Folate-functionalized polymeric micelles for tumor targeted delivery of a potent multidrug-resistance modulator FG020326

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Abstract: To overcome multidrug resistance (MDR) existing in tumor chemotherapy, polymeric micelles encoded with folic acid on the micelle surface were prepared with the encapsulation of a potent MDR modulator, FG020326. The micelles were fabricated from diblock copolymers of poly(ethylene glycol) (PEG) and biodegradable poly(ε-caprolactone) (PCL) with folate attached to the distal ends of PEG chains. The folate-conjugated copolymers, folate-PEG-PCL, were synthesized by multistep chemical reactions. First, allyl-terminated copolymer (allyl-PEG-PCL) was synthesized through a ring-opening polymerization of ε-caprolactone in bulk employing monoallyl-PEG as a macroinitiator. Second, the allyl terminal groups of copolymers were converted into primary amino groups by a radical addition reaction, followed by conjugation of the carboxylic group of folic acid. *In vitro* studies at 37°C demonstrated that FG020326 release from micelles at pH 5.0 was faster than that at pH 7.4. Cytotoxicity studies with MTT assays indicated that folate-functionalized and FG020326-loaded micelles resensitized the cells approximately five times more than their folate-free counterparts ($p < 0.01$) in human KB_{v200} cells treated with vincristine (VCR). The *in vitro* Rhodamine 123 efflux experiment using MDR KB_{v200} cells revealed that when cells were pretreated with folate-attached and FG020326-loaded micelles, the P-glycoprotein (P-gp) drug efflux function was significantly inhibited. J Biomed Mater Res 86A: 48–60, 2008

Key words: multidrug resistance; polymeric micelles; poly (ethylene glycol)-poly(ε-caprolactone); folate functionalization; tumor targeting

INTRODUCTION

The resistance of cancer cells to multiple structurally unrelated chemotherapeutic drugs termed "multidrug resistance (MDR)" has been recognized as a major cause of failure in human cancer chemotherapy. It is well known that various mechanisms are involved in drug resistance in cancer, chief among them the classical efflux mechanism associated with the function of P-glycoprotein (P-gp). P-gp is an ABC transporter and a plasma membrane glycoprotein encoded by the human *MDR1* gene, which acts as a drug efflux pump that extrudes a wide range of structurally and mechanistically different chemotherapeutic drugs out of cancer cells.[1–3] The mechanism of action of various drugs, including vinca alkaloids, anthracyclines, epipodophyllotoxins, taxanes and other natural products, have been reported to be affected by classical MDR.[1] Hence, a common way to circumvent P-gp-based MDR is to use MDR modulators that inhibit P-gp-mediated drug efflux, resulting in resensitization of MDR cancer cells to treatment with chemotherapeutic agents when coadministered with MDR modulators.[4] Recently, several effective MDR modulators have been reported including verapamil, quinidine, PSC833, etc.[5] One of the authors (LF) of the present work and coworkers have demonstrated that the small molecular FG-020326, an imidazole derivative (E)-methyl 3-(4-(4,5-bis(4-(isopropyl(methyl)amino)phenyl)-1*H*-imidazol-2-yl)phenyl)acrylate as shown in Figure 1, can significantly inhibit the function of P-gp, and consequently increase the intracellular accumulation and

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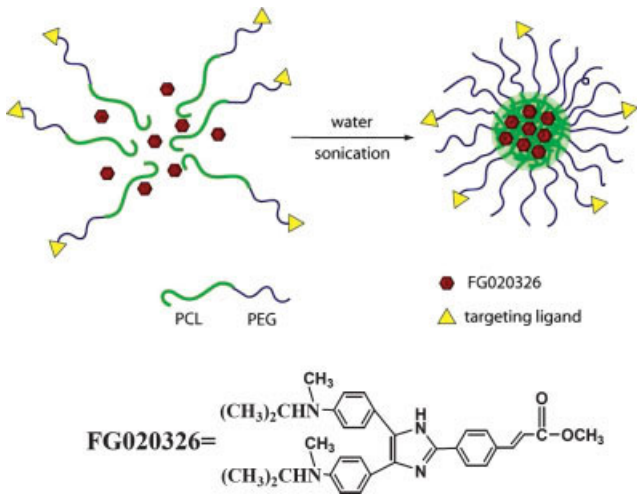


Figure 1. Schematic formation of FG020326-encapsulated micelle by adding THF solution containing FG020326 and copolymer to water under sonication, followed by evaporation of THF. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

efficacy of vincristine (VCR), a commonly used anticancer agent. Notably, FG020326 increased the sensitivity of KB_{v200} cells to VCR by ~52-fold.[6,7]

Despite remarkable promise for concomitant delivery of FG020326 and anticancer drugs, the low water solubility of the unprotonated FG020326 remains a problem for traditional *in vivo* administration. Moreover, the implementation of P-gp inhibition in cancer treatment with MDR modulators has faced two major obstacles. Firstly, it is often associated with exacerbated toxicity of anticancer drugs because this approach may also block the excretory functions of P-gp expressed in healthy tissues, and thus markedly reduces clearance of anticancer agents from these sites.[8] Secondly, MDR modulators, even if soluble in physiological aqueous media, undergo quick renal clearance following *in vivo* administration, resulting in short blood retention that prevents tumors from receiving therapeutic concentrations.

Several studies over the past decade have extensively documented the advantages of polymeric micelles as anticancer drug carriers, specifically their ability to overcome limitations stemming from toxicity, and agent solubilization and long circulation time.[9,10] Polymer micelles consist of a hydrophobic core that acts as a carrier compartment that accommodates hydrophobic agents, and the soluble shell consists of a brush-like protective corona that stabilizes the nanoparticles in aqueous solution. Upon encapsulation within the micelles, solubility limits for hydrophobic drugs can be exceeded.[11] Until now, most reported micelle systems have focused on the delivery of hydrophobic anticancer drugs, with examples including micelles based on PEGylated polyesters such as poly(ethylene glycol)-*b*-poly(D,L-lactide) (PEG-PLA) and poly(ethylene glycol)-*b*-poly(ε-caprolactone) (PEG-PCL), which have been reported for the delivery of doxorubicin and paclitaxel.[12,13] Owing to their nanoscaled dimension, these micellar particles are also expected to escape the quick kidney excretion. Despite several advantages, a significant challenge for micelle delivery systems is how to achieve high targeting efficiency at the tumor sites and associated cells. One strategy, adopted by many researchers is the functionalization of the micelles with a ligand that can selectively bind to a specific receptor over-expressed on the cell surface (i.e. active targeting). However, only a handful of reports have dealt with the modification of micelles with an active targeting ligand, specifically PEG-PCL micelles, in this fashion. Among them, a cyclic pentapeptide, cRGDfK, which is specific to the αvβ3 receptor, was demonstrated to significantly enhance the uptake of micelles by SLK tumor endothelial cells.[14] Furthermore, Lee and coworkers showed that PEG-PCL micelles, with folate acid conjugated to the PCL block, demonstrated a folate-directed targeting of micelles to MCF-7 cells and HeLa 229 cells for the delivery of doxorubicin.[15]

