4 °C and 37 °C. However, 4 °C showed profound inhibition effect on the uptake of M($Cur_{0.095}$/Gem/RhB).This result further proved the above mentioned endycytosis of M($Cur_{0.095}$/Gem/RhB).

3.6. In vitro evaluation of drug pairs curcumin/M(Cur) and gemcitabine/M(Gem)

We then initiated the evaluation of two drug pairs curcumin/M(Cur) and gemcitabine/M(Gem) *in vitro* on HCT-116 cancer cells via MTT assay. The results were shown in Figure 4a and Figure 4b. M(Cur) was slightly better than curcumin as far as the toxicity was concerned. Gemcitabine and M(Gem) did not show any great different in inhibiting growth of HCT-116 cells.

3.7. In vitro evaluation of free drug combinations

As mentioned in the previous part, we obtained four M(Cur/Gem) nanoparticles with four different ratios of curcumin/gemcitabine. To compare the free drug based combinations and the micellar nanoparticles based combinations, we therefore first mix curcumin/gemicitabine at four ratios, ie, Cur/Gem=0.095:1,0.46:1,0.89:1 and 1.75:1 respectively. Then we tested the four combinations via MTT assay and the results were shown in Figure 4c. Shown in Figure 4c, the X-axis is the concentration of gemcitabine in curcumin/gemicitabine combinations. As the combination ratio increases from 0.095:1 to 1.75:1 which suggests the amount of curcumin relative to gemcitabine is increased, the dose dependent curve shifted down, denoting greater enhancement of drug efficacy. For example, at a gemcitabine concentration of 12.5 μM, the cell viability of Cur/Gem=0.095:1,0.46:1,0.89:1 and 1.75:1 were 59%, 40%, 35% and 23% respectively, which clearly shows adding more curcumin leads to better efficacy. The results here imply possibility of further combining the two drugs in micellar nanoparticles.

3.8. In vitro evaluation of nanoparticle based combination of M(Cur/Gem)

Then, for comparison of free combinations, M(Cur/Gem) at four ratios were tested on HCT-116 cancer cells at 72 h as shown in Figure 4d. Similarly, the dose dependent curve of M(Cur/Gem) also shifted down when the ratio of Cur/Gem increases, which also suggesting that by adding more curcumin to the nanoparticles, the efficacy of M(Cur/Gem) becomes better. To make it clearer, at a gemcitabine concentration of 12.5 μM, the cell viability of M($Cur_{0.095}$/Gem), M($Cur_{0.46}$/Gem), M($Cur_{0.89}$/Gem) and M($Cur_{1.75}$/Gem) were 65%, 34%, 23% and 12.5% respectively, which showed greater drug efficacy enhancement by increasing curcumin ratios. The result here gives light to combination of curcumin and gemcitabine in nanoparticles as possible benefit appears.

3.9. IC_{50} values of drug combination and combination index calculations

To give a quantitative insight into drug combinations, the IC_{50} values of free combinations and micelle based combinations on HCT-116 cells at 72 h were listed in Figure 5a and Figure 5b. The IC_{50} values of $Cur_{0.095}$/Gem, $Cur_{0.46}$/Gem, $Cur_{0.89}$/Gem and $Cur_{1.75}$/Gem decreased from 16.9 μM, 9.7 μM, and 5.1 μM to 3.3 μM. Similarly, the IC_{50} values of M($Cur_{0.095}$/Gem), M($Cur_{0.46}$/Gem), M($Cur_{0.89}$/Gem) and M($Cur_{1.75}$/Gem) also decreased from 21.3 μM,7.5 μM,4.1 μM to 1.6 μM. It is clearly shown that adding curcumin to gemcitabine can enhance the drug efficacy in both free combinations and micelle based combinations. It should be noted that the IC_{50} values of M(Cur/Gem) are lower than those of free combinations with an exception when the ratio was 0.095:1.

Then, the drug interactions of curcumin and gemcitabine were evaluated by combination index as previously described [31,32]. The combination index, short for CI, is larger than 1, equal to 1 or lower than 1 means antagonism, additivity and synergy respectively. At a certain drug effect, for example, a certain drug inhibition rate, the CI values were different. Here, to simplify the study, we only show the CI values at 50% cell inhibition rate (IC_{50}). Results were shown in Figure 5c and Figure 5d. The CI values of both free combinations and micelle based combinations of curcumin/gemcitabine were almost lower than 1 and decreased from 0.9 to 0.34 and 1.04 to 0.18 respectively when the ratio of curcumin/gemcitabine increases, denoting the synergy effect of combining curcumin and gemcitabine in treating HCT-116 cancer cells. It should be also pointed out that the CI values of M(Cur/Gem) were almost lower than those of free combinations of curcumin/gemcitabine, which means greater synergy when drugs were combined in a micellar platform.

