

RETRACTED ARTICLE: The therapeutic effect of methotrexate- conjugated Pluronic-based polymeric micelles on the folate receptor-rich tumors treatment

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Abstract: The therapeutic effect of methotrexate (MTX)-conjugated Pluronic-based polymeric mixed micelles (F127/P105-MTX) on the folate receptor-overexpressing tumors treatment was investigated in this study. Due to its high structural similarity to folic acid and the high expression of folate receptor in most solid tumors, MTX serves not only a cytotoxic agent but also a homing ligand. Cellular uptake and the endocytic mechanism studies of MTX-conjugated mixed micelles were performed in folate receptor-rich KBv and folate receptor-deficient A-549 cancer cells. Additionally, the efficacy and safety studies of F127/P105-MTX in KBv tumor-bearing mice were evaluated. Results indicate that F127/P105-MTX significantly enhanced the cellular uptake in KBv cells as compared to that of conventional non-MTX-conjugated mixed micelles. Moreover, the results showed that F127/P105-MTX can be internalized by both caveolae- and clathrin-mediated endocytosis in energy-dependent and folate receptor-dependent manner. The *in vitro* and *in vivo* antitumor efficacies of F127/P105-MTX were significantly enhanced in comparison with MTX-entrapped mixed micelles. Furthermore, no acute toxicity to hematology system and major organs have been observed after intravenous administration during the regimen. Therefore, our results suggest that F127/P105-MTX could be an effective and safe nano-drug delivery system for cancer therapy, especially for the folate receptor-rich cancer treatment.

Keywords: methotrexate-conjugated, mixed micelles, folate receptor, Pluronic, KBv cells

Introduction

Recently, research interest has been raised in the application of block copolymer micelles as the nanocarrier system in the field of drug delivery because of the hydrophobic drug-loading capacity of the inner core as well as the unique *in vivo* disposition characteristics.¹ Polymeric micelles have small particle size (<100 nm) and unique core-shell architecture, where hydrophobic segments are segregated from the aqueous exterior to form an inner core surrounded by a palisade of hydrophilic segments. The narrow size range is similar to those of viruses and lipoproteins and natural mesoscopic-scaled vehicle systems, and has been considered as a crucial factor in determining their *in vivo* disposition, especially when enhanced permeation and retention (EPR) effect is involved.² In our previous studies, polymeric micelles prepared with Pluronic F127 and P105 have been demonstrated to enhance anticancer efficacy of docetaxel and methotrexate (MTX) against multidrug-resistant (MDR) cancer cells *in vitro* and *in vivo*.³⁻⁵ Pluronic P105 is presented as PEO₃₇-PPO₅₆-PEO₃₇ triblock copolymer with high MDR tumor-sensitizing ability, and Pluronic F127 is presented as PEO₁₀₀-PPO₆₅-PEO₁₀₀ triblock copolymer with long poly(ethylene oxide) (PEO) hydrophilic

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chain which can improve the physical stability and increase the blood circulation time in vivo of the polymeric micelles. Compared to single Pluronic P105 micelles, the micelles comprising Pluronic P105 and F127 can be more stable, due to the lowered critical micelle concentration conferred by the high PEO–poly(propylene oxide) ratio of F127, which will contribute to the long retention of drug-loaded micelles in blood, and eventually achieve higher drug accumulation at the target site (tumor) through the EPR effect.^{6–8} It is noteworthy that doxorubicin-loaded mixed micelles composed of Pluronic L61 and F127 (SP1049C) have already reached Phase III stage.⁹

However, a major obstacle to the conventional drug delivery systems has been found to be the nonspecific uptake by reticuloendothelial system (RES). Thus, the ability to avoid recognition by RES is ideal for advanced drug delivery systems to achieve longevity in the blood circulation.¹⁰ To this end, research efforts are being placed on the development of nanomicelles to date.¹¹ Functionalization of the outer space of the polymeric nanomicelles to modify its physicochemical or biological properties is of great value from the perspective of design and development of intelligent nano-drug delivery systems, which can be accomplished by constructing micelles with a variety of end-functionalized copolymers. To date, strategic modification of polymeric micelles with functional peptides, saccharides, antibodies, and aptamers on their periphery has been reported,^{12,13} with attempt to enhance tumor accumulation, penetration, and retention. The folate receptor, which is expressed normally at the luminal surface of polarized epithelium,^{14,15} has been found to be overexpressed in some solid tumors such as ovarian, endometrial, colorectal, breast, lung, and anal cell carcinoma as well as brain metastases derived from epithelial cancer, and neuroendocrine carcinoma.^{16–19} Because of this distinctive feature between normal and cancer cells, folic acid (FA) has emerged as an effective targeting ligand for selective delivery.²⁰ Interestingly, the classic antimetabolite drug MTX has a similar chemical structure to FA, and ceases the intracellular metabolism of FA, leading to the impairment of tumor growth and induction of cell death. Similar to FA, MTX also displays a hydrophilic property due to its two carboxylic acid groups that are ionized at physiological pH ($pK_a = 3.8, 4.8$). Thus, MTX is unable to cross the cell membrane passively unless being uptaken in an active mechanism which is mediated by cell surface proteins like folate receptor and reduced folate carrier.²¹ Thus, MTX may be used as not only an anticancer drug but also a potential targeting ligand.^{22–24} Therefore, in this manuscript, based

on the findings from previous studies,^{3,4} we have further elaborated on the mechanism of the affinity between F127/P105-MTX and folate receptor. Besides, both in vitro and in vivo safety evaluation and therapeutic efficacy have been systemically performed. To achieve this goal, the cellular uptake and internalization mechanism of F127/P105-MTX in KBv cells, and the distribution and accumulation of F127/P105-MTX in KBv tumor spheroids were evaluated. The in vivo biodistribution and anticancer efficacy of F127/P105-MTX were also investigated in subcutaneous xenograft mice model. Additionally, in vivo toxicity was assessed in terms of changes in the body weight, hematology parameters, as well as common serum biochemical levels after the treatment with different MTX formulations.

Materials and Methods

Materials and animals

MTX was purchased from Zibo Puxin Pharm & Chemical Co. Ltd. (Zibo, People's Republic of China). Samples of Pluronic P105 and F127 were kindly supplied by BASF Ltd. (Shanghai, People's Republic of China). Pluronic P105-MTX was synthesized as described before.⁴ Briefly, amine-terminated Pluronic P105 (P105-NH₂), MTX, *N*-hydroxy succinimide, and *N,N'*-dicyclohexylcarbodiimide were dissolved in dimethylsulfoxide in the presence of triethylamine. The mixture was stirred in a nitrogen atmosphere at room temperature overnight. Then, the mixture was diluted with deionized water and centrifuged. The supernatant was further purified by dialysis against deionized water, followed by lyophilization. F127-MTX, P105-FA, F127-FA, and P105-fluorescein isothiocyanate (FITC) were synthesized as described previously.^{25,26} Propidium iodide (PI), chlorpromazine hydrochloride (CPZ), sucrose, filipin, genistein, cytochalasin D, 5-(*N,N*-dimethyl) amiloride hydrochloride (DMA), NaN₃, monensin, and FA were purchased from Sigma (St Louis, MO, USA). Low-melting-point agarose was obtained from Yixin Biotechnology Co., Ltd. (Shanghai, People's Republic of China). Bicinchoninic acid protein assay kit and Triton X-100 were purchased from Beyotime Biotechnology Co., Ltd. (Nantong, People's Republic of China). Dioctadecyl-3,3',3'-tetramethylindotricarbocyanine iodide (DIR) was purchased from Biotium (Life Technologies, Carlsbad, CA, USA). Penicillin–streptomycin, Roswell Park Memorial Institute (RPMI)-1640, fetal bovine serum (FBS), and 0.25% (w/v) trypsin–0.03% (w/v) ethylenediaminetetraacetic acid (EDTA) solution were purchased from Gibco BRL (Gaithersburg, MD, USA). Purified deionized water was prepared by Milli-Q plus system (Millipore Co.,

Billerica, MA, USA). All other solvents were analytical- or chromatographic grade.

The human MDR KBv carcinoma cell line was purchased from Nanjing KeyGen Biotech. Co. Ltd. (Nanjing, People's Republic of China). The human lung adenocarcinoma cell line A-549 was obtained from Cell Resource Center of the China Science Academy (Shanghai, People's Republic of China). Culture plates and dishes were purchased from Corning Inc. (New York, NY, USA). The cells were cultured in RPMI-1640 medium, supplemented with 10% FBS, 100 IU/mL of penicillin, and 100 µg/mL of streptomycin sulfate. The cells were cultured in the incubator maintained at 37°C with 5% CO₂ under fully humidified conditions.

Male BALB/c nude mice (20±2 g), supplied by Department of Experimental Animals, Fudan University (Shanghai, People's Republic of China), were acclimated at 25°C and 55% of humidity under natural light/dark conditions for 1 week before the experiments. All the animal experiments were carried out in accordance with guidelines evaluated and approved by the ethics committee of Fudan University.

Preparation of MTX-loaded Pluronic-based polymeric mixed micelles

F127/P105-MTX was prepared by thin-film hydration method according to the procedure described previously. Briefly, a thin polymeric film was formed in a round-bottom flask by removing the organic solvents from 300 mg of mixed solution composed of P105-MTX (86.7 mg), P105 (180 mg), and F127 (33.3 mg) dissolved in 5 mL of methanol by rotary evaporation. This film was further dried under vacuum overnight at room temperature to remove any traces of remaining solvents. Then, the dry polymeric film was hydrated with 5 mL of deionized water. The mixture was stirred at 750 rpm for 45 minutes to obtain a transparent yellowish micellar solution.

FA-conjugated mixed micelles (F127/P105-FA) was prepared in the same way as F127/P105-MTX except that P105-FA was used instead of P105-MTX.