The purpose of the present work is to propose a novel, tumor-targeted polymeric micelle platform for the delivery of FG020326. Until now, only passive targeting of P-gp inhibitor Cyclosporin A (CsA) by PEO-b-PCL micelles has been reported by Lavasanifar et al.[16,17] We propose micelle particles fabricated from the biodegradable copolymer poly(ethylene glycol)-*b*-poly(ε-caprolactone) (PEG-PCL) containing FG020326 and functionalized with folate on their surface layer to target tumor cells possessing an over-expression of folate receptors on the cellular membrane. In contrast to the strategy by Lee and coworkers in which folate was conjugated to the PCL block, we attached folate to the PEG distal ends in the present work in hopes that the targeting ligand would have a better chance of being presented out of the surface layer of micelles for a further enhancement of targeting effect of the folate-bearing PEG-PCL micelles. In the present work, copolymers for fabrication of targeting micelles were synthesized via multistep, sequential syntheses of allyl-PEG-PCL by anionic reaction, NH_2-PEG-PCL by radical addition reaction in water with potassium persulfate ($K_2S_2O_8$) as a catalyst, and folate-PEG-PCL by conjugation of folic acid. FG020326 loading and its *in vitro* release were investigated. Efficient cell internalization of FG020326-loaded targeting micelles was demonstrated using a Rhodamine 123 efflux assay. As hypothesized, FG020326 transported with folate-bearing micelles rather than folate-free micelles remarkably resensitized MDR KB_{v200} cells to VCR as demonstrated by cell culture experiments. Taking into consideration the fact that folate recep-

tors are known to over-express in various types of tumors, including ovarian, lung, colorectal, renal, breast cancers, and non-Hodgkin's lymphomas,[18] we expect that this novel platform of folate-directed delivery of MDR modulators is of great potential for cancer treatment purposes.

MATERIALS AND METHODS

Materials

ε-Caprolactone (ε-CL, from Sigma-Aldrich) and allyl alcohol (from Guangzhou Chemical Reagent Factory, China) were both purified by vacuum distillation over calcium hydride (CaH_2). Tetrahydrofuran (THF, from Sigma-Aldrich) was dried by refluxing over a sodium-potassium alloy and distilled under dry argon. 18-Crown-6 (from Sigma-Aldrich) was vacuum-dried overnight at 46°C. 2-Aminoethanethiol hydrochloride, folic acid, *N*-hydroxysuccinimide (NHS), naphthalene, potassium persulfate ($K_2S_2O_8$), dicyclohexylcarbodiimide (DCC), azobisisobutyronitrile (AIBN) were purchased from Sigma-Aldrich and used as received. Ethylene oxide (EO, purity 99%) stored inside a gas tank were obtained from Foshan Kedi Gas Chemical Industry (China) and used as received. All organic solvents are of analytic grade. Phosphate-buffered solutions (PBS, pH 7.4 and 5) were prepared in our laboratory. Potassium naphthalide solution was prepared by adding potassium into an anhydrous THF solution of naphthalene and then stirring the mixture at room temperature for 1 h under dry argon, just prior to use. FG020326 was synthesized according to a previously published procedure in the literature.[19,20] VCR was purchased from ShenZhen Main Luck Pharmaceuticals, China. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was purchased from Sigma-Aldrich. Dulbecco's modified Eagle's medium (DMEM) and RPMI-1640 were purchased from Gibco BRL (USA). KB_{v200} is a human epidermoid carcinoma MDR cell line with high expression of P-gp as well as folate receptors on the cell membrane. KB_{v200} cells were cultured with RPMI-1640 culture medium containing 10% FBS at 37°C in a humidified atmosphere of 95% air plus 5% CO_2.

Synthesis of allyl-terminated diblock copolymer of PCL and PEG (allyl-PEG-PCL)

The copolymer was synthesized by sequential anionic ring-opening polymerization of EO and ε-CL in one pot using potassium alkoxide as an initiator. THF solution (4 mL) of potassium naphthalide was allowed to mix with 0.5-mL allyl alcohol, and then the mixture was stirred for 15 min into a flame-dried reaction flask equipped with a magnetic stirring bar and two capillary gas inlets for EO and argon, respectively. Subsequently, 20 mL anhydrous THF and 1.5 g 18-crown-6 predissolved in 5-mL anhydrous THF in another flamed flask were then transferred into the first reaction flask under argon. After stirring for another 15 min, the mixture was cooled with a salted ice-water bath of −5°C. A precalculated amount of dry EO was slowly blown and condensed into the reaction mixture. Afterwards, the EO polymerization was conducted at 0°C for 24 h and then at room temperature for 3 days to ensure a thorough conversion of EO. In the second step, a predesigned amount of ε-CL was injected into the reaction flask under argon protection and then polymerized at room temperature for 48 h. The polymerization was finally quenched by adding a small amount of acetic acid. The crude copolymer collected by precipitation in hexane was redissolved in dichloromethane and added to ten-fold diethyl ether under vigorous stirring. A white powder was sequentially isolated by filtration and washed with hexane and diethyl ether.

Conversion of allyl-PEG-PCL into NH_2-PEG-PCL

This procedure was carried out by a radical addition reaction of 2-aminoethanethiol hydrochloride, which was modified based on a previously reported method by Kataoka and coworkers for converting allyl-PEG into NH_2-PEG.[21] The reaction was conducted in an aqueous micelle solution, which was prepared by slowly adding a THF solution (2 mL) of allyl-PEG-PCL (0.5 g) into distilled water (20 mL) under stirring, and then allowing evaporation of THF and formation of micelles as previously reported.[22] The micelle solution was first bubbled with nitrogen for 1 h to remove oxygen, and then $K_2S_2O_8$ (0.8 molar equivalent of allyl-PEG-PCL)and 2-aminoethanethiol hydrochloride (10-fold molar equivalent of allyl-PEG-PCL) were added into the above solution. Subsequently, the micelle solution was sealed in a nitrogen atmosphere and stirred for 5 h at 52°C. Unreacted 2-aminoethanethiol hydrochloride and $K_2S_2O_8$ were removed by dialysis against water for 24 h at room temperature (MW cut-off: 8000 Da). Lithium hydroxide solution (1*M*) was then added in a dropwise fashion into the micelle solution to adjust the pH from 7.4 to 9.4, which converts the terminal amine salt into primary amino groups. The obtained micelle solution was immediately freeze-dried. After lyophilization, the micelle powder was redissolved in THF, filtered across a 220-nm pore-size membrane to remove lithium chloride and unreacted lithium hydroxide, and finally precipitated into hexane to recover pure copolymer (yield > 78%). Conversion rate of allyl- to NH_2- was calculated based on the analysis of NMR spectra of allyl-PEG-PCL and NH_2-PEG-PCL, as shown in Figure 2. Integral values of absorption peaks "a" and "h" in Figure 2(a), "d" and "h" in Figure 2(b) (denoted as I_{a-2a}, $I_{h-2a,}$ I_{d-2b}, and I_{h-2b}, respectively) were used for the calculation. The conversion rate of allyl-PEG-PCL into NH_2-PEG-PCL was calculated as $(I_{d-2b}/I_{h-2b})/(I_{a-2a}/I_{h-2a})$.