To further elucidate the benefit of combination the gemcitabine and curcumin in one nanoparticle, we also tested the IC_{50} values and calculated the CI index for just combination of two separate nanoparticles M(Cur) and M(Gem) as shown in Figure 5e and Figure 5f. M(Cur/Gem) is different from M(Cur)+M(Gem) as in the former formulation, the two drugs were co-assembled in the same nanoparticles, however, in the later, the two drugs were in separate nanoparticles. The IC_{50} values of

M(Cur)+M(Gem) at a Cur/Gem molar ratio of 0.095:1, 0.46:1 and 1.75:1 are 25.7, 9.1, 6.2 and 2.7 μM.Compared to those were found on M(Cur/Gem), IC_{50} are higher with increased CI values , indicating M(Cur/Gem) combinations can surpass the M(Cur)+M(Gem).This demonstrated definitely the benefit of co-assembly the two drugs in one nanoparticles. As M(Cur/Gem) are better than M(Cur)+M(Gem), the following *in vivo* study will not include M(Cur)+M(Gem).

In vivo antitumor efficacy of M(Cur/Gem)

To further prove our micelle based combination of curcumin and gemcitabine can bright clinic benefit, here first M(Cur/Gem) were tested on mice bearing HCT-116 xenograft tumors. When the tumor nodule grew to 40~50 mm^3, mice were treated with curcumin (20 mg/kg), gemcitabine (10 mg/kg), free combination of curcumin/gemcitabine(curcumin, 20 mg/kg plus gemcitabine, 10 mg/kg) and M(Cur/Gem) (curcumin, 20 mg/kg plus gemcitabine,10 mg/kg) via tail vein at day 0 and day 6.The day that the mice received the first injection of drugs was set as day 0. Mice treated with PBS were used as a control. The tumor volume change and relative body weight change were monitored for a period of ca. 24 days shown in Figure 6a and Figure 6b. Curcumin (20 mg/kg) had some effect on tumor inhibition as compared to the PBS control group. However, the tumor growth resembles the PBS treated mice though the rate is lower. Gemcitabine (10 mg/kg) had shown better tumor inhibition effect than curcumin (20 mg/kg), as the tumor increased much slower. As shown in Figure 6a, mice treated with a combination of curcumin and gemcitabine at a dose of 20 mg curcumin/kg and 10 mg gemcitabine/kg showed increased antitumor efficacy compared to mice treated with curcumin and gemcitabine alone. At last, it can be clearly seen that M(Cur/Gem) had the best tumor inhibition effect. The tumor growth almost halted over this long period of 24 days. The result here indicated possible clinic benefit of M(Cur/Gem) over the free combination of curcumin and gemcitabine for better drug efficacy.

To further gain better insight into M(Cur/Gem), the systemic toxicity as judged by the relative body weight change was evaluated as shown in Figure 6b.

The relative body weight was increasing over the time period of 24 days for PBS treated mice following by the group treated by curcumin (20 mg/kg). Mice treated with gemcitabine (10 mg/kg) lose their body weight from day 2 to day 12, and gained back the body weight gradually from day 12 on. As the drug injection was performed on day 0 and day 6, this suggests that gemcitabine exerted great toxicity on mice after injection. At last, free combination of curcumin/gemcitabine (curcumin, 20 mg/kg plus gemcitabine, 10 mg/kg) led to the gradual decreasing of the body weight over the observed time. However, mice treated with M(Cur/Gem) (curcumin, 20 mg/kg plus gemcitabine,10 mg/kg) almost did not lose body weight in first 4 days. After that, the mice gradually increased their body weight, which suggests minimum toxicity of M(Cur/Gem).

To take a closer look at the tumor inhibition efficacy of the drugs, at day 24, there radom mice in each group were scarified and the tumor weight were measured as shown in Figure 6c. The tumor mass for M(Cur/Gem) was only 0.135 g, which accounted for 29.3% of the tumor for free drug combination group and 11.1% of the tumor for PBS treated group. Curcumin and gemcitabine had limited antitumor effect, as the tumor weights were 0.865 and 0.754 g respectively, which accounted for 71.2% and 62.1% of the tumor for PBS treated group. The results here cleared showed the benefit of combining curcumin and gemcitabine in the micellar nanoparticle formulation, which could in the near future lead to clinic translation.

4. Conclusion

We have shown here using a biodegradable polymer gemcitabine conjugate for encapsulating curcumin to prepare nanoparticles with both drugs for combinational therapy. The as-prepared nanoparticles showed spherical structures with mean diameter ranging from 118 nm to 149 nm by DLS. *In vitro*, M(Cur/Gem) almost showed greater synergy than free combination of curcumin/gemcitabine. *In vivo*, better antitumor effect and lower systemic toxicity of M(Cur/Gem) were observed. The present study provides the possibility of combining curcumin and gemcitabine in nanoparticle formulation, and translation of this combination may bring benefit in the further clinic use.