For preparation of physically entrapped MTX mixed micelles (F127/P105-MTX), the mixture (300 mg) of P105 and F127 (8:1, w/w), 50 µL of triethylamine, and MTX (10 mg) were used to prepare the thin polymeric film as described earlier. The resultant physical-entrapped micelles were filtrated through 0.22 µm filter membrane to remove the unentrapped MTX before further use. Blank Pluronic mixed micelles (F127/P105) were prepared by the same method shown earlier except that P105-MTX was replaced by P105.

The preparation of DIR-labeled mixed micelles (F127/P105/DIR and F127/P105-MTX/DIR) was performed in a similar way, except that 80 µL of DIR (1 mg/mL of stock solution in dichloromethane) was added into the mixture of methanol and Pluronic before removing the organic solvents. After preparation, the free DIR was removed via CL-4B column chromatography (Hanhong Chemica Co. Ltd., Shanghai, People's Republic of China).

MTX or FA modification was performed by conjugating MTX or FA to the functionalized end of long PEO chain of Pluronic F127, and the corresponding mixed micelles (MTX-F127/P105, FA-F127/P105) were prepared using the same method as that of F127/P105, except that Pluronic F127 was substituted by adding a mixture of 20 wt% F127-MTX or F127-FA and 80 wt% F127.²⁶

FITC was used as the fluorescent probe for in vitro studies. FITC-labeled Pluronic micelles were prepared in the same way described above except that 6.7 mg of P105-FITC was used to replace the same amount of P105.

Particle size distribution, and zeta potential of the polymeric micelles were determined by dynamic light scattering using a zeta plus analyzer (Zetasizer Nano ZS, Malvern Instruments Ltd, Malvern, UK). The MTX-loading efficiency was measured by high-performance liquid chromatography as previously described.⁴ F127/P105-MTX and F127/P105-MTX were lyophilized and stored at 4°C for 6 months. The drug content and particle size were also monitored during the stability period.

Cellular uptake of FITC-labeled Pluronic-based polymeric mixed micelles

KBv and A-549 cells were seeded at a density of 1×10⁴ cells per well in 24-well plates and allowed to grow until 80% confluence reached. The old culture medium was replaced by 250 µL of FITC-labeled mixed micelles in fresh medium at a concentration of 100 µg/mL. After 1-hour incubation, the medium was removed, and the cells were washed three times with cold pH 7.4 phosphate-buffered saline (PBS) and then visualized under fluorescent microscope (Leica DMI 4000B, Leica Microsystems, Solms, Germany). To qualitatively evaluate whether FA could hinder the receptor-mediated endocytosis, cells were preincubated with FA (1 mM) for 2 hours, and then washed three times with cold pH 7.4 PBS before the exposure to FITC-labeled mixed micelles.

For quantitative analysis of the cellular uptake of different polymeric mixed micelles, KBv and A-549 cells were seeded at a density of 1×10⁵ cells per well in six-well plates and allowed to grow until reaching 80% confluence. Then, cells

were incubated with FITC-labeled mixed micelles directly or preincubated with FA (1 mM) for 2 hours before exposure to FITC-labeled mixed micelles. The cells were washed three times with pH 7.4 PBS, and then trypsinized and centrifuged at 2,000 rpm for 4 minutes to obtain the cell pellets, which were subsequently re-suspended in pH 7.4 PBS and analyzed using a flow cytometer (FACSCalibur, BD Biosciences, San Jose, CA, USA). Cells without treatment served as the control group. Living cells were defined by gating the major population of the cells, and only cells within this gate were used for analysis. The mean fluorescence intensity was calculated using the histogram plot.

Cellular internalization mechanism of FITC-labeled mixed micelles in KBv cells

Cells were seeded at a density of 1×10^4 cells per well in 24-well plates and incubated for 24 hours. After checking the cell confluence and morphology, different inhibitors including CPZ (10 $\mu\text{g/mL}$), sucrose (0.45 M), filipin (5 $\mu\text{g/mL}$), genistein (0.2 M), cytochalasin D (3 μM), DMA (10 mM), NaN_3 (1 $\mu\text{g/mL}$), and monensin (3 μM) were added into each well separately and incubated for 30 minutes. Then, the compounds were withdrawn, and 200 $\mu\text{g/mL}$ of FITC-labeled mixed micelles along with different compounds (concentration of each compound was equal to that described above) in fresh serum-free RPMI-1640 medium was added. After 2-hour incubation, the treatment solution was discarded and the cells were washed three times with cold pH 7.4 PBS, trypsinized, and centrifuged at 2,000 rpm for 4 minutes to collect the cell pellets, which were subsequently re-suspended in pH 7.4 PBS and analyzed by a flow cytometer (FACS Calibur, BD Biosciences). The KBv cells without any treatment represent the maximum cellular uptake of FITC-labeled mixed micelles, which served as the control group.

In vitro KBv tumor spheroid formation

The ex vitro KBv tumor spheroids were developed using liquid overlay technique as reported earlier,²⁷ in order to evaluate the solid tumor diffusion capacity of MTX-conjugated mixed micelles. Briefly, agarose was heated at 80°C for 30 minutes, and diluted to 2% (w/v) using serum-free RPMI-1640. Each well in 24-cell culture plates was coated with a thin layer (300 μL) of sterilized agarose-based RPMI-1640. Then, KBv cells were seeded into each well at a density of 1×10^3 cells per well (in complete medium), agitated for 10 minutes, and incubated at 37°C for 7 days. The culture medium was changed every 2 days.

The uniform and compact multicellular spheroids were selected for further studies.

Cell viability in KBv tumor spheroids

PI was used as the fluorescent probe to distinguish the dead cells in treated tumor spheroids. Briefly, the KBv tumor spheroids were incubated separately with RPMI-1640, 10 $\mu\text{g/mL}$ of MTX, F127/P105/MTX, or F127/P105-MTX for 7 days. Then, the tumor spheroids were incubated with PI (50 $\mu\text{g/mL}$) for 30 minutes at 4°C. After incubation, the tumor spheroids were washed three times with cold pH 7.4 PBS and lysed with 1% Triton X-100. Fluorescence intensity of the cell lysate was measured by a microplate reader (TECAN M1000, Tecan Group Ltd, Männedorf, Switzerland). The protein content in tumor spheroids was determined using the bicinchoninic acid protein assay kit in accordance with the method specified by the manufacturer.

Growth inhibition of KBv tumor spheroids

The influence of various treatments on the growth of KBv tumor spheroids was also investigated.^{27,28} To this end, the serum-free RPMI-1640 medium containing MTX, F127/P105/MTX, or F127/P105-MTX was applied to the tumor spheroids which have been incubated for 7 days. Tumor spheroids only incubated in RPMI-1640 were used as the control group. After different treatments, tumor spheroids were observed under an inverted microscope (Chongqing Optical and Electrical Instrument Co. Ltd., Chongqing, People's Republic of China) every day. The major (d_{max}) and minor (d_{min}) diameters of each selected spheroid were determined, and the corresponding spheroid volume (V) was calculated using Equation 1.

$$V = \frac{\pi \times d_{\text{max}} \times d_{\text{min}}}{6} \quad (1)$$

The tumor spheroid volume ratio (R) was calculated using Equation 2:

$$R = \frac{V_i}{V_0} \times 100\% \quad (2)$$

where V_i is the KBv tumor spheroid volume at the i th day after treatment and V_0 is the tumor spheroid volume prior to the treatment.

Tissue distribution studies

In vivo fluorescence imaging system was used to evaluate the biodistribution of mixed micelles in tumor-bearing mice.³

The KBv xenograft mice model was established by inoculating 5×10^6 KBv cells (in 0.2 mL of pH 7.4 PBS) subcutaneously into the right hind leg. When tumor size reached 1 cm³ in diameter, tumor-bearing mice were injected with 100 μ L of DIR-labeled mixed micelles (DIR content: 0.2%) via the tail vein. After 48 hours postinjection, the mice were sacrificed, and the tumor and major organs were excised. Each organ was rinsed with PBS (pH 7.4) three times and placed onto the board of fluorescence imaging system for fluorescence detection. The biodistribution of the micelle system was semi-quantified by measuring the ratio of DIR fluorescence intensity recorded as total photons per centimeter square per dissected organs.

In vivo antitumor efficacy and safety evaluation

The antitumor efficacy of different MTX formulations was evaluated in KBv tumor-bearing mice model, which was established by inoculating 0.2 mL of PBS containing 5×10^6 KBv cells subcutaneously in the right hind leg of the mice. The first dose was scheduled at 24 hours after tumor inoculation^{3,4} (designated as day 0). Tumor-bearing mice were randomly divided into four groups and treated with 100 μ L of saline, MTX, F127/P105/MTX, or F127/P105-MTX at a 5 mg/kg dose of MTX every 4 days for six continuous dosing. On day 24, the mice were sacrificed by cervical dislocation, and the tumor mass was harvested and weighed. The inhibitory rate of tumor (IRT) was calculated using Equation 3:

$$\text{IRT (\%)} = \frac{W_s - W_t}{W_s} \times 100\% \quad (3)$$

where W_s is the weight of tumor in the same group and W_t is the weight of tumor in the treatment group.

Major organs and blood samples were also collected for hematologic analysis and histochemistry observation. Tissue samples were fixed with 4% paraformaldehyde for 48 hours and embedded in paraffin. Each section was cut into 5 μ m, processed for routine hematoxylin and eosin staining, and then visualized under microscope (Leica DMI 4000B). Blood was collected in tubes containing EDTA-2K, and a portion of blood sample was used for the measurement of white blood cell, red blood cell, and platelet. In order to collect the serum, the rest of the blood sample was allowed to stand for 30 minutes at room temperature and then centrifuged at 3,000 rpm for 15 minutes at 4°C. The serum aspartate transaminase, alanine transaminase, total bilirubin, urea nitrogen, and creatinine levels were analyzed. All the blood

and serum measurements were conducted at the Shanghai Institute for Food and Drug Control.