Preparation of folate-conjugated copolymer (folate-PEG-PCL)

Folate was first activated with NHS according to a previously published procedure.[23] Briefly, folic acid (1 g) dissolved in anhydrous DMSO (30 mL) was reacted overnight with NHS (0.9 g) in the presence of DCC (0.5 g) under argon at room temperature, and the major byproduct, 1,3-dicyclohexylurea (DCU), was removed by filtration. Subse-

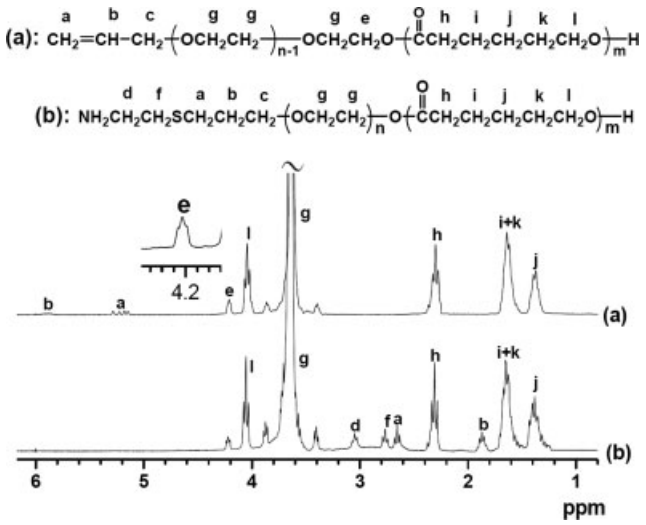


Figure 2. ^{1}H NMR spectra of allyl-PEG3k-PCL1k (a) and NH_2-PEG3k-PCL1k (b) in $CDCl_3$. The expanded peak "e" at ~4.2 ppm indicates the successful synthesis of block copolymer.

quently, the above activated folate solution (3 mL) was added to a DMSO solution (5 mL) containing NH_2-PEG-PCL (0.4 g) and triethylamine (0.05 mL). The reaction was performed at room temperature for 10 h under argon. The resulting solution was centrifuged and filtered. The filtrate thus obtained was dialyzed against water for 24 h (MW cut-off: 1000 Da). The aqueous solution inside the dialysis bag was then freeze-dried. The powdery sample was redissolved in THF (3 mL), and the filtrate was added dropwise to distilled water under stirring. After overnight evaporation of THF, the resultant micelle solution was dialyzed against water for 5 days to completely remove unreacted folic acid and any residual THF. The micelle solution was finally freeze-dried to yield a solid powder (yield > 82%). To evaluate the conversion rate of NH_2-PEG-PCL into folate-PEG-PCL, copolymer was dissolved in DMSO and folate absorbance at 363 nm was measured by a Unico UV-2000 UV–Vis spectrophotometer to quantify the folate mass content in the sample. Absorbance of folate at 363 nm in DMSO with various concentrations was measured to generate a calibration curve.

Characterization of copolymers

Nuclear magnetic resonance (NMR) spectra were recorded on a Varian 300-MHz NMR spectrometer in deuterated water (D_2O), chloroform ($CDCl_3$), or DMSO depending on sample solubility at room temperature. Gel permeation chromatography (GPC) was employed to determine molecular weight and molecular weight distribution. GPC analysis was carried out using a SHODEX® 7.8 mm × 300 mm column with chloroform as an eluent (1 mL/min) and polystyrene standards for column calibration. Twenty microliter samples were injected with a microsyringe, and the eluent was analyzed with a differential refractive index (RI) detector Waters 2414 from Waters (USA). High performance liquid chromatograph (HPLC) was performed in pH 7.4 PBS using an Agilent(HP)1100 chromatographic instrument to analyze the content of free folate in the folate-conjugated copolymer samples. Folate solutions with various concentrations were first analyzed with HPLC to generate a calibration curve. The total folate amount in each sample was determined by measuring the absorbance of folate at 363 nm in DMSO with a Unico UV-2000 UV–Vis spectrophotometer.

Determination of critical micellization concentration

The critical micellization concentration (CMC) of folate-targeted copolymers was determined by fluorescence measurements using pyrene as an extrinsic probe, as previously reported.[24] The fluorescence spectra of pyrene were measured at varying polymer concentrations using a Shimadzu RF-5301PC fluorescence spectrometer (Japan) at 25°C. The excitation wavelength was adjusted to 339 nm, and the detection of fluorescence was performed at 375 and 385 nm, which correspond to the wavelength of the (0,0) band and the (0,2) band of pyrene fluorescence, respectively. CMC was measured from the onset of a rise in the intensity ratio of peaks at 385 nm to peaks at 375 nm in the fluorescence spectra of pyrene plotted *versus* the logarithm of polymer concentration.

Preparation of FG020326-loaded micelles

Polymeric micelles containing FG020326 were prepared as follows: 10 mg of copolymer (allyl-PEG-PCL or folate-PEG-PCL) and 2 mg of FG020326 were codissolved in 2-mL THF in a glass vial. The solution was then added to pure water (20 mL) under sonication using a UP 50H Dismembrator (Hielscher, Germany). THF was allowed to slowly evaporate overnight, leading to the formation of micelles (Fig. 1). Residual THF was completely removed by vacuum evaporation at room temperature with a rotary evaporator. The micelle solution, concentrated to 5 mL, was filtered with a syringe filter (pore size: 0.22 μm) to eliminate polymer and FG020326 aggregates, and then dialyzed against pure water for 24 h to remove unencapsulated FG020326 (MW cut-off: 8000 Da). The micelles thus obtained were characterized with photon correlation spectroscopy, which was performed at 25°C on a BI-200 SM dynamic laser scattering system from Brookhaven Instruments. Scattered light was detected at 90° and collected on an autocorrelator. For each sample, data obtained from five measurements were averaged to yield the size and size distribution. The polymer concentration in size measurement studies was $\sim 10^{-3}$ mg/mL. Solid micelle samples for NMR, GPC and UV–Vis experiments were obtained by lyophilization of micelle solutions.

Determination of drug-loading content

The drug-loading content (DLC) was defined as the weight percentage of FG020326 in the micelle. DLC was quantified by determining the absorbance at 411.5 nm

using a Unico UV-2000 UV–Vis spectrophotometer. The freeze-dried micelle samples were redissolved in THF for the UV–Vis measurement. FG020326 solutions of various concentrations in THF were prepared, and the absorbance at 411.5 nm was measured to generate a calibration curve for the DLC calculations for various micelles. The extinction coefficient of FG020326 at THF is $\varepsilon_{411.5} = 1.67 \times 10^4\ M^{-1}\ cm^{-1}$. The drug-loading content was calculated using the following equation:

$$\text{DLC} = \frac{\text{Amount of FG020326 in micelle (mg)}}{\text{Amount of FG020326} - \text{loaded micelle (mg)}} \times 100$$

In vitro release of FG020326 from micelles

Freeze-dried micelle samples (10 mg each) were resuspended in PBS (pH 7.4 or 5) and transferred into a dialysis bag (MW cut-off: 8000 Da). The bag was placed into 25 mL PBS (pH 7.4 or 5). The release study was performed at 37°C in a Shanghai Yiheng Scientific DKZ incubator shaker. At selected time intervals, solution outside the dialysis bag was removed for UV–Vis analysis and replaced with fresh buffer solution. FG020326 concentration was calculated based on the absorbance intensity of FG020326 at 411.5 nm. In the assessment of drug release behavior, the cumulative amount of the released drug was calculated, and the percentages of drug released from each micelle were plotted against time. Release of free drug from the dialysis bag at both pHs were performed as control following the same procedure as described above.