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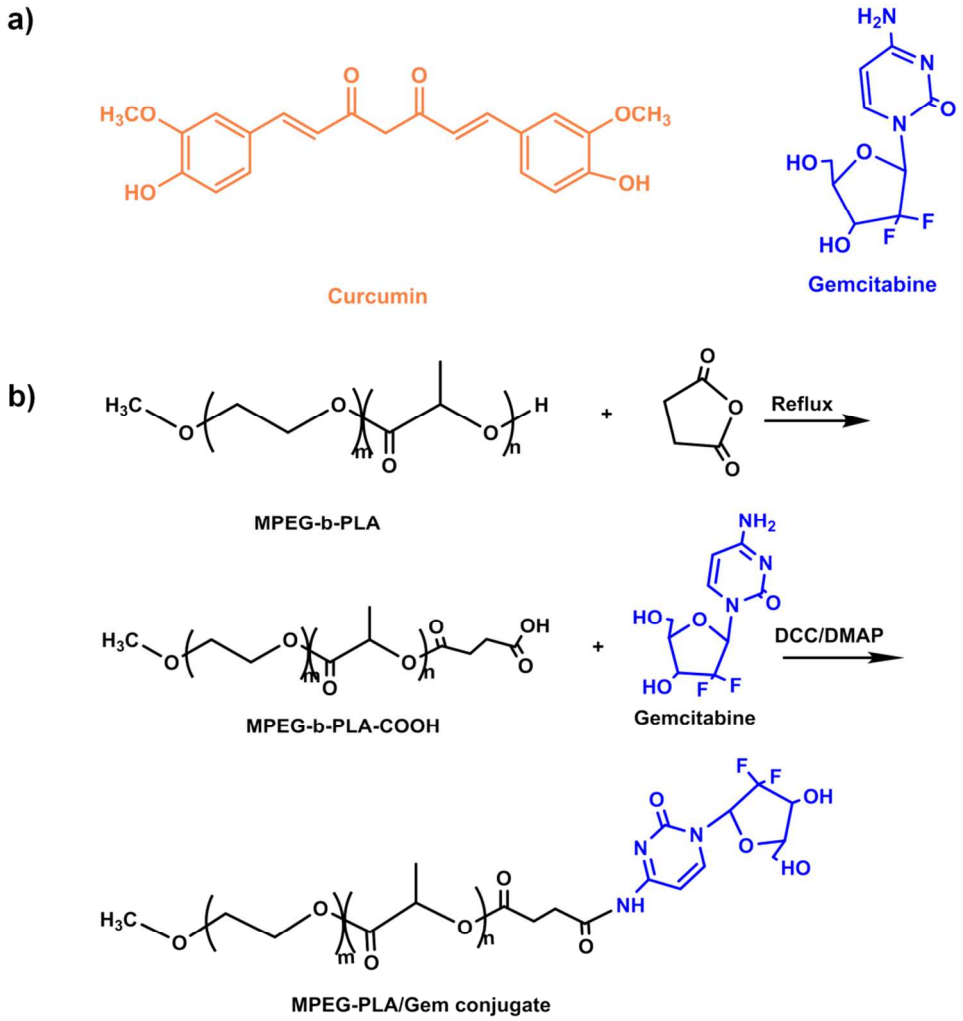

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568 **Scheme 1.** Synthesis of MPEG-b-PLA/Gem conjugates and preparation of
569 nanoparticles with both gemcitabine and curcumin using the MPEG-b-PLA/Gem
570 conjugates. (a) Chemical structure of curcumin and gemcitabine, (b) illustration of
571 synthetic route for MPEG-b-PLA/Gem conjugates and (c) encapsulation of curcumin
572 with MPEG-b-PLA/Gem conjugates for preparation of the M(Gem/Cur).