Data analysis

Data are presented as mean $\pm$ standard deviation. One-way analysis of variance was used to determine the significance among groups, after which post hoc tests with the Bonferroni correction were used for comparison between individual groups. A value of $P < 0.05$ was considered to be significant.

Results and discussion

Preparation and characterization of MTX-loaded polymeric mixed micelles

The constructions of MTX-entrapped polymeric mixed micelles and MTX-conjugated polymeric mixed micelles are schematically illustrated in Figure 1. To trace the micelles qualitatively and quantitatively, FITC was used as the fluorescent probe to label micelles through conjugation with Pluronic copolymer via covalent coupling. In the process of micelle preparation, 10% (w/w) Pluronic P105 was replaced by P105-FITC. Table 1 summarizes the average particle size of different mixed micelles. The average particle size of mixed micelles prepared with MTX-P105 (F127/P105-MTX) and FA-P105 (F127/P105-FA) was about 100 nm, which was larger than those of blank F127/P105 mixed micelles (F127/P105), physically entrapped MTX mixed micelles (F127/P105/MTX), and mixed micelles prepared with MTX-F127 (MTX-F127/P105) or FA-F127 (FA-F127/P105) (around 20 nm). The larger diameter of MTX- or FA-conjugated mixed micelles could be probably due to the enlarged inner hydrophobic core by P105 terminal conjugation. The MTX-loading coefficients of both conjugated and entrapped MTX mixed micelles were kept at 2.8%. F127/P105/MTX and F127/P105-MTX were lyophilized and stored at 4°C for 6 months. As presented in Table 2, 95.50% and 98.08% of MTX remained in F127/P105/MTX and F127/P105-MTX, respectively, after 6-month storage, suggesting the good stability of lyophilized micelle formulation. The particle size of F127/P105-MTX after reconstitution was slightly increased from 105.1 nm to 106.4 nm, which might be due to the slight aggregation of hydrophobic micelle cores during the storage period.

Cellular uptake

In order to evaluate the role of folate receptor in the cellular uptake of MTX-conjugated mixed micelles, FA-overexpressing MDR KBv cells were selected in the study,

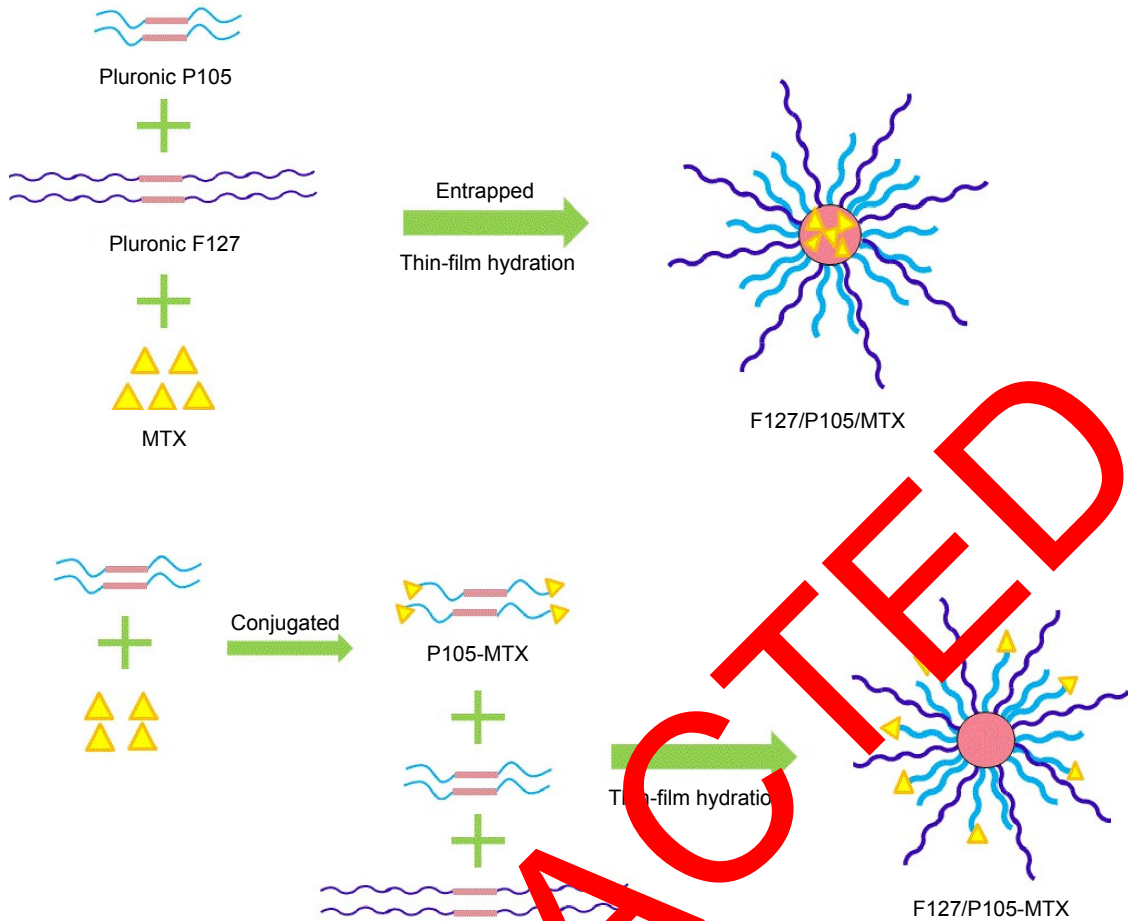


Figure 1 The schematic illustration of construction of MTX-entrapped polymeric mixed micelles and MTX-conjugated polymeric mixed micelles.
Abbreviation: MTX, methotrexate.

while A-549 cells were chosen as folate receptor-deficient cell model.^{29,30} FITC was used to label the micelles and as shown in Figure 2A, KBv cells treated with FITC-labeled F127/P105-MTX exhibited higher fluorescence intensity than FITC-labeled F127/P105. For quantitative analysis (Figure 2B), compared with cells treated with non-MTX-conjugated mixed micelles, the mean fluorescence intensity of FITC-labeled F127/P105-MTX was increased significantly ($P<0.05$), suggesting MTX-conjugated MTX in micelles aqueous layer could facilitate the uptake of micelles to some

extent than non-MTX-conjugated ones. When adding free FA, cellular uptake of F127/P105-MTX was significantly reduced ($P<0.01$). By contrast, both FITC-labeled F127/P105 and FITC-labeled F127/P105-MTX have shown less cellular uptake in A-549 cells, regardless of whether free FA was added or not ($P>0.05$). The intracellular uptake of F127/P105-MTX could be competitively inhibited by free FA, which further proved that the uptake of MTX-conjugated micelles was at least partly mediated by the interaction of MTX and folate receptors overexpressed in KBv cells.

Table 1 Characterization of FA-modified and MTX-loaded mixed micelles

Formulation	Size (nm)	Polydispersity	Zeta potential (mV)
F127/P105	22.5±1.30	0.12±0.02	-4.73±1.12
F127/P105-FA	102.2±4.53*	0.18±0.01	-3.02±1.52
MTX-F127/P105	26.9±0.45	0.12±0.02	-2.67±0.59
FA-F127/P105	28.4±3.26	0.16±0.02	-3.33±1.24
F127/P105/MTX	22.8±1.14	0.11±0.03	-2.04±0.25
F127/P105-MTX	105.1±2.35*	0.14±0.04	-2.69±2.16

Notes: Mean ± standard deviation (n=3). * $P<0.01$, compared with F127/P105.
Abbreviations: FA, folic acid; MTX, methotrexate.

Table 2 Particle sizes and MTX amounts of lyophilized mixed micelles kept at 4°C for 6 months

Parameters	Formulation	
	F127/P105/MTX	F127/P105-MTX
Particle size (nm)	27.9±0.52	106.4±1.44
MTX amounts (%)	95.50±1.30	98.08±0.78

Note: Mean ± standard deviation (n=3).

Abbreviation: MTX, methotrexate.

As shown in Figure 3, KBv cells treated with FITC-labeled F127/P105-MTX and FITC-labeled F127/P105-FA also exhibited higher fluorescence intensity than that of FITC-labeled F127/P105 ($P<0.01$), and there was no obvious difference in FITC fluorescence intensity between these two groups ($P>0.05$). It was found that when MTX or FA was conjugated on the outer surface of mixed micelles (F127 with long PEO chain), FA-F127/P105 showed more (1.36-fold) cellular fluorescence intensity than that of MTX-F127/P105 group ($P<0.05$) (Figure 4). Based on these results, it was suggested that although MTX has relatively lower affinity to folate receptor compared with FA ($K_D \approx 20\text{--}100\text{ nM}$ vs $K_D(\text{FA}) \approx 1\text{ nM}$),^{31,32} MTX-conjugated micelles might bind to folate receptor through polyvalent interaction as reported recently.^{21,33,34}