MTT cytotoxicity assay

The inhibition of cell growth was evaluated by the MTT method using triplicate assays. Human epidermoid carcinoma cell lines KB_{v200} cells were harvested during logarithmic growth phase, seeded in 96-well plates at 0.3×10^4 cells/well, and then adjusted to a final volume of 170 μL with RPMI-1640 culture medium containing 10% FBS. After 24 h incubation, the cells were incubated with culture media containing VCR and FG020326-free or FG020326-loaded micelles at predesigned concentrations in each well. FG020326 concentration in FG020326-containing media, i.e. media containing either free FG020326 or FG-020326-loaded micelle, was set to 2 μmol/L. Cell incubation with VCR and copolymers below the CMC was also performed as control. VCR and micelle at various gradient concentrations in the culture media were obtained by media dilution. After 72 h, 10 μL of MTT solution (5 mg/mL in 0.9% NaCl saline) was added to each well and the incubation was continued for an additional 4 h in a 37°C incubator containing 5% CO_2, allowing the viable cells to reduce the yellow MTT into dark-blue formazan crystals, which were then dissolved in 100 μL of DMSO. The absorbance in individual wells was determined at 540 and 655 nm by a microplate reader (BIO-RAD). The VCR concentration required to inhibit cell growth by 50% (IC_{50}) was calculated from the cytotoxicity curves (Bliss's software). Free FG020326 in its protonated form was also tested as a control. The MDR reversal effect was assessed by quantifying the VCR IC_{50} values with or without the presence of FG020326 in the culture media.

Rhodamine 123 efflux studies

KB_{v200} cells were exposed for 2 h to media containing one of the following: blank micelles, copolymers below the CMC, FG020326-loaded micelles, and free FG020326. In a control experiment, KB_{v200} cells were first incubated with free folate (10 m*M*) for 1 h, and then coincubated with folate-functionalized and FG020326-loaded micelles for 2 h. FG020326 concentration in media containing FG020326-loaded micelles or free FG020326 was 2 μmol/L. The cells were collected and washed once and then resuspended in 1 mL RPMI-1640 at the concentration of 1×10^5 cells/mL. The cells were then loaded with 5 μg/mL of Rhodamine 123 for 30 min at 37°C. After one wash with dye-free RPMI-1640, cells were allowed to efflux the dye for 10 min in dye-free RPMI 1640 at 37°C. The cells were then washed out and suspended in 1 mL RPMI 1640, after which flow cytometric analysis was carried out using a FACScan flow cytometer to evaluate the MDR strength of KB_{v200} cells.

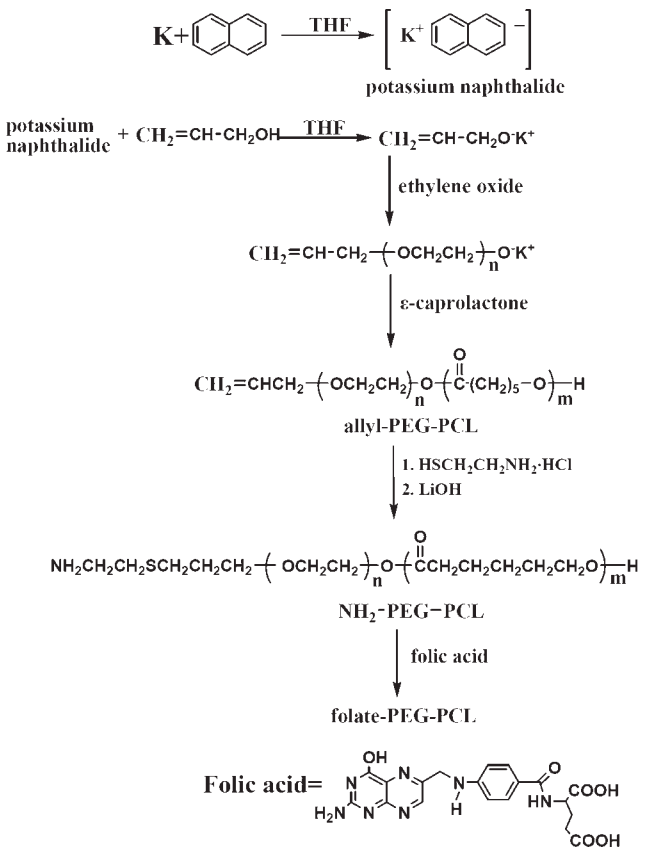
Statisistical analysis

The experimental data were measured as mean values with standard deviations. Statistical analysis was carried out using the Student's *t*-test. Differences were considered statistically significant with $p < 0.05$.

RESULTS AND DISCUSSION

Syntheses of block copolymers

Synthesis of allyl-terminated PEG using allyl alkoxide as an initiator during anionic polymerization of EO, and subsequent conversion of allyl groups to primary amino groups by radical addition reaction of 2-aminoethanethiol hydrochloride, has been reported earlier by Kataoka and coworkers.[21] Using a similar approach, we synthesized primary amino group-capped PEG-PCL for folate conjugation. The synthetic route for the targeting copolymer, folate-PEG-PCL, as shown in Scheme 1, outlines three major reaction steps involved in the synthesis. The synthesis of allyl-terminated diblock copolymer (allyl-PEG-PCL) by stepwise anionic ring-opening polymerization of EO and ε-CL was performed in a well controlled manner regarding the copolymer molecular weight as well as the length of individual blocks. To carry out the conversion of allyl groups of copolymers into primary amino groups, we first attempted the radical addition reaction of 2-aminoethanethiol hydrochloride with copolymer in DMF at 70°C using AIBN as an initiator, according to the



Scheme 1. Synthetic approach to folate-PEG-PCL.

method reported by Kataka et al. for the conversion of allyl-ended PEG into PEG mono-amine. However, the loss of PCL blocks of copolymers was noted under such reaction conditions for neat PEG, which is likely due to the susceptibility of the PCL carbonyl group to a nucleophilic attack of 2-aminoethanethiol hydrochloride at the elevated temperature 70°C. Indeed, the primary amino groups, even if protonated, have been reported to show some nucleophilic activity.[25] Therefore, we modified the reaction conditions of the above radical addition reaction, which allowed us to achieve complete allyl conversion while avoiding loss of integrity of PCL blocks. The major difference between our procedure and Kataoka's is that, in the present work, the radical addition of thiol groups to the allyl double bonds was performed at a lower temperature (50°C) in an aqueous micelle solution, in which the PCL blocks are insoluble and thus well protected, rather than in an organic phase that may dissolve both PEG and PCL, and as a result, water-soluble $K_2S_2O_8$ rather than AIBN was used as an initiator. After the radical addition reaction, 1*M* lithium hydroxide solution was added dropwise to deprotonate the terminal amine chloride salt into the primary amino group. After freeze-drying of the micelle solution, micelle powder was dissolved in THF and filtered to remove lithium chloride and unreacted lithium hydroxide. About 100% conversion of allyl to amino group has been obtained for both allyl-PEG3k-PCL1k and allyl-PEG3k-PCL2k. There is no evidence in the GPC and NMR measurements that the above process caused PCL cleavage (Table I). Finally, folic acid was activated and then conjugated to the PEG distal ends of copolymers. Although folic acid possesses α- and γ-carboxylic acid groups, which could both be activated by NHS, the γ-carboxylic acid group has much higher reactivity.[26,27] Therefore, copolymers conjugating folate through the γ-carboxylic acid reaction should be produced predominantly. We obtained a conversion rate of 82.2% for folate-PEG3k-PCL1k, and 81.5% for folate-PEG3k-PCL2k respectively from their NH_2-PEG-PCL intermediates.