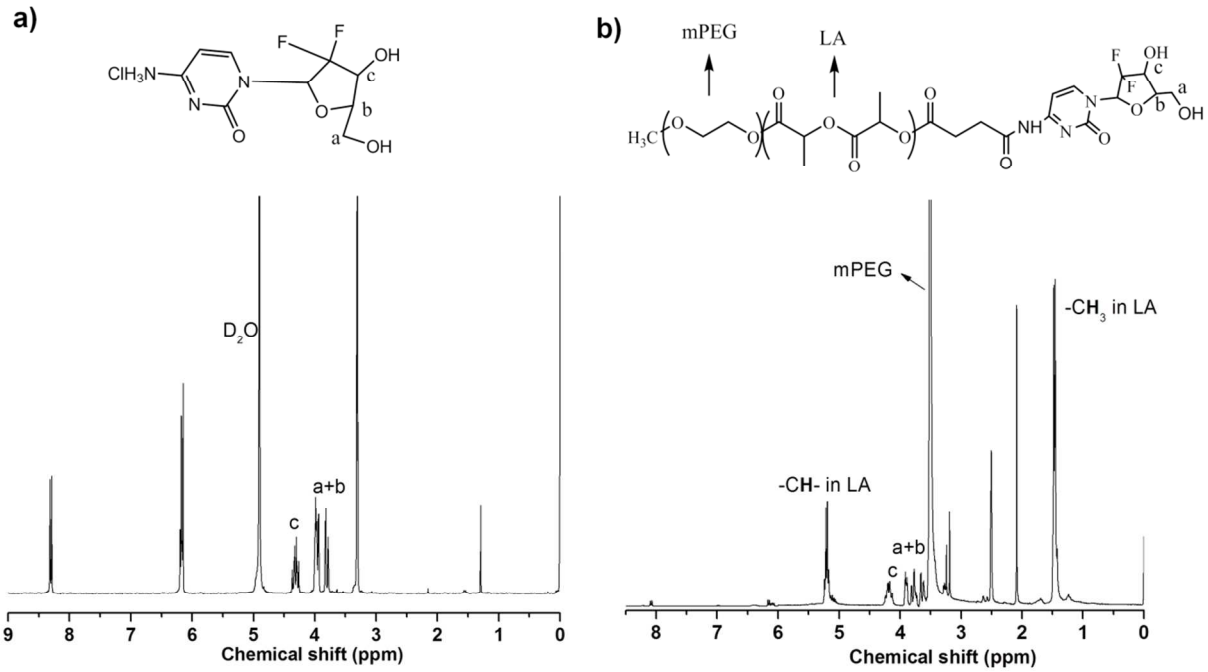

Figure 1. ^{1}HNMR of gemcitabine (a) and MPEG-b-PLA/Gem(b) in D_2O(a) and $CDCl_3$(b) respectively at room temperature.

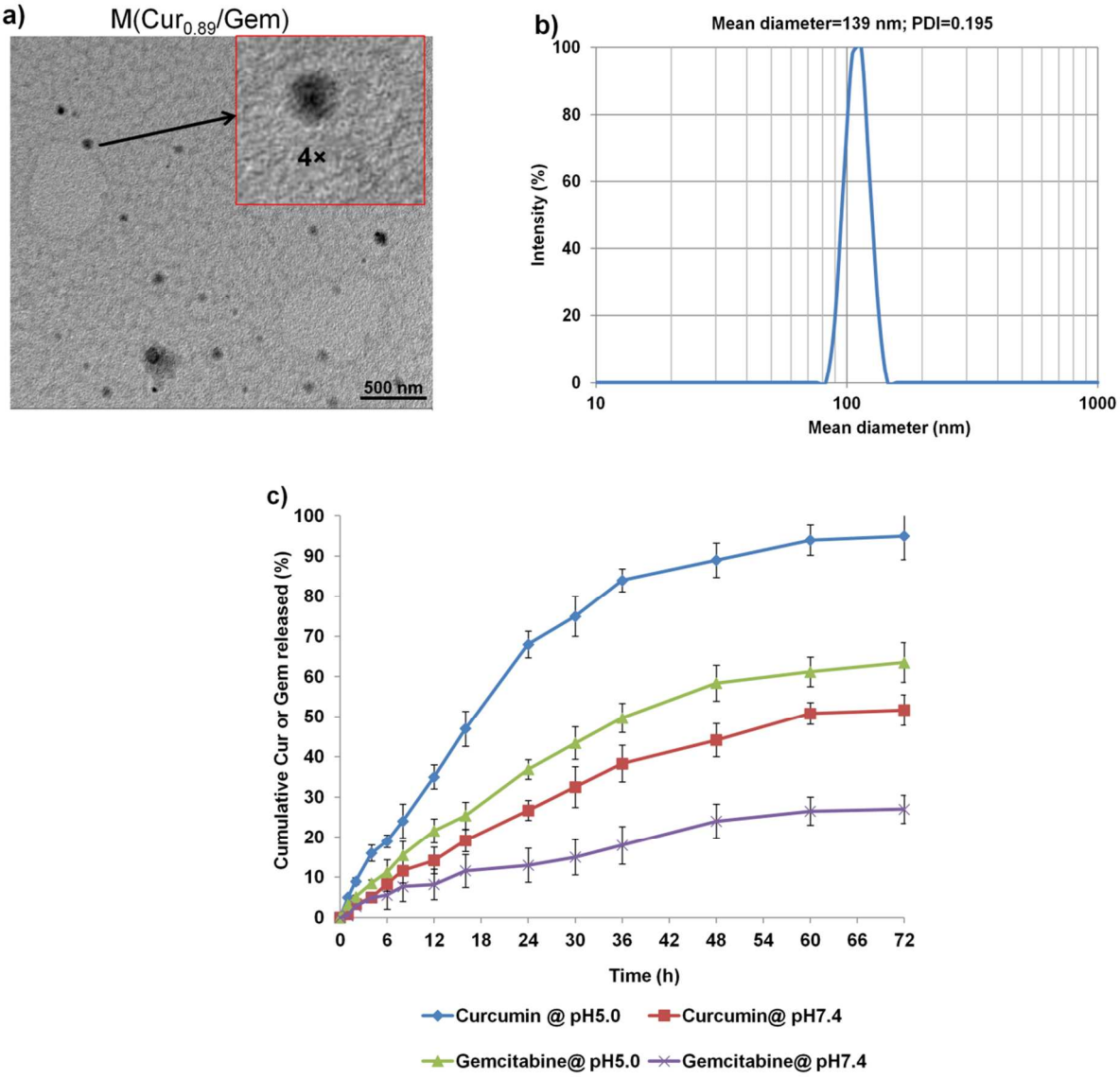

Figure 2. Characterization of the nanoparticles and drug release. Representative characterization of the M($Cur_{0.89}$/Gem) micelles by TEM. The insets show the extended views by 4 times (a) and DLS(b) were shown and drug release of curcumin and gemcitabine from the two drug loaded nanoparticles were studied at pH5.0 and pH7.4(c).Data were shown as mean values ±S.D.