Cellular uptake mechanism of FITC-labeled mixed micelles in KBv cells

In order to elucidate the internalization mechanism of mixed micelles in KBv cells, the effects of endocytosis inhibitors, ATP depletion, and ligands of folate receptor on the cellular uptake were evaluated quantitatively (Figure 5). Endocytosis occurs in most cells as pinocytosis, and normally represents four basic mechanisms: clathrin-mediated endocytosis, caveolae-mediated endocytosis, macropinocytosis, as well as both clathrin- and caveolae-independent endocytosis.³⁵ The effect of clathrin-mediated endocytosis on the internalization of the mixed micelles was evaluated using CPZ and sucrose,^{36,38} a kind of clathrin-coated pits formation blocking agent. Filipin and genistein were used to evaluate the effect of caveolae-mediated endocytosis on the cellular uptake.^{39,40} Both types of endocytosis inhibitors significantly reduce the cellular uptake of mixed micelles in KBv cells ($P<0.05$), suggesting the involvement of both clathrin- and caveolae-mediated endocytosis in the cellular uptake of F127/P105 and F127/P105-MTX in KBv cells. Cytochalasin D and DMA were used as microtubule-disrupting agents^{41,42} to evaluate the effect of macropinocytosis on the internalization. It was found that there was no influence on the cellular uptake of both mixed micelles ($P>0.05$), indicating the minimal

contribution of macropinocytosis to the internalization of F127/P105 and F127/P105-MTX in KBv cells. During pre-incubation with monensin or NaN_3 , it was found that the intracellular ATP was significantly depleted, and the cellular uptake of mixed micelles was decreased to some extent, suggesting that internalization of the F127/P105 and F127/P105-MTX in KBv cells occurred through an energy-dependent endocytosis process. Free FA was also used to investigate the receptor-blocking effect, and the results showed that free FA can significantly reduce the cellular uptake of F127/P105-MTX in KBv cells, indicating that folate receptor-mediated endocytosis was involved in the internalization of F127/P105-MTX. Collectively, results suggest that the MTX-conjugated mixed micelles could be recognized by folate receptors in KBv cells via MTX on the terminal of P105, and then were energy-dependently internalized by KBv cells through a multiple endocytic pathway including both clathrin- and caveolae-mediated endocytosis.

Cytotoxicity in KBv tumor spheroid studies

The ex vivo 3D tumor spheroids model has become the most commonly used tool to evaluate the effect of drug delivery systems, suggestive of the extracellular matrix of tumors in vivo.^{43,44} In this study, we first constructed the KBv tumor spheroids by the liquid overlay technique. After 7-day culture, the compact and homogeneous tumor spheroids were acquired (Figure 6A and B). The fluorescence intensity of PI was reported to be specifically related to the DNA of dead cell.^{28,45} Thus, dead cells in the KBv tumor spheroids after treatment with MTX-loaded mixed micelles were stained by PI, a fluorescent probe. As presented in Figure 6C, the fluorescence intensity values of PI in the tumor spheroids treated with RPMI-1640, MTX, F127/P105/MTX, and F127/P105-MTX were $4.45 \times 10^5/\text{mg protein}$, $8.43 \times 10^5/\text{mg protein}$, $17.6 \times 10^5/\text{mg protein}$, and $27.4 \times 10^5/\text{mg protein}$, respectively, suggesting that F127/P105-MTX can induce a statistically significant increase in the cell death of the KBv tumor spheroids as compared to those of free MTX and conventional F127/P105/MTX ($P<0.05$).

KBv tumor spheroid growth inhibition effect

The KBv tumor spheroids growth inhibition by different MTX-loaded mixed micelles treatment was also investigated. Figure 7 represents the KBv tumor spheroid volume ratios (of the control, %) after different treatments of MTX, F127/P105/MTX, and F127/P105-MTX at different MTX concentrations.

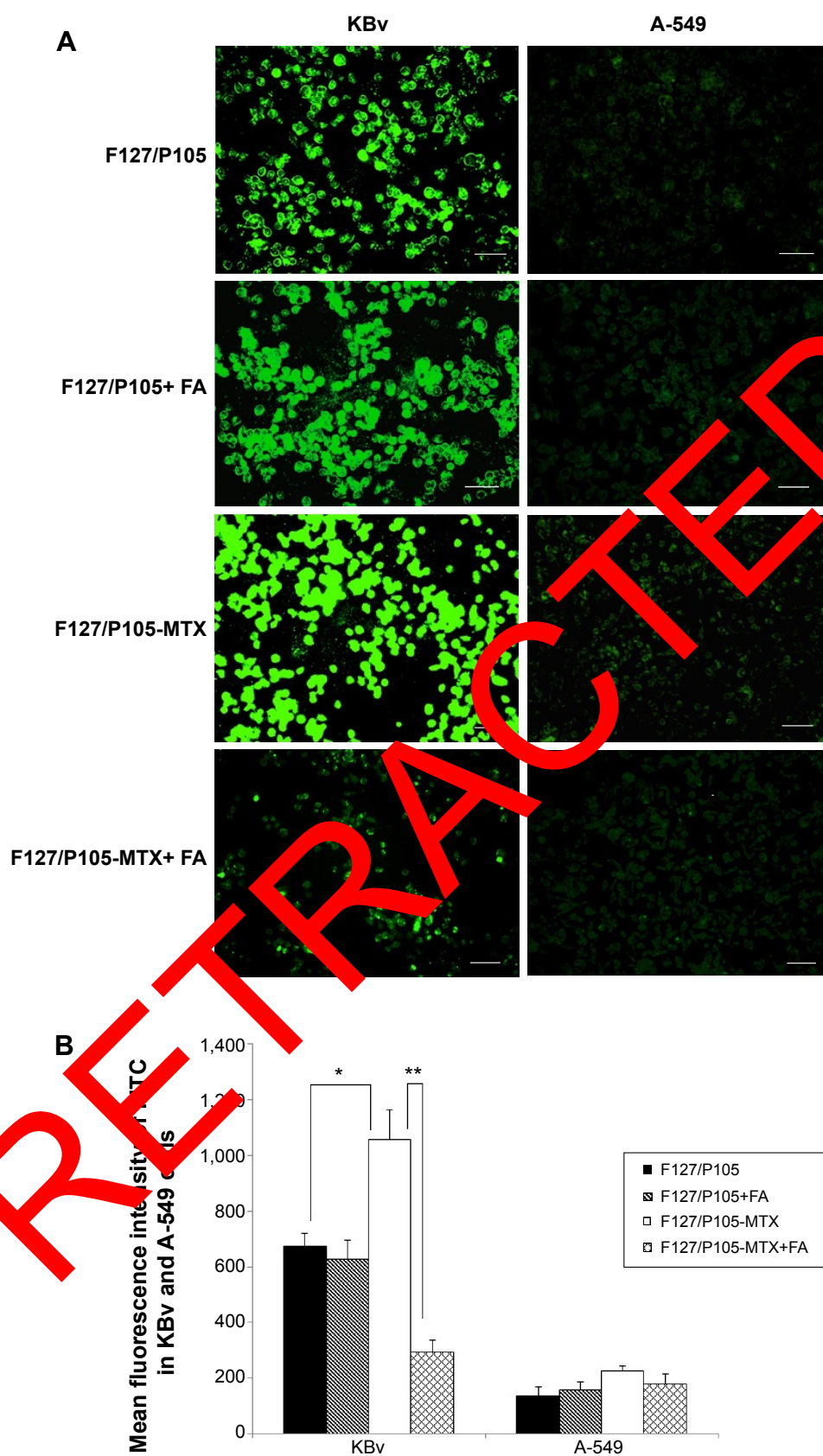


Figure 2 The cellular uptake in KBv and A-549 cells.

Notes: Cellular uptake studies in KBv and A-549 cells were examined by fluorescent microscopy (**A**) and flow cytometry (**B**), respectively, after a 1-hour treatment with FITC-labeled F127/P105 mixed micelles, FITC-labeled F127/P105-MTX, or 2-hour pre-incubation with 1 mM of free FA and then exposure to FITC-labeled F127/P105 mixed micelles or FITC-labeled F127/P105-MTX. Mean $\pm$ standard deviation ($n=3$). * $P<0.05$ and ** $P<0.01$. Green: FITC-labeled mixed micelles. Bar: 30 μ m.

Abbreviations: FITC, fluorescein isothiocyanate; MTX, methotrexate; FA, folic acid.

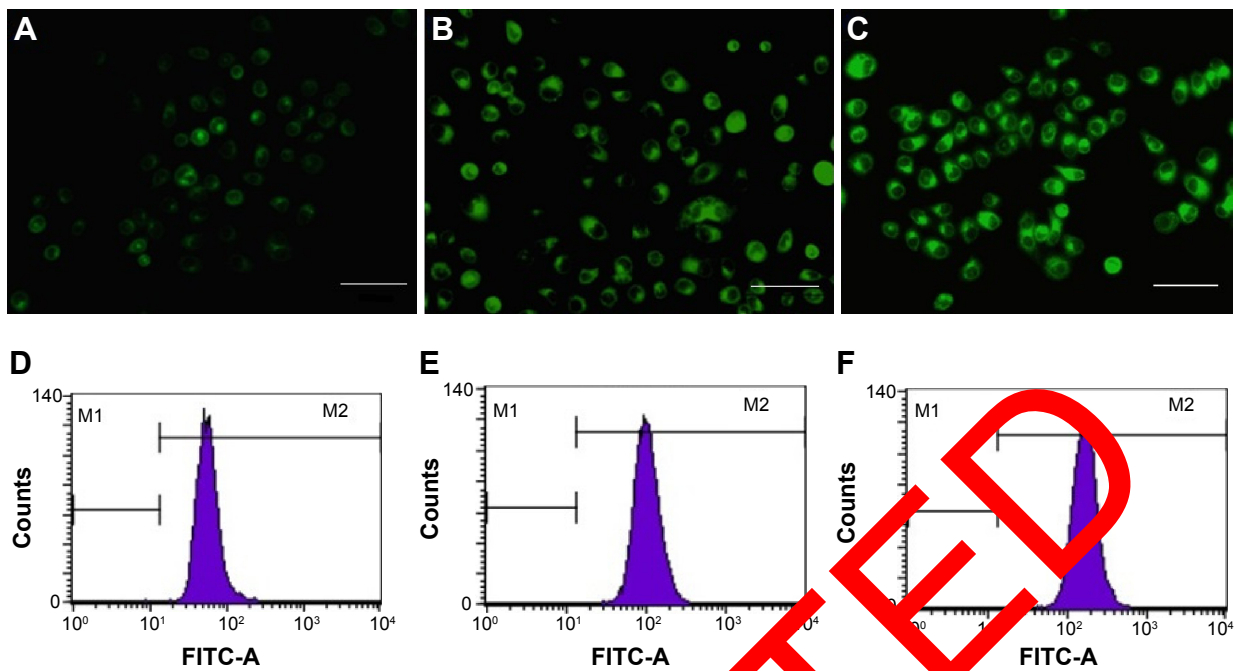


Figure 3 The cellular uptake in KBv cells, of P105 terminal modified mixed micelles.