Spectroscopic methods were employed to characterize diblock intermediates (i.e., allyl- and NH_2-terminated copolymers) and folate-PEG-PCLs. As shown in Figures 2 and 3, the copolymer structures were confirmed by ^{1}H NMR measurements in which the characteristic resonances of both PCL and allyl-ended PEG were observed, indicating the coexistence of both blocks in the purified samples. Notably, the ^{1}H NMR absorption around 4.2 ppm in Figure 2, i.e., the triplet peak "e," strongly demonstrated the linkage structure between PCL and PEG blocks, complying with the expected copolymer structure in Scheme 1. By comparing the integrals of characteristic peaks of PEG blocks (e.g., the singlet of —O*CH*$_2$— at 3.65 ppm) and PCL blocks (e.g., the triplet of —C(=O)—*CH*$_2$— at 2.25 ppm) with that of characteristic peaks of the terminal allyl groups (e.g., the multiplet of CH$_2$=*CH*— at 4.90 ppm) in the ^{1}H NMR spectrum, the length of PEG and PCL blocks was calculated and reported as molecular weights in Table I.

GPC measurements also demonstrated the successful synthesis of the diblock copolymer by revealing a unimodal molecular weight distribution in the GPC chromatograms (data not shown). Only a negli-

TABLE I
Characteristics of the Prepared Block Copolymers

Code	Polymer	M_n[a]	M_n[b]	M_w/M_n[a]
1	Allyl-PEG3k-OH	2943	NA	1.106
2	Allyl-PEG3k-PCL1k	3808	3724	1.097
	NH_2-PEG3k-PCL1k	4018	3870	1.215
	Folate-PEG3k-PCL1k	5090	NA	1.261
3	Allyl-PEG3k-PCL2k	5125	5047	1.092
	NH_2-PEG3k-PCL2k	5224	5026	1.132
	Folate-PEG3k-PCL2k	6079	NA	1.271

NA: data not available.
[a]Determined by GPC with RI detector.
[b]Calculated based on ^{1}H NMR spectra. The GPC-determined molecular weight of PEG block, 2943, was used for calculation.

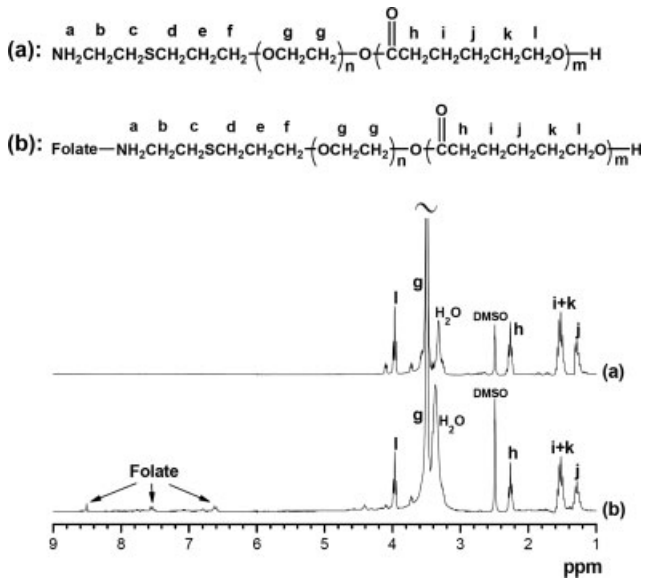


Figure 3. ^{1}H NMR spectra of NH_2-PEG3k-PCL1k (a) and folate-PEG3k-PCL1k (b) in DMSO-d_6.

gible increase in molecular weight of copolymer upon radical addition process was detected (Table I). By comparison, folate conjugation induced an increase in the copolymer molecular weight in GPC measurements (about 800–1000), due to the molecular weight addition effect of folate molecules (M_n of folic acid: 441). This molecular weight increase in GPC measurements is acceptable considering the detection variation of GPC analysis. GPC measurements with the UV detector did not detect a secondary peak in the chromatogram of the folate-conjugated copolymers (data not shown). Furthermore, the amount of unattached folate molecules determined by HPLC measurement in these copolymers is negligible (i.e. less than 0.9 wt % of the total amount of folate in each sample). This small residual amount of free folic acid should have little effect on micelle targeting to KB_{v200} cells. On the whole, both NMR and GPC measurements suggested that the radical addition reaction of aminoethanethiol hydrochloride with the allyl-bearing copolymer did not lead to a significant increase in copolymer molecular weight, implying that radical polymerization of allyl groups was suppressed to a great extent under our modified reaction conditions.

The critical micellization concentration determined by fluorescence measurements using pyrene as a probe was 9.6×10^{-6} g/mL for folate-PEG3k-PCL1k, and 3.8×10^{-6} g/mL for folate-PEG3k-PCL2k.

Micelle properties

Similar to the earlier observations for the doxorubicin or paclitaxel-loaded PEG-PCL micelles,[22,28] Figure 4 demonstrates that FG023026 was encapsulated inside the PCL core of the core-shell-structural micelles. In $CDCl_3$, prominent resonance peaks (about 1.2, 2.08 ppm) of FG020326 were clearly observed in the ^{1}H NMR spectrum, in addition to those of PCL and allyl-PEG blocks, indicating that the micelle contains both copolymer and FG020326. However, in D_2O, only the PEG resonance peaks were detected while both the PCL and FG020326 resonance peaks were hardly observed. Based on these phenomena, we reasoned that the micelle shells consisting of PEG blocks were solvated in D_2O and therefore showed clear ^{1}H NMR signals. In contrast, resonance peaks of PCL blocks and FG020326 confined inside the solid micelle core were hardly observed due to their insufficient chain mobility in D_2O.