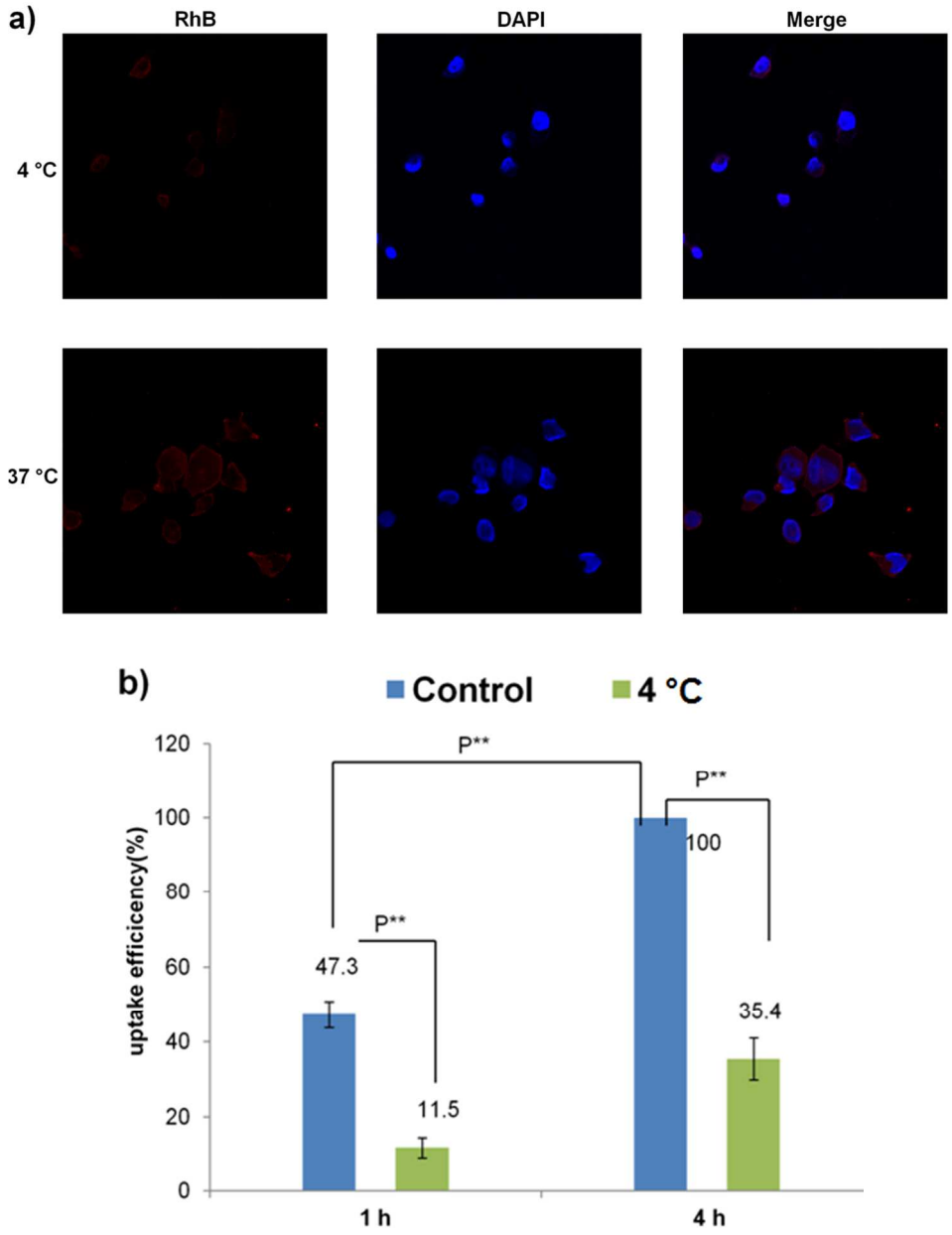

Figure 3. M(Cur/Gem) nanoparticles can be effectively internalized by HCT-116 cancer cells via endocytosis.The micelles M($Cur_{0.89}$/Gem) were labeled with a hydrophobic fluorescent molecule Rhodamine B(RhB).(a) uptake of M($Cur_{0.89}$/Gem/RhB) was studied by confocal laser scanning at 1 h at 4˚C and 37˚C; (b) the relative uptake efficiency of M($Cur_{0.89}$/Gem/RhB) at 4 ˚C and 37 ˚C for 1 h and 4 h. Uptake of drugs by cells treated with M($Cur_{0.89}$/Gem/RhB) at 37 ˚C in the incubator for 4 h were set as 100%. Data were shown as mean values ±S.D.(P*<0.05 and P** < 0.01).