Notes: Cellular uptake studies in KBv cells were examined by fluorescent microscopy (A–C) and flow cytometry (D–F) after a 1-hour treatment with FITC-labeled F127/P105 mixed micelles (A and D), FITC-labeled F127/P105-MTX (B and E), or FITC-labeled F127/P105-FA (C and F). Green: FITC-labeled mixed micelles. Bar: 30 μ m.

Abbreviations: FITC, fluorescein isothiocyanate; MTX, methotrexate; FA, folic acid.

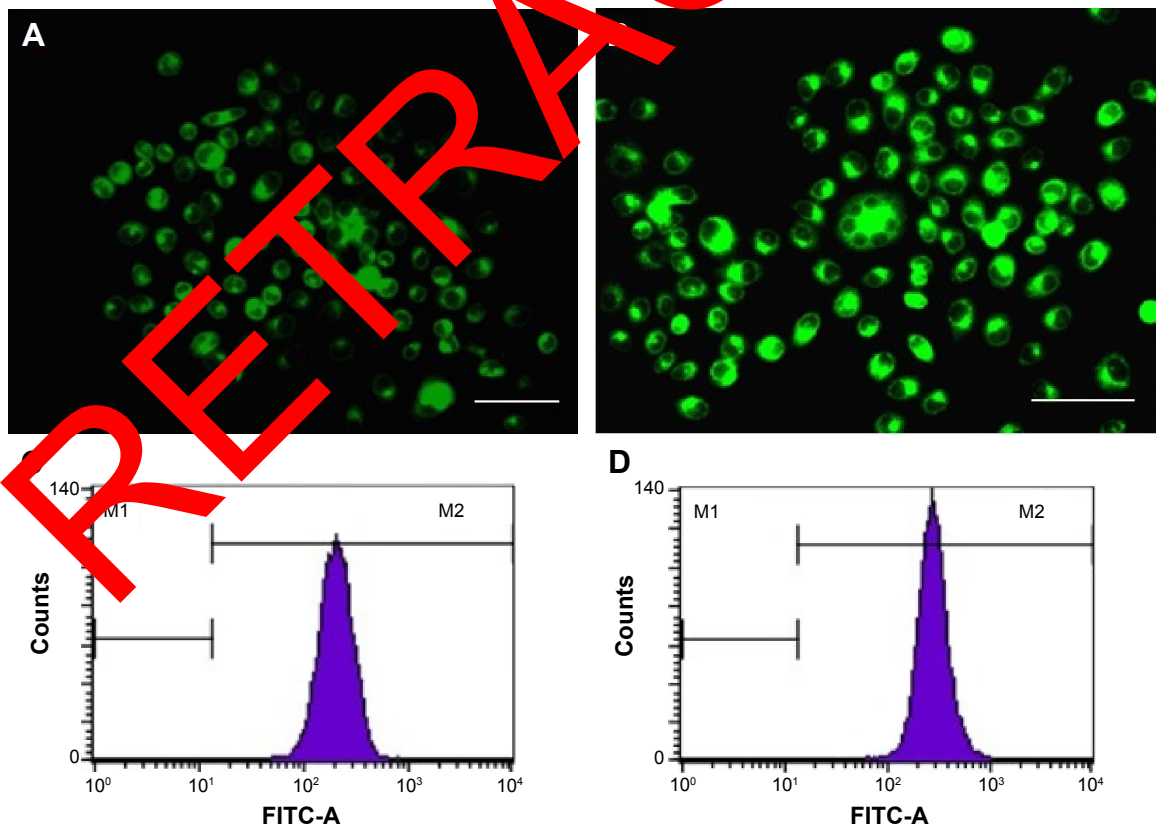


Figure 4 The cellular uptake in KBv cells, of F127 terminal modified mixed micelles.

Notes: Cellular uptake studies in KBv cells were examined by fluorescent microscopy (A and B) and flow cytometry (C and D) after a 1-hour treatment with FITC-labeled MTX-F127/P105 mixed micelles (A and C) or FITC-labeled FA-F127/P105 (B and D). Green: FITC-labeled mixed micelles. Bar: 30 μ m.

Abbreviations: FITC, fluorescein isothiocyanate; MTX, methotrexate; FA, folic acid.

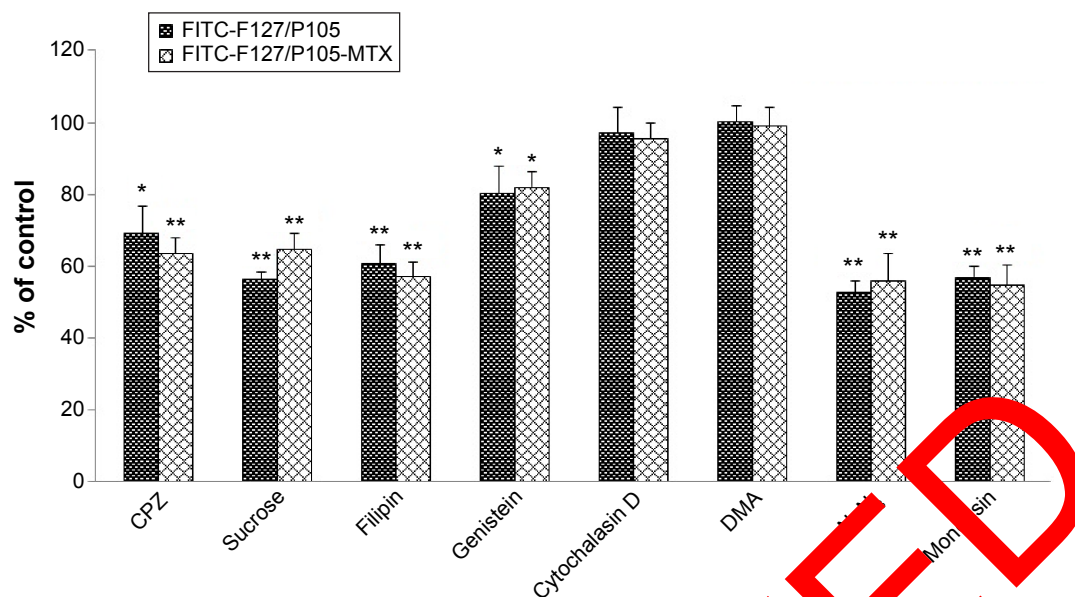


Figure 5 Effects of different inhibitors on cellular uptake of FITC-F127/P105 and FITC-F127/P105-MTX in KBv cells. **Notes:** Fluorescence intensity of FITC-F127/P105 and FITC-F127/P105-MTX in untreated cells, representing maximum internalized amount for each formulation, was measured as control. Mean $\pm$ standard deviation (n=3). * $P < 0.05$ and ** $P < 0.01$, compared with control.

Abbreviations: FITC, fluorescein isothiocyanate; MTX, methotrexate; CPZ, chlorpromazine hydrochloride; DMA, 1-(4-(N-dimethyl) amiloride hydrochloride).

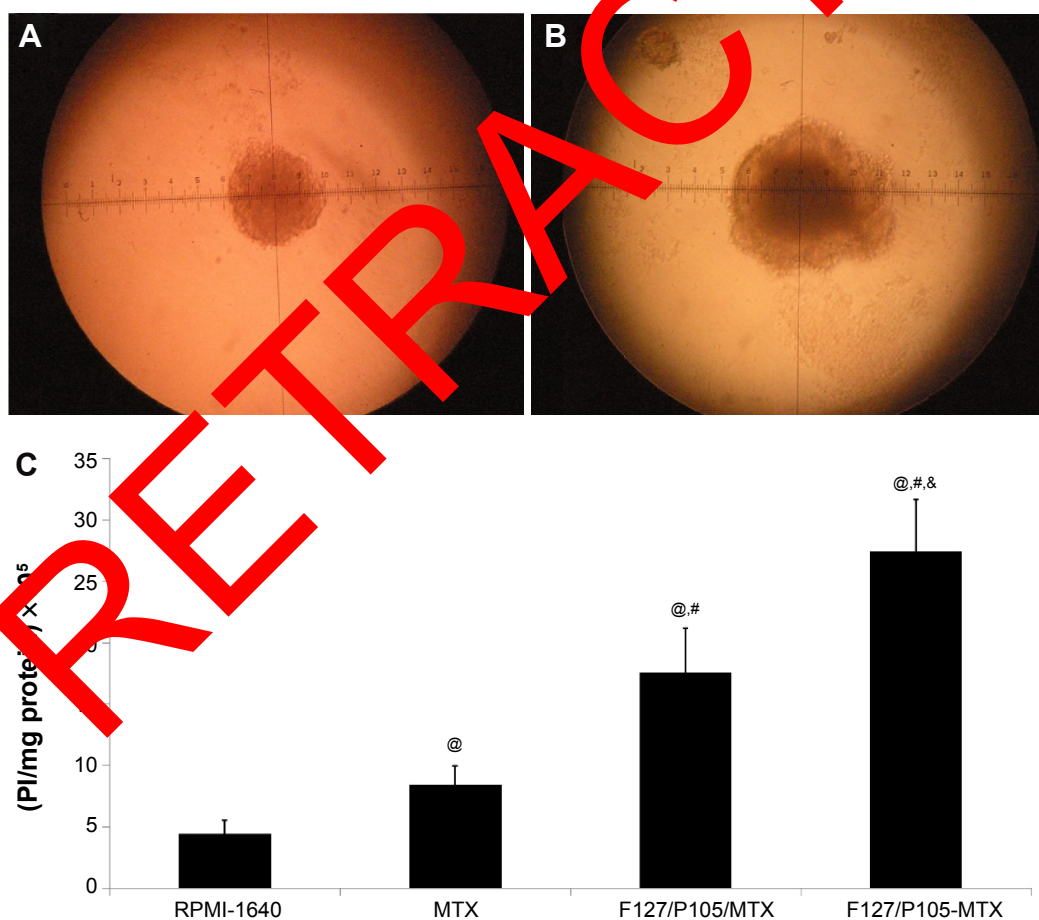


Figure 6 Cell viability in KBv tumor spheroid.