As shown in Table II, micelles predominantly less than 100 nm in diameter were successfully prepared, and the micelle size appears to be dependent on both copolymer composition and drug-loading. In general, similar to the doxorubicin or paclitaxel-loaded PEG-PCL micelles reported earlier,[22,28] copolymers with longer PCL blocks formed larger micelle particles. Folate functionalization of copolymers does not lead to an obvious change in micelle size. However, unlike the previously reported data for the doxorubicin or paclitaxel-loaded PEG-PCL micelles, where micelle size is mostly independent

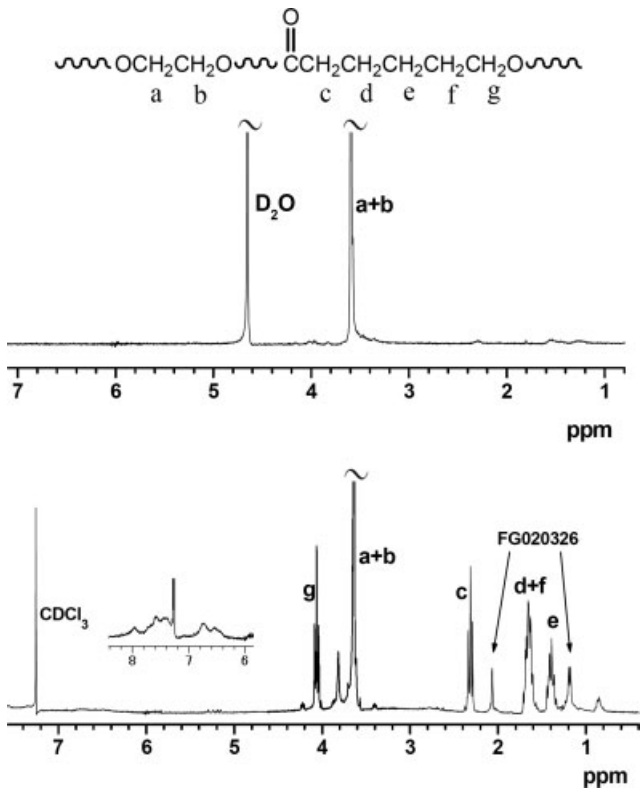


Figure 4. ^{1}H NMR spectra of FG020326-loaded allyl-PEG3k-PCL2k micelles in D_2O (a) and $CDCl_3$ (b).

TABLE II
Influence of Copolymer Composition on Micellar Properties

Copolymers	Micelle Size (nm)		Drug Loading Content (%)
	FG020326 Unencapsulated	FG020326-Loaded	
Allyl-PEG3k-PCL1k	51.4 ± 2	76.7 ± 3	5.3 ± 0.2
Allyl-PEG3k-PCL2k	82.4 ± 2	98.8 ± 3	6.1 ± 0.3
Folate-PEG3k-PCL1k	54.1 ± 2	73.6 ± 2	5.6 ± 0.2
Folate-PEG3k-PCL2k	81.8 ± 3	117.8 ± 4	6.4 ± 0.4

of drug-loading, FG020326-loading apparently led to the increase in particle size for both folate-free and folate-bearing micelles. FG020326-loading for the most part did not change the narrow size distributions of micelles. In addition, the loading content of FG020326 (DLC) in folate-PEG-PCL micelles increased from 5.6% to 6.4% with an increase of PCL molecular weight from 1 to 2 kDa, likely due to the fact that longer PCL brings about more micellar hydrophobic space for the drug to embed in, as well as stronger hydrophobic interaction between copolymer and FG020326.

In vitro FG020326 release properties

In the control experiments, free FG020326 quickly diffused out of the dialysis bag at both pH values [Fig. 5(a)]. The release of free FG020326 reached 100% in 3.5 h. The *in vitro* release profiles of FG020326-loaded micelles in two different buffered solutions (PBS at pH 7.4 and 5.0) were shown in Figure 5(b). The considerably slower release rate of micelle encapsulated drug than that of the free drug indicates the negligible effect of the dialysis bag on the detected rate of drug release from the micelles. The release behavior of FG020326 from the PEG-PCL micelles is similar to that of doxorubicin from the same type of micelles.[22] Briefly, a relatively rapid release in the first phase, followed by a sustained and slower release over a prolonged time up to several weeks, was observed in both solutions. Furthermore, in comparison with the release at pH 5.0, FG020326 release from micelles at pH 7.4 was obviously slower ($p < 0.05$ for both formulations). It is important to note that FG020326, like doxorubicin, can be protonated at low pH resulting in an increase of drug solubility in aqueous solutions. Indeed, we determined the solubility values of FG020326 at pH5 and at pH7.4 to be 9.2×10^{-5} g/mL and 2.4×10^{-5} g/mL, respectively. Therefore, it is expected that FG020326 can be released faster when the micelle solution becomes acidic, e.g. at pH 5. Another reason causing the faster release of FG020326 at pH 5 might be that the micelle core hydrolyzed faster at lower pH. For our FG020326-loaded micelles, the amount of released FG020326 for 24 h was about 15 wt % at pH 7.4, while it reached about 30 wt % at pH 5.0. This type of pH-dependent releasing behavior of drugs is of particular interest for tumor-targeted drug delivery using polymeric micelles. In the present case, a fast FG020326 release can be anticipated to occur once the micelle particles are internalized inside the tumor cells via folate-mediated endocytosis and trapped inside the lysosomal compartments where the pH value is known to drop to around 5.0.[29] Consequently, much enhanced bioavailability of the micelle-transported FG020326 is likely to occur, which is essential for achieving the desirable MDR reversal effect. FG020326 release rates

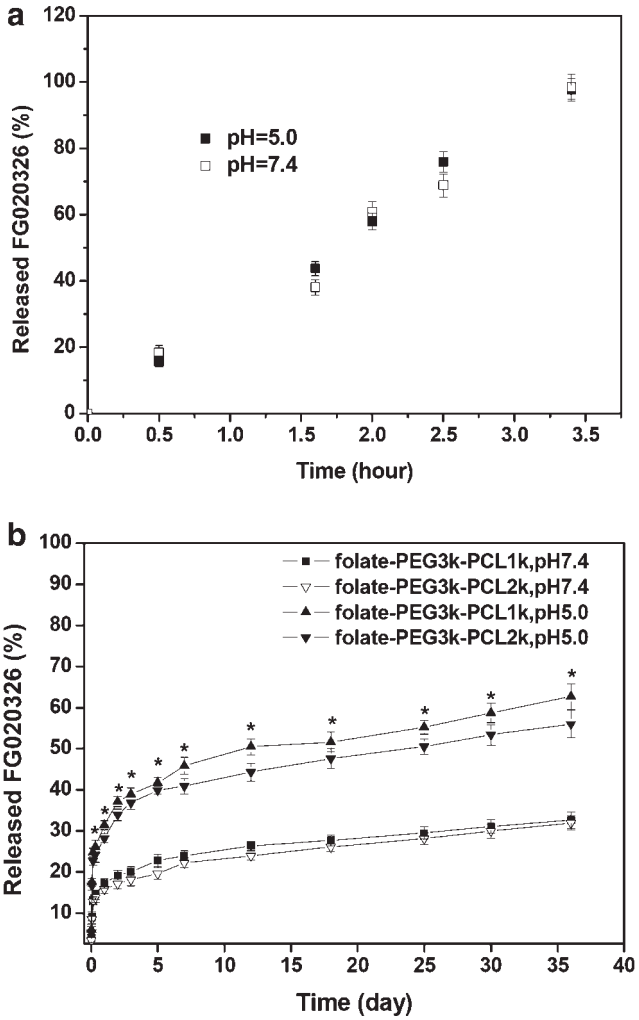


Figure 5. (a) Release profiles of free FG020326 from saturated solutions at pH 5.0 and pH 7.4. Data are presented as mean ± SD ($n = 3$). (b) *In vitro* FG020326-release profiles for two micelle formations, folate-PEG3k-PCL1k and folate-PEG3k-PCL2k, at neutral (pH 7.4) and acidic (pH 5.0) conditions at 37°C. Data are presented as mean ± SD ($n = 3$). *Denotes a significant difference between FG020326 release at that time point ($p < 0.05$, same formulation at different pH).