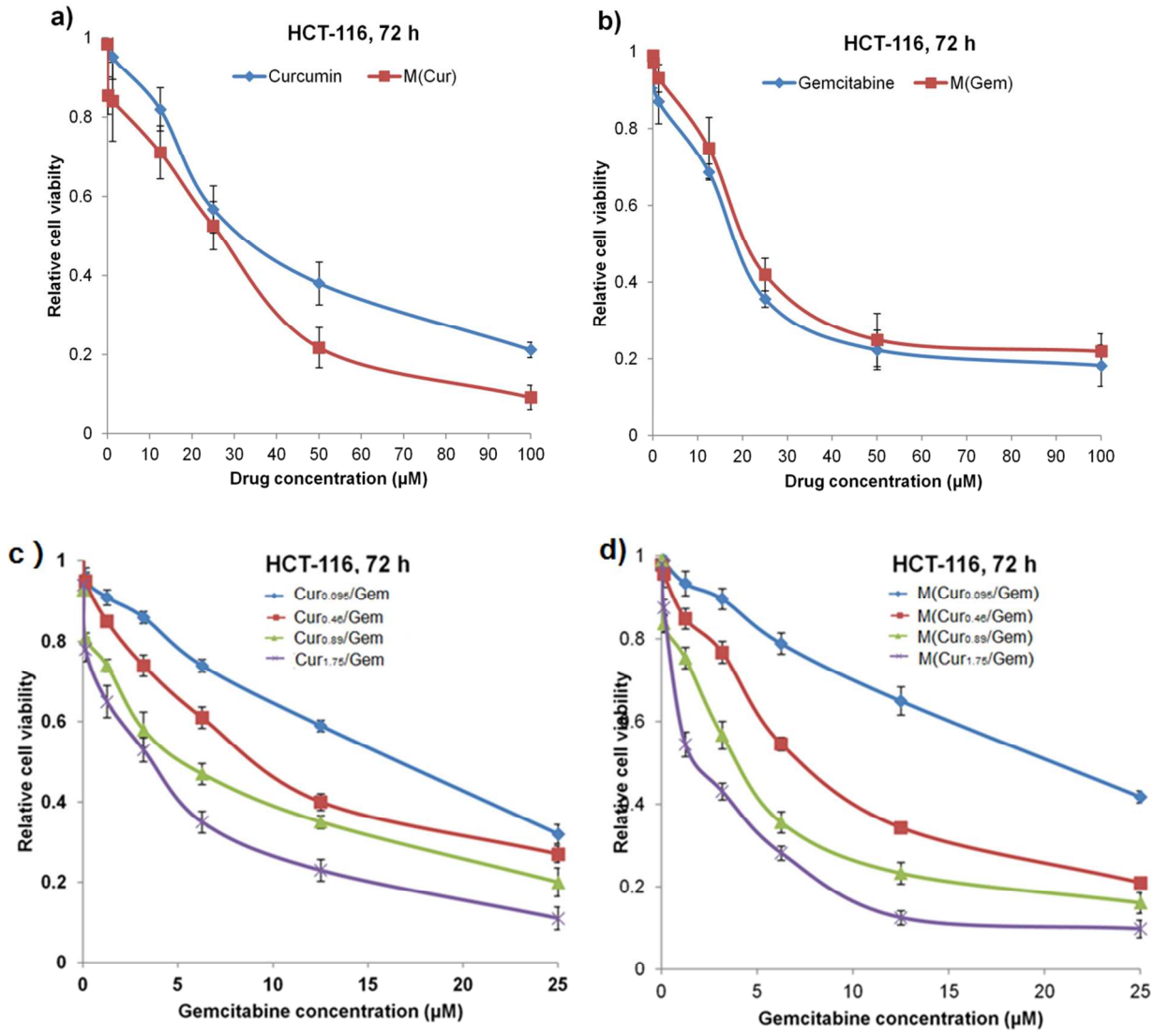

Figure 4. *In vitro* evaluation of various drugs. MTT assay of single used drug pairs of curcumin and M(Cur)(a), and gemcitabine and M(Gem)(b) at 72 h as well as *in vitro* evaluation of free curcumin and gemcitabine combination (c) and micellar nanoparticles based combination M(Cur/Gem)(d) at various drug ratios against HCT-116 cancer cells at 72 h. Data were shown as mean values ±S.D.