Notes: KBv tumor spheroids at day 3 (A) and day 7 (B) after cells were seeded. Accumulation of PI in KBv tumor spheroids subjected to different treatments (C). PI can stain the DNA of dead cells, and thus, its fluorescence intensity relates to dead cell population. Mean $\pm$ standard deviation (n=3). @ $P < 0.05$, compared with RPMI-1640. # $P < 0.05$, compared with MTX. & $P < 0.05$, compared with F127/P105/MTX. Original magnification: $\times 10$.

Abbreviations: PI, propidium iodide; DNA, deoxyribonucleic acid; RPMI, Roswell Park Memorial Institute; MTX, methotrexate.

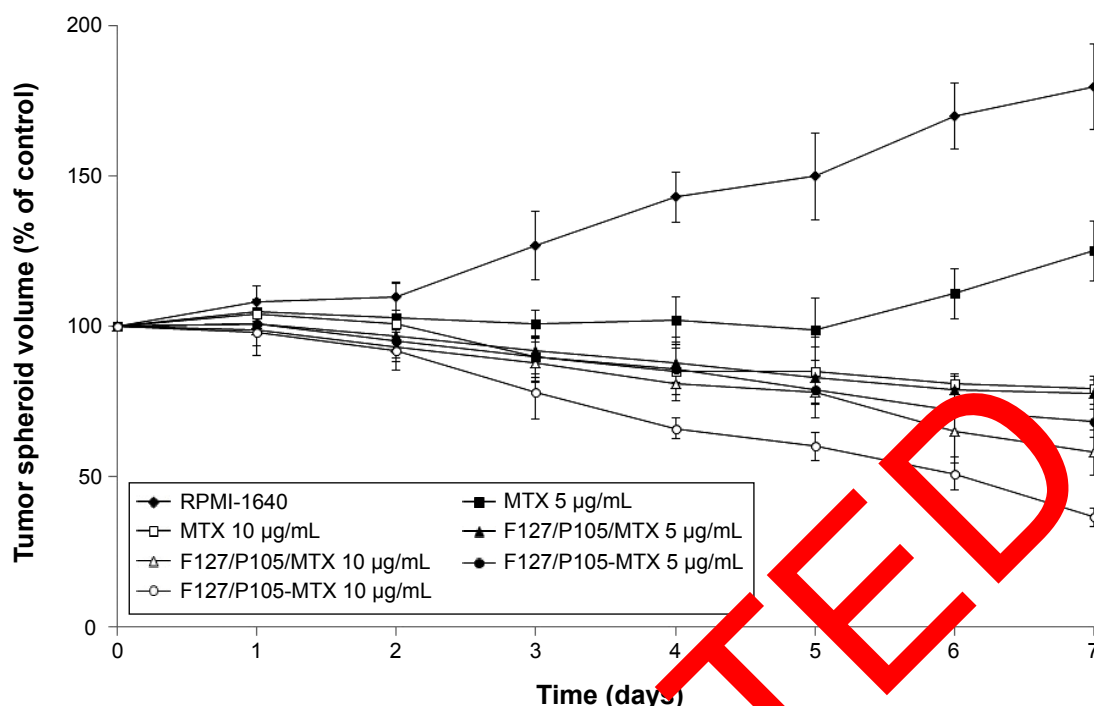


Figure 7 Growth inhibition curves of KBv tumor spheroids after treatment with MTX or MTX-loaded mixed micelles at different concentrations of MTX.

Notes: The diameter of tumor spheroids was measured using a microscope equipped with an ocular micrometer, and the corresponding volume of tumor spheroids was calculated by Equation 2. Mean $\pm$ standard deviation ($n=3$).

Abbreviations: MTX, methotrexate; RPMI, Roswell Park Memorial Institute.

It was observed that tumor spheroids continued to grow in the absence of drug (179.8% of the control after 7 days). No reduction in the volume of tumor spheroids was observed in MTX group (5 $\mu\text{g/mL}$) after 7 days of treatment, indicating that KBv tumor spheroids were resistant to MTX to some extent. The calculated KBv tumor spheroid volume ratios were 125.2%, 77.8%, and 68.5% at 5 $\mu\text{g/mL}$ and 98.3%, 57.9%, and 36.4% at 10 $\mu\text{g/mL}$ for MTX, F127/P105-MTX, and F127/P105-MTX, respectively. Results showed that F127/P105-MTX significantly improved the growth inhibitory effect than that of F127/P105-MTX.

Tissue distribution study

Tissue distribution study was carried out using subcutaneous KBv tumor-bearing nude mice. Semi-quantitative analysis of dissected organs (the heart, liver, spleen, lung, kidney, and tumor) at 48 hours postinjection of DIR-labeled mixed micelles was performed. The fluorescence intensity of tumor tissues treated with F127/P105-MTX was about 1.46 times higher than that of nonspecific F127/P105 mixed micelles (F127/P105-MTX) ($P<0.05$), suggesting that the conjugated MTX could facilitate the accumulation of mixed micelles in tumor tissue. Moreover, F127/P105-MTX in tumor was found to be more than in the liver, spleen, and kidney ($P<0.05$) (Figure 8).

In vivo antitumor activity and safety evaluation

In vivo antitumor efficiency of different MTX-loaded mixed micelles was validated in subcutaneous xenograft-bearing mice. As shown in Figure 9 and Table 3, there was no serious body weight loss observed in mice treated with F127/P105-MTX or F127/P105-MTX during the treatment period, and the IRT was 59.1% and 71.4% on the 24th day, respectively, for F127/P105-MTX and F127/P105-MTX. The results showed that F127/P105-MTX exhibited stronger antitumor efficacy in KBv tumor-bearing mice model than that of F127/P105-MTX. A synergistic effect of the passive targeting and enhanced cellular accumulation in folate receptor-rich tumor could be the main reason for the significant tumor depression in F127/P105-MTX group. The in vivo tumor growth inhibitory effect of MTX-loaded mixed micelles was consistent well with the in vitro 3D tumor spheroids results.

It is well known that most of the intravenously injected nanoparticles will be taken up and eliminated by macrophage-related organs based on our results of biodistribution study, which urge us to further investigate the potential pathological lesions induced by MTX-loaded mixed micelles.²⁴ To reveal any potential toxic effect of MTX-loaded mixed micelles on the treated tumor-bearing mice, hematology and blood biochemistry analysis were carried out. The histological

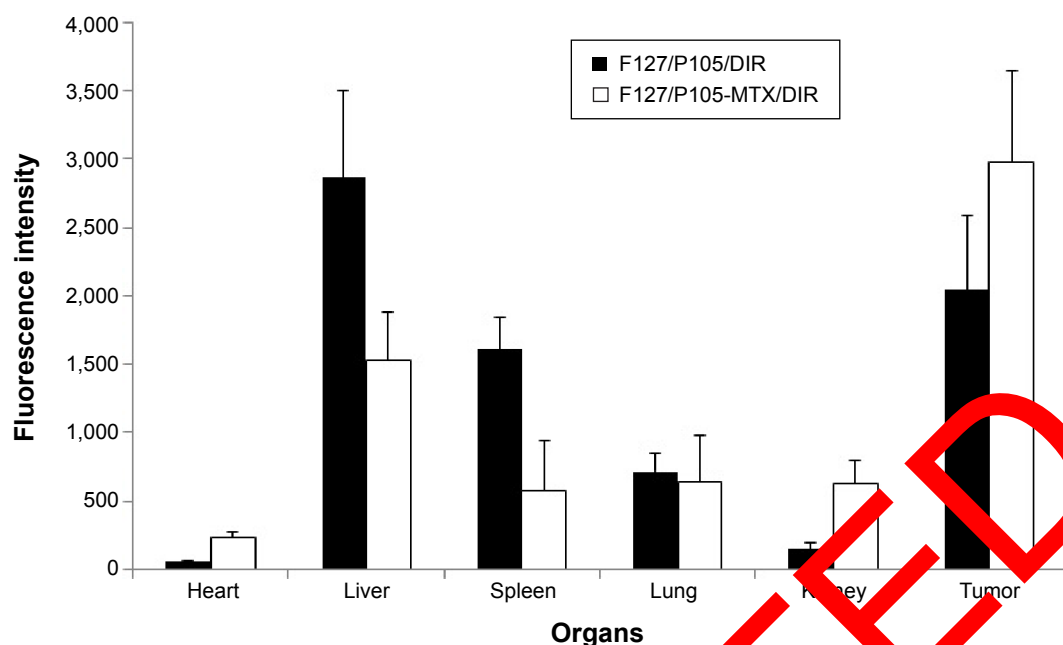


Figure 8 Fluorescence intensity of F127/P105/DIR and F127/P105-MTX/DIR in dissected organs of subcutaneous tumor-bearing mice sacrificed 48 hours after intravenous administration.