from the two micelles at pH 7.4 are similar within 35 h, which is to some extent consistent with the observation that CsA release from PEO-b-PCL micelles was almost not affected by the PCL length.[16] At pH 5, FG020326 release from the PEG3k-PCL2k micelle appears to be slower than that from the PEG3k-PCL1k micelle ($p < 0.05$ for most time points starting from 24 h). At this condition, it is likely that FG020326 traversing the larger micelle particle may be retarded because of the longer diffusion path.[30]

Although polymeric micelles provide an effective solution for the delivery of poorly soluble drugs, drug leaking out from micelles at the initial hours after injection is a commonly existing problem. Our research indicates that, at pH 7.4, which is close to the pH of normal biological fluids, the percentage of FG020326 released in 24 h for both formulations is about 15%, i.e. 85% of the encapsulated FG020326 is still trapped in micelles after 24 h. Note that micelle in solution can be freeze-dried to keep its intactness before use and 24 h is considerably long compared to the circulation time of small molecular drugs in blood. Although not perfect, these micelle systems still provided a biologically meaningful platform for FG020326 delivery. Further research in our labs is underway to get more desirable FG020326 release profile, i.e. reducing release at first hours and enhancing the release rate in the following sustained release stage, by varying the carrier polymer structure.

MDR reversal

A number of experimental and clinical approaches have been studied to overcome MDR effects in cancer cells.[31] For instance, either conjugating drug to a soluble polymer carrier like *N*-(2-hydroxypropyl)methacrylamide (pHPMA) or transporting drug with a polymeric micelle was demonstrated as an effective approach to overcome MDR. Unlike free drugs which cross the membrane by diffusion, a pathway that is affected by P-gp in MDR cells, polymer-drug conjugates or drug-encapsulated polymeric micelles are most likely transported into the cells via endocytosis, which is not affected by P-gp.[8,32,33] In addition, the use of MDR modulators, e.g. cyclosporine A and some surfactants such as Pluronic® block copolymer below the CMC, were demonstrated to be a common and effective approach to inhibit P-gp-mediated drug efflux.[31,34] Passive targeting of P-gp inhibitor Cyclosporin A by PEO-b-PCL micelles has been reported recently by Lavasanifar and coworkers. Their study focused on the pharmacokinetics and biodistribution of micelle-transported drug, and no information is available on the MDR reversal effect of this system.[16,17]

In the present work, the MDR reversal potency was evaluated by MTT assays that revealed the cytotoxicity of anticancer VCR with and without the presence of FG020326 in the culture media. Previous studies have demonstrated that Pluronic® block copolymers below the CMC, i.e. block unimers of poly(polyethylene oxide) and poly(propylene oxide), are very effective P-gp function inhibitors, and Pluronic micelles do not possess such function of P-gp inhibition.[31] To clarify whether PEG-PCL itself may inhibit the P-gp function like Pluronic unimers do, we tested the MDR reversal effect of these copolymers without the presence of FG020326. Cells were treated with copolymer solutions either above or below the polymer CMC. Our results indicate that in either case, above or below CMC, neither folate-PEG3k-PCL1k nor folate-PEG3k-PCL2k obviously improved the cytotoxicity of VCR to KB_{v200} cells [Table III and Fig. 6(a,b)]. This implies that folate-PEG-PCL alone cannot suppress the P-gp drug efflux function of KB_{v200} cells regardless of whether it is in unimer or micellar form. By contrast, FG020326 and

TABLE III
IC_{50} of VCR in KB_{v200} Cells Determined by MTT Assay

Modulator Used in VCR Toxicity Test			$IC_{50} \pm SD$
FG020326-loaded allyl-PEG3k-PCL1k micelle[a]			0.0202 ± 0.0051
FG020326-loaded allyl-PEG3k-PCL2k micelle[a]			0.0207 ± 0.0042
FG020326-loaded folate-PEG3k-PCL1k micelle[a]			0.0046 ± 0.0031[b]
FG020326-loaded folate-PEG3k-PCL2k micelle[a]			0.0089 ± 0.0022
Free FG020326[a]			0.0059 ± 0.0013
None			0.3157 ± 0.1102
FG020326-free unimers or micelles	Folate-PEG3k-PCL1k	9.6×10^{-5} (>CMC)	0.3523 ± 0.0923
		1.0×10^{-6} (<CMC)	0.3489 ± 0.1045
	Folate-PEG3k-PCL2k	3.8×10^{-5} (>CMC)	0.3681 ± 0.0758
		1.0×10^{-7} (<CMC)	0.3315 ± 0.0824

Various experiments were conducted to clarify the performance of the FG020326-loaded micelles in terms of the MDR reversal.

[a] KB_{v200} cells were incubated at the same FG020326 concentration of 2 μmol L^{-1}.

[b] $p < 0.01$ (folate-PEG3k-PCL1k *vs.* allyl-PEG3k-PCL1k).