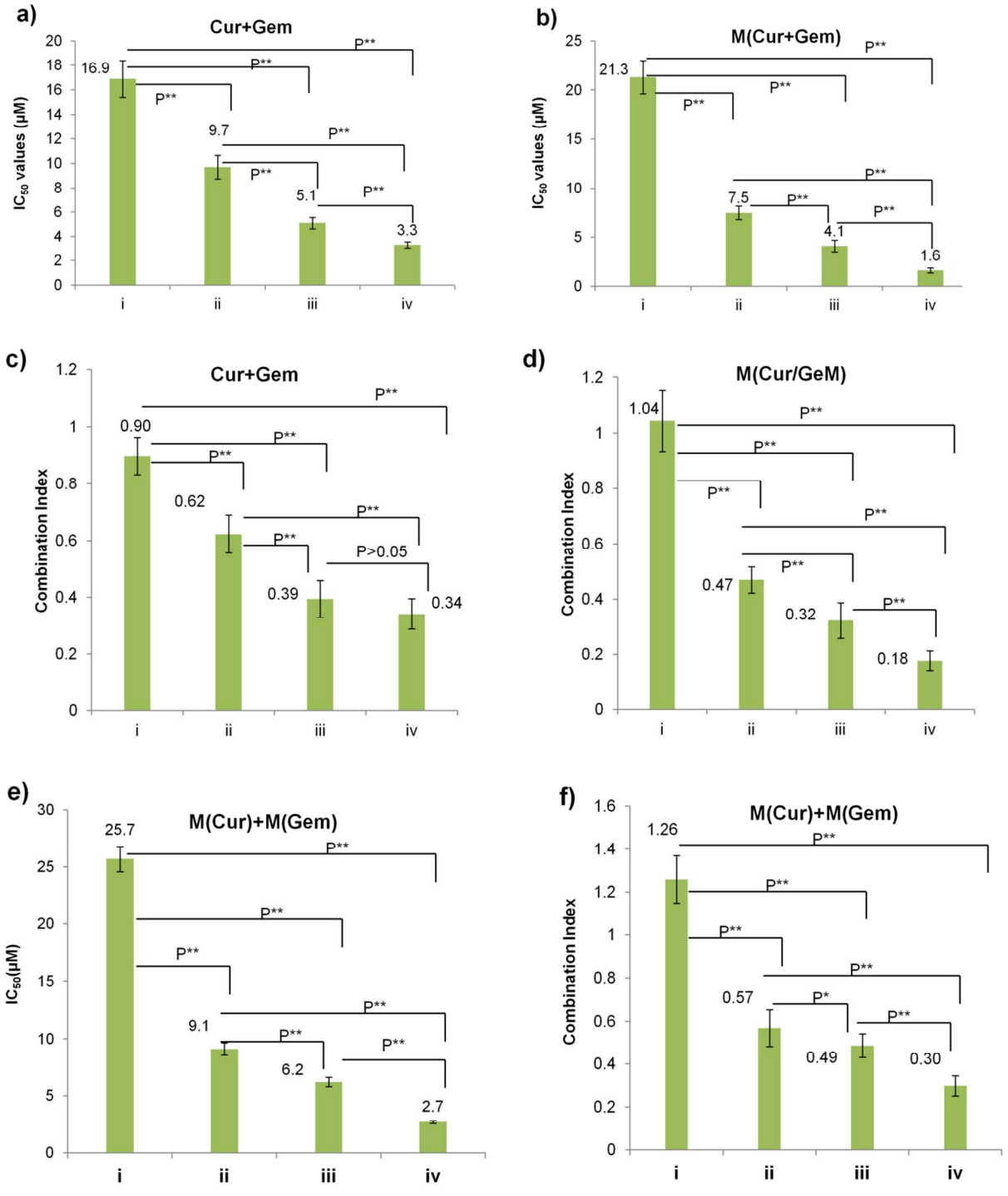

Figure 5. IC_{50} values of various drugs and analysis of the drug interactions via combination index calculations. IC_{50} values of free curcumin and gemcitabine combinations: i) $Cur_{0.095}$/Gem, ii) $Cur_{0.46}$/Gem, iii)$Cur_{0.89}$/Gem and iv) $Cur_{1.75}$/Gem (a) and micellar naoparticle based combinations: i) M($Cur_{0.095}$/Gem), ii)M($Cur_{0.46}$/Gem), iii)M($Cur_{0.89}$/Gem) and iv) M($Cur_{1.75}$/Gem) (b) at 72 h on HCT-116 cancer cells. Based on the collected IC_{50} values of the drugs, analysis of the drug interactions via

combination index of free combination of curcumin and gemcitabine: i) $Cur_{0.095}$/Gem, ii) $Cur_{0.46}$/Gem, iii)$Cur_{0.89}$/Gem and iv) $Cur_{1.75}$/Gem (c) as well as the micellar nanoparticles based combinations: i) M($Cur_{0.095}$/Gem), ii)M($Cur_{0.46}$/Gem), iii)M($Cur_{0.89}$/Gem) and iv) M($Cur_{1.75}$/Gem) (d). For comparison, drug combination in one nanoparticle platform in the form of M(Cur/Gem) was compared with the combination with the two micelles in which the drugs were not in the same micelle. The result of the IC50 values (e) and the combination index (f) of M(Cur)+M(Gem) at a molar ratio 0.095:1, 0.46:1, 0.89:1 and 1.75:1(i-iv) were shown. Data were shown as mean values ±S.D. ($P^*<0.05$ and $P^{**} < 0.01$).