Note: Mean $\pm$ standard deviation (n=3).

Abbreviations: DIR, dioctadecyl-3,3',3'-tetramethylindotricarbocyanine iodide; MTX, methotrexate.

analysis of major tissues including the heart, liver, spleen, lung, and kidney was conducted, and observation results revealed no obvious histopathological abnormalities or lesions in all groups (Figure 10). Liver function markers including alanine aminotransferase, aspartate aminotransferase, as well as total bilirubin, and the kidney function markers such as creatinine and blood urea nitrogen were used to further evaluate the safety of drug-loaded polymeric micelles (Table 4). No obvious hepatic or renal toxicity

was observed in the mice treated with MTX-loaded mixed micelles, indicating that multiple dosing of MTX-loaded micelles had minimal impact on the function of the liver and kidney. For the hematological assessment, we chose the following important hematology markers: white blood cell, red blood cell, and platelet count (Table 5). All the hematology parameters in the treated groups appeared to be normal in comparison with the control group. It indicates that multiple dosing of MTX-loaded mixed micelles did not



Figure 9 The dissected tumor tissue images after intravenous administration of 5 mg/kg of MTX, F127/P105/MTX (5 mg/kg, MTX equivalent), or F127/P105-MTX (5 mg/kg, MTX equivalent) which was injected every 4 days for six continuous times.

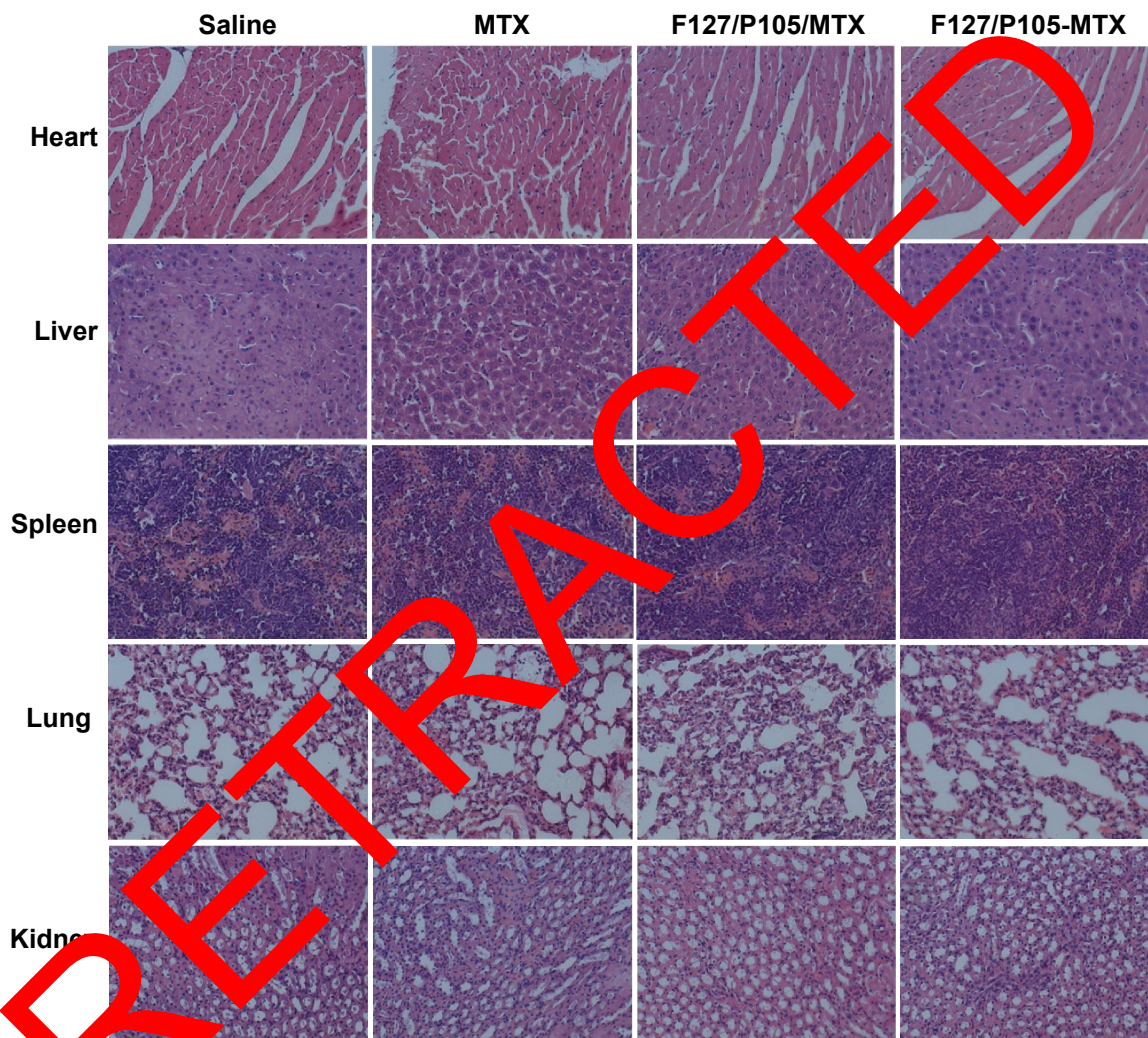
Abbreviation: MTX, methotrexate.

Table 3 The changes in the body weight and tumor weight after different MTX treatments in KBv tumor-bearing mice

Groups	Body weight (g)		Tumor weight (g)	IRT (%)
	Day 1	Day 24		
Saline	20.88±0.77	25.60±0.33	0.88±0.16	0
MTX	21.97±1.70	25.17±1.86	0.67±0.21	23.86
F127/P105/MTX	22.60±1.10	24.17±2.27	0.36±0.04 ^a	59.09
F127/P105-MTX	20.67±1.12	23.62±2.67	0.25±0.08 ^{ab}	71.42

Notes: Mean ± standard deviation (n=6). ^aP<0.01, when compared with free MTX group. ^bP<0.05, when compared with F127/P105/MTX group.

Abbreviations: MTX, methotrexate; IRT, inhibitory rate of tumor.

**Figure 10** Histology analysis of the heart, liver, spleen, lung, and kidney sections stained with hematoxylin and eosin after treatment with different formulations.

Note: Original magnification: ×20.

Abbreviation: MTX, methotrexate.

Table 4 Serum biochemical levels after treatment with different MTX formulations

Groups	ALT (IU/L)	AST (IU/L)	TBIL (μmol/L)	BUN (mmol/L)	CRE (μmol/L)
Saline	55.0±10.3	117.6±18.5	1.13±0.52	1.04±0.38	15.1±3.24
MTX	50.8±12.1	114.9±14.1	1.06±0.57	1.12±0.41	14.5±3.90
F127/P105/MTX	52.1±14.7	122.5±13.8	1.10±0.37	1.06±0.73	14.8±4.57
F127/P105-MTX	51.3±18.2	120.5±15.3	1.02±0.46	1.17±0.66	14.4±3.03

Notes: Mean ± standard deviation (n=3). There was no significant difference in the above-mentioned parameters among different MTX formulations when compared with the saline group (P>0.05).

Abbreviations: MTX, methotrexate; ALT, alanine transaminase; AST, aspartate transaminase; TBIL, total bilirubin; BUN, blood urea nitrogen; CRE, creatinine.

Table 5 The hematological results in mice after treatment with different MTX formulations

Groups	WBC ($10^9/L$)	RBC ($10^{12}/L$)	PLT ($10^9/L$)
Saline	5.34±1.47	6.28±1.52	1,249±233
MTX	5.11±1.18	7.33±1.05	1,254±217
F127/P105/MTX	5.27±0.84	6.89±1.41	1,295±370
F127/P105-MTX	5.49±1.03	7.37±0.94	1,327±197

Notes: Mean ± standard deviation (n=6). There was no significant difference in the above-mentioned parameters among different MTX formulations when compared with the saline group ($P>0.05$).

Abbreviations: MTX, methotrexate; WBC, white blood cell; RBC, red blood cell; PLT, platelet.

cause acute toxicity to the hematological system and major organs in mice.

Conclusion

In this report, the therapeutic effect of MTX-conjugated Pluronic-based polymeric micelles (F127/P105-MTX) and conventional MTX-entrapped polymeric micelles (F127/P105/MTX) was evaluated in vitro and in vivo. F127/P105-MTX displayed higher cellular uptake ability in KBv cells than that of F127/P105/MTX, through folate receptor-mediated transcytosis. Both F127/P105-MTX and F127/P105/MTX were found to be internalized by clathrin- and caveolae-mediated endocytosis in KBv cancer cells, and free FA can significantly reduce the cellular uptake of F127/P105-MTX in KBv cells. The antitumor efficacy of F127/P105-MTX was significantly enhanced in comparison with F127/P105/MTX in 3D tumor spheroid cell-based xenograft nude mice-based models. Preliminary safety evaluation revealed that no acute hematological toxicity or histopathological abnormalities were observed after treatment. Our results showed the favorable in vitro and in vivo efficacy and a good biological safety of covalent conjugated MTX-based micelle system, which could be attributed to the receptor-mediated active targeting effect in folate receptor-overexpressed cancerous cells.

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Disclosure

The authors report no conflicts of interest in this work.

References

- Kataoka K, Harada A, Nagasaka M. Block copolymer micelles for drug delivery: design, characterization and biological significance. *Adv Drug Deliv Rev*. 2001;47(1):1-21.
- Matsumura Y, Maeda H. A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs. *Cancer Res*. 1986;46(12):6387-6392.
- Chen Y, Sha X, Zhang W, et al. Pluronic mixed micelles overcoming methotrexate multidrug resistance: in vitro and in vivo evaluation. *Int J Nanomedicine*. 2013;8:1474-1476.
- Chen Y, Zhang W, Gu J, et al. Enhanced antitumor efficacy by methotrexate conjugated Pluronic mixed micelles against KBv multidrug resistant cancer. *J Pharm*. 2013;452(1-2):421-433.
- Chen Y, Sha X, Zhang X, Chen Y, Ren Q, Fang X. Pluronic P105/F127 mixed micelles for the delivery of docetaxel against Taxol-resistant non-small cell lung cancer: optimization and in vitro, in vivo evaluation. *Int J Nanomedicine*. 2013;8:73-84.
- Zhang W, Shi Y, Chen Y, et al. Enhanced antitumor efficacy by paclitaxel-loaded pluronic P123/F127 mixed micelles against non-small cell lung cancer based on passive tumor targeting and modulation of drug resistance. *Eur J Pharm Biopharm*. 2010;75(3):341-353.
- Wang Y, Hao J, Li Y, et al. Poly(caprolactone)-modified Pluronic P105 micelles for reversal of paclitaxel-resistance in SKOV-3 tumors. *Biomaterials*. 2012;33(18):4741-4751.
- Wei Z, Hao J, Yuan S, et al. Paclitaxel-loaded Pluronic P123/F127 mixed polymeric micelles: formulation, optimization and in vitro characterization. *Int J Pharm*. 2009;376(1-2):176-185.
- Armstrong A, Brewer J, Newman C, et al. SP1049C as first-line therapy in advanced (inoperable or metastatic) adenocarcinoma of the oesophagus: a phase II window study. *ASCO Annual Meeting Proceedings Part 1, J Clin Oncol*. 2006;24(suppl 18):4080.
- Kataoka K, Kwon GS, Yokoyama M, Okano T, Sakurai Y. Block copolymer micelles as vehicles for drug delivery. *J Control Release*. 1993;24(1-3):119-132.
- Kwon GS, Kataoka K. Block copolymer micelles as long-circulating drug vehicles. *Adv Drug Deliv Rev*. 2012;64(suppl):237-245.
- Mlinar LB, Chung EJ, Wonder EA, Tirrell M. Active targeting of early and mid-stage atherosclerotic plaques using self-assembled peptide amphiphile micelles. *Biomaterials*. 2014;35(30):8678-8686.
- Deng C, Jiang Y, Cheng R, Meng F, Zhong Z. Biodegradable polymeric micelles for targeted and controlled anticancer drug delivery: promises, progress and prospects. *Nano Today*. 2012;7(5):467-480.
- Leamon CP, Low PS. Folate-mediated targeting: from diagnostics to drug and gene delivery. *Drug Discov Today*. 2001;6(1):44-51.
- Leamon CP, Reddy JA. Folate-targeted chemotherapy. *Adv Drug Deliv Rev*. 2004;56(8):1127-1141.
- Sudimack J, Lee RJ. Targeted drug delivery via the folate receptor. *Adv Drug Deliv Rev*. 2000;41(2):147-162.

17. Elnakat H, Ratnam M. Distribution, functionality and gene regulation of folate receptor isoforms: implications in targeted therapy. *Adv Drug Deliv Rev.* 2004;56(8):1067–1084.
18. Sonvico F, Mornet S, Vasseur S, et al. Folate-conjugated iron oxide nanoparticles for solid tumor targeting as potential specific magnetic hyperthermia mediators: synthesis, physicochemical characterization, and in vitro experiments. *Bioconj Chem.* 2005;16(5):1181–1188.
19. Marchetti C, Palaia I, Giorgini M, et al. Targeted drug delivery via folate receptors in recurrent ovarian cancer: a review. *Onco Targets Ther.* 2014;7:1223–1236.
20. Low PS, Henne WA, Doorneweerd DD. Discovery and development of folic-acid-based receptor targeting for imaging and therapy of cancer and inflammatory diseases. *Acc Chem Res.* 2008;41(1):120–129.
21. Thomas TP, Joice M, Sumit M, et al. Design and in vitro validation of multivalent dendrimer methotrexates as a folate-targeting anticancer therapeutic. *Curr Pharm Des.* 2013;19(37):6594–6605.
22. Duthie SJ. Folic-acid-mediated inhibition of human colon-cancer cell growth. *Nutrition.* 2001;17(9):736–737.
23. Rosenholm JM, Peuhu E, Bate-Eya LT, Eriksson JE, Sahlgren C, Lindén M. Cancer-cell-specific induction of apoptosis using mesoporous silica nanoparticles as drug-delivery vectors. *Small.* 2010;6(11):1234–1241.
24. Rosenholm JM, Peuhu E, Eriksson JE, Sahlgren C, Lindén M. Targeted intracellular delivery of hydrophobic agents using mesoporous hybrid silica nanoparticles as carrier systems. *Nano Lett.* 2009;9(9):3308–3311.
25. Wang Y, Yu L, Han L, Sha X, Fang X. Difunctional Pluronic copolymer micelles for paclitaxel delivery: synergistic effect of folate-mediated targeting and Pluronic-mediated overcoming multidrug resistance in tumor cell lines. *Int J Pharm.* 2007;337(1–2):63–73.
26. Zhang W, Shi Y, Chen Y, Ye J, Sha X, Fang X. Multifunctional Pluronic P123/F127 mixed polymeric micelles loaded with paclitaxel for the treatment of multidrug resistant tumors. *Biomaterials.* 2011;32(11):2894–2906.
27. Jiang X, Sha X, Xin H, et al. Integrin-facilitated transcytosis for enhanced penetration of advanced gliomas by poly(trimethylene carbonate)-based nanoparticles encapsulating paclitaxel. *Biomaterials.* 2013;34(12):2969–2979.
28. Xin H, Sha X, Jiang X, Zhang W, Chen L, Fang X. Antitumor efficacy and safety of paclitaxel-loading poly(hep)-conjugated dual targeting PEG-PCL nanoparticles. *Biomaterials.* 2012;33(32):8167–8176.
29. Stella B, Marsaud V, Arpicco A, et al. Biological characterization of folic acid-conjugated poly(M2NPEGCA-co-PCLDCA) nanoparticles in cellular models. *J Drug Target.* 2007;15(2):141–153.
30. Zheng Y, Cai Z, Song X, et al. Preparation and characterization of folate conjugated trimethyl chitosan nanoparticles as protein carrier targeting folate receptor. *in vitro studies.* *J Drug Target.* 2009;17(4):294–300.
31. Jackman AL, Theti DS, Gibbs DD. Antifolates targeted specifically to the folate receptor. *Adv Drug Deliv Rev.* 2004;56(8):1111–1125.
32. Li MH, Choi SK, Thomas TP, et al. Dendrimer-based multivalent methotrexates as dual acting nanoconjugates for cancer cell targeting. *Eur J Med Chem.* 2012;47:560–572.
33. Kiessling LL, Gestwicki JE, Strong LE. Synthetic multivalent ligands in the exploration of cell-surface interactions. *Curr Opin Chem Biol.* 2000;4(6):696–703.
34. Thomas TP, Huang B, Choi SK, et al. Polyvalent dendrimer-methotrexate as a folate receptor-targeted cancer therapeutic. *Mol Pharm.* 2012;9(9):2669–2676.
35. Liu J, Shapiro JI. Endocytosis and signal transduction: basic science update. *Biol Res Nurs.* 2003;5(2):117–128.
36. Roger E, Lagarce F, Garcion E, Benoit JP. Lipid nanocarriers improve paclitaxel transport throughout human intestinal epithelial cells by using vesicle-mediated transcytosis. *J Control Release.* 2009;140(2):174–181.
37. Tahara K, Sakai T, Yamamoto H, Takeuchi T, Hirashima N, Kawashima Y. Improved cellular uptake of chitosan-modified PLGA nanospheres by A549 cells. *Int J Pharm.* 2009;387(1–2):198–204.
38. Abu Lila AS, Kizuki T, Doi Y, Saito T, Kawanishi T, Kiwada H. Oxalipatin encapsulated in PEG-coated cationic liposomes induces significant tumor growth suppression via a dual-targeting approach in a murine solid tumor model. *J Control Release.* 2009;137(1):8–14.
39. Aoki T, Yoshida R, Fujimoto T. Tyrosine phosphorylation of caveolin-1 in the endothelium. *Exp Cell Res.* 1999;253(2):629–636.
40. Liu P, Anderson JG. Spatial organization of EGF receptor translocation by PDGF. *Biochem Biophys Res Commun.* 1999;261(3):695–700.
41. Xu Z, Chen H, Gu W, et al. The performance of docetaxel-loaded solid lipid nanoparticles targeted to hepatocellular carcinoma. *Biomaterials.* 2009;30(2):226–232.
42. Kim H, Kwon SM, Chung H, et al. Cellular uptake mechanism and intracellular fate of hydrophobically modified glycol chitosan nanoparticles. *J Control Release.* 2009;135(3):259–267.
43. Ong SM, Zhao Z, Arooz T, et al. Engineering a scaffold-free 3D tumor model for in vitro drug penetration studies. *Biomaterials.* 2010;31(6):1180–1190.
44. Mehta G, Hsiao AY, Ingram M, Luker GD, Takayama S. Opportunities and challenges for use of tumor spheroids as models to test drug delivery and efficacy. *J Control Release.* 2012;164(2):192–204.
45. Bahmani P, Schellenberger E, Klohs J, et al. Visualization of cell death in mice with focal cerebral ischemia using fluorescent annexin A5, propidium iodide, and TUNEL staining. *J Cereb Blood Flow Metab.* 2011;31:1311–1320.

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