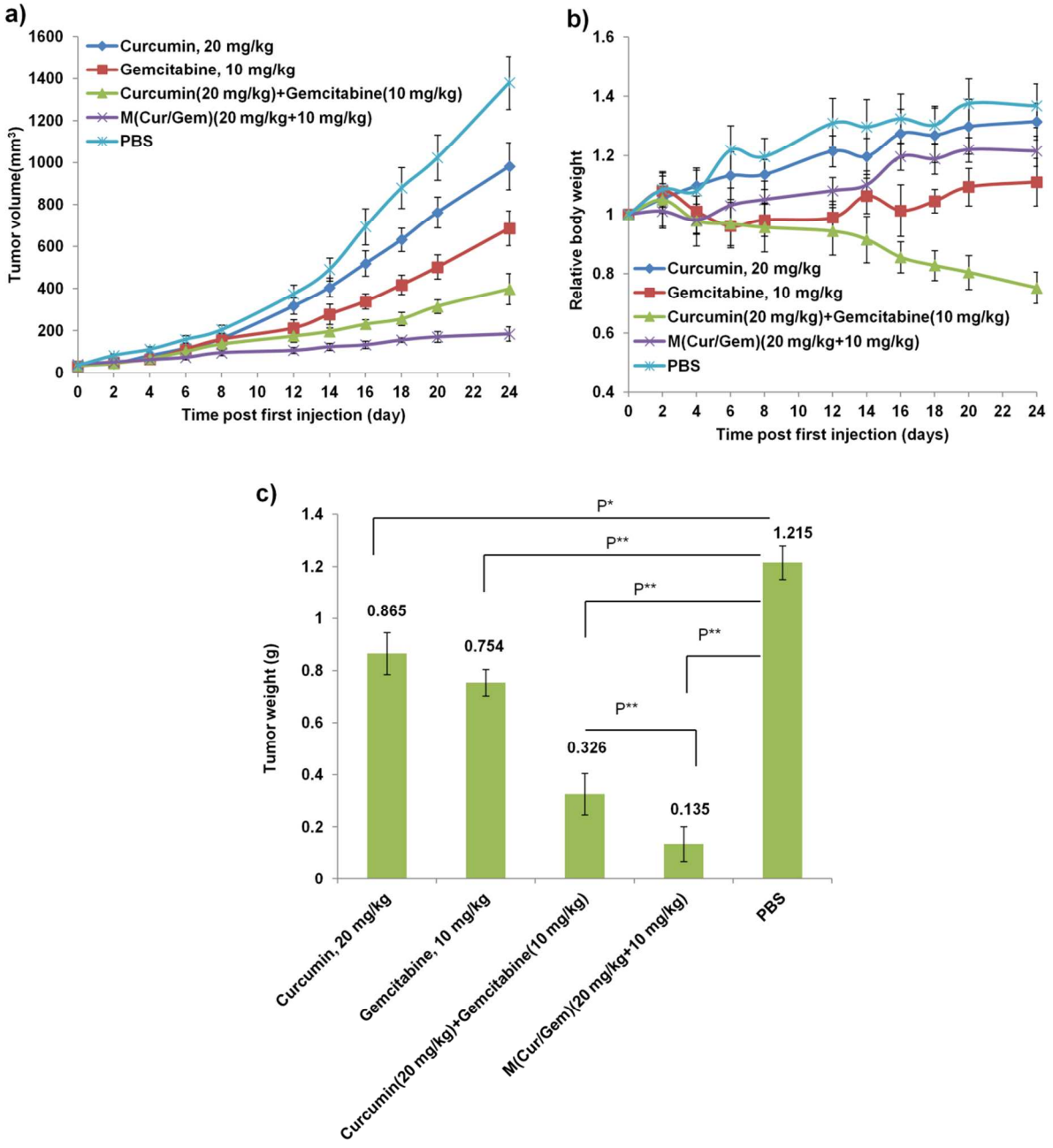

Figure 6. *In vivo* animal study of M(Cur/Gem) against mice bearing HCT-116 xenograft tumors. The tumor volume change (a) and relative body weight change (b) were monitored. The drug doses were listed in the insets. Drugs (100 μL) and PBS (100 μL) were given via tail vein at day 0 and day 6.The day that the mice received the first injection of drugs was set as day 0. The tumor volume and body weight were monitored from day 0 for a period of ca. 24 days. The tumor volume were calculated by using the following formula, $V = 0.5 \times a \times b^2$, where a and b are the length and width of tumor, respectively. The tumor volume and relative body weight data were shown as mean value ± standard deviation of 6 mice at each time points. The tumor weight was

657 measured at day 24 by sacrificing three random mice in each group as shown in (c).
658 Data were shown as mean values ±S.D. (P*<0.05 and P** < 0.01).

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660 **Table 1.** Physical characterization of M(Gem), M(Gur) ,M(Cur/Gem) micelles by DLS, zeta potential (ZP) .

Micelles	DLS(nm)	TEM(nm)	PDI	ZP(mV)
M(Gem)	78	61	0.157	-3.5
M(Cur)	93	77	0.178	-8.6
M($Cur_{0.095}$/Gem)	118	97	0.134	-2.1
M($Cur_{0.46}$/Gem)	127	105	0.211	-1.6
M($Cur_{0.89}$/Gem)	139	114	0.195	-3.4
M($Cur_{1.75}$/Gem)	149	125	0.132	-4.9

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