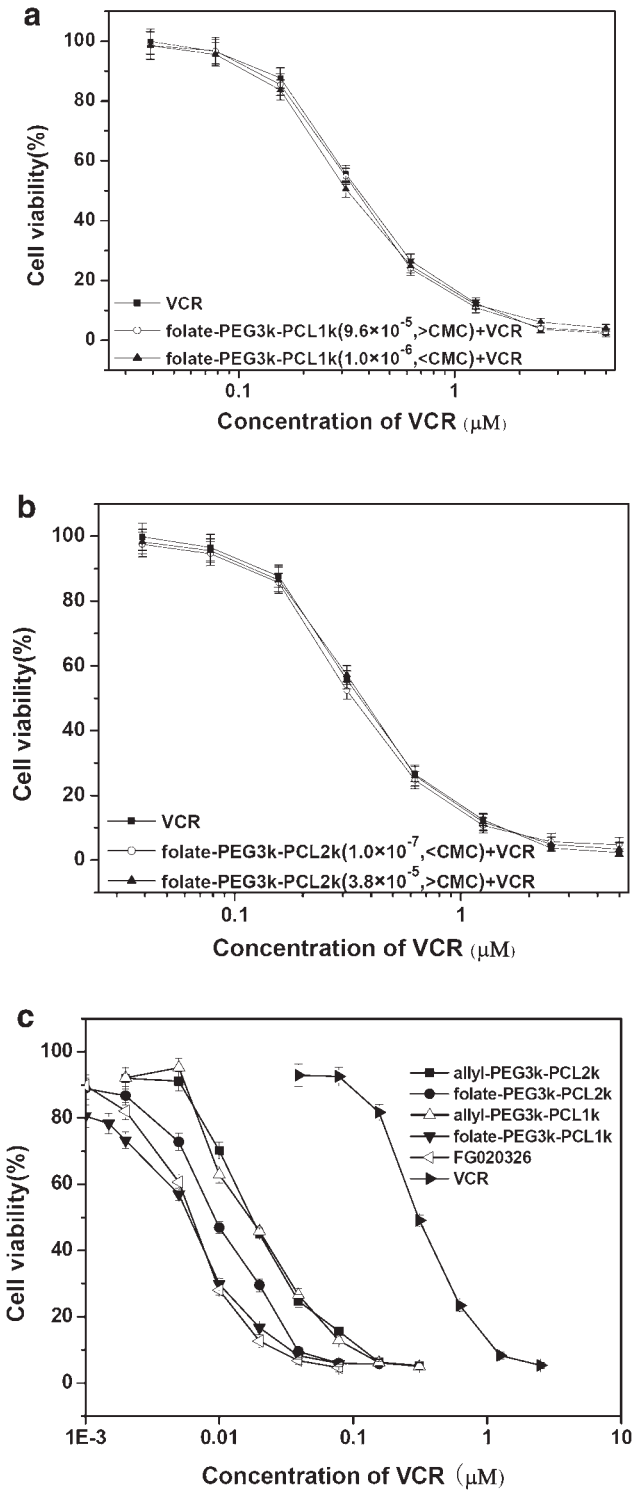


Figure 6. (a, b) Effect of FG020326-free unimers (below CMC) and micelles (above CMC) on the cytotoxicity of VCR in KB_{v200} cells. Data are presented as mean ± SD ($n = 3$). (c) Effect of FG020326 or FG020326-loaded polymeric micelles on the cytotoxicity of VCR in KB_{v200} cells. Cells were incubated at the same FG020326 concentration 2 μmol L^{-1}. Data is presented as mean ± SD ($n = 3$).

FG020326-loaded micelles showed significant MDR reversal effect on KB_{v200} cells. It is known that FG020326 re-sensitizes KB_{v200} cells by inhibiting the function of P-gp associated with the resistance of cancerous cells to anticancer drugs.[6] As can be seen, FG020326 transported by micelles functioned the MDR reversal of KB_{v200} cells.

In the VCR concentration-effect curves shown in Figure 6(c), the concentration yielding half-maximal inhibition to cell growth (IC_{50}) was detected at 0.316 μ*M* for KB_{v200} cells not treated with FG020326 or FG020326-containing micelles (i.e. the negative control group). In comparison, IC_{50} values lower than 0.021 μ*M* were detected for cells treated with FG020326 or FG020326-containing micelles, which is at least one order of magnitude lower than that for the negative control. Moreover, in comparison with the cells exposed to the folate-free and FG020326-loaded micelles, cells exposed to the folate-bearing and FG020326-loaded micelles are apparently even more sensitive to VCR treatment. For example, MTT assays with the PEG3k-PCL1k micelles revealed a significant IC_{50} decrease from 0.02 to 0.0046 μ*M* upon folate functionalization of micelles. A major cause for this significant IC_{50} decrease of VCR in MTT assay upon folate-functionalization of micelles may be the augmented internalization of FG020326-loaded micelles into KB_{v200} cells, which overexpress the folate receptors on the cell membrane, i.e. more FG020326 transported into the cells by targeting micelles resulted in better suppression of the P-gp-mediated drug efflux. This hypothesis was supported by the Rhodamine 123 efflux study as will be discussed in the next section. In addition, cellular attachment of drug-loaded micelles was enhanced when the micelles were functionalized with folate. Even if the micelles attached to KB_{v200} cells did not undergo further endocytosis, enhanced cellular attachment of micelles will still increase FG020326 amounts in the replaced culture media, which will likely lead to better MDR reversal as well.

Although the strong MDR reversal activity of FG020326 was demonstrated previously,[6,7] the mechanism of this MDR modulator was not completely understood. P-gp is a plasma membrane protein that mediates the ATP-dependent efflux of cancer therapeutics. On the basis of its amino acid sequence, P-gp is predicted to consist of two similar halves, each containing six putative transmembrane segments and a nucleotide-binding domain.[34] In a previously published report, an MDR modulator OC144-093, which is a substituted diarylimidazole ($M = 495$) and structurally very similar to FG020326, was found to inhibit the P-gp ATPase activity and block the binding of [^{3}H]azidopine to P-gp.[35] Moreover, [^{3}H]azidopine was known to block the binding of VCR to P-gp,[36] and inhibition of the ATPase activity

means that higher concentrations of intracellular ATP would be required for functioning of P-gp.[31] Based on these literature reports, we assume that there might exist two pathways that the internalized FG020326 led to the MDR reversal of KB_{v200} cells. First, FG020326, like [^{3}H]azidopine, may share a common P-gp binding site for VCR as well. Consequently, FG020326 transported into the cells may interact with this P-gp binding site and block the P-gp binding of VCR from the cytoplasm. Second, like OC144-093, FG020326 may inhibit the intracellular ATPase activity resulting in the suppression of P-gp functioning. FG020326 transported by micelles via endocytosis are believed to release in the lysosome and afterwards may act in the same way as free FG020326 taken up by the cells. If this be the case, it is not difficult to understand why the enhanced endocytosis of micelles by attachment of a targeting ligand would lead to better MDR reversal effects of micelle-transported FG020326. However, future studies are needed to confirm these hypotheses, and it must be noted that in our study, free FG020326 still showed the best MDR reversal than any micellar formulation in the present study.

It is noteworthy that, in the MTT assays, only the folate-PEG3k-PCL1k micelle-delivered FG020326 resulted in a VCR cytotoxicity comparable to that induced by free FG020326, and FG020326 transported by other micelles appeared to be less effective [Fig. 6(c)]. One underlying reason for the lower reversal potency of FG020326-loaded micelles compared to that of free FG020326 is likely the sustained release of FG020326 from micelles. Although FG020326 loaded inside the PEG-PCL micelles is believed to release quickly in acidic lysosomal compartments based on the *in vitro* FG020326 release data, it still may not be utilized as quickly as free FG020326 inside the cells. Furthermore, we assume that the considerably high VCR cytotoxicity induced by the folate-PEG3k-PCL1k micelle-delivered FG020326 is that, in this particular formulation, the cellular uptake of micelles have been significantly enhanced due to the folate-functionalization and relative small micelle size, resulting in a strong compensation to the above mentioned drawback in the bioavailability of the micelle encapsulated drug.

Rhodamine 123 efflux studies

Rhodamine 123 has been known to be the P-gp substrate, and MDR modulators could affect intracellular Rhodamine 123 levels by inhibiting the function of P-gp in human MDR cancer cells.[37] Pretreatment of cells with blank copolymer in either concentrations (above or below CMC) did not increase the amount of Rhodamine 123 remaining in KB_{v200} cells, which is in good agreement with the cytotoxicity data that blank copolymer did not improve VCR cytotoxicity. The results shown in Figure 7(a) demonstrate that the amount of Rhodamine 123 remaining in KB_{v200} cells was obviously increased upon pretreatment of the cells with either FG020326 or FG020326-loaded micelles. Moreover, the accumulation of Rhodamine 123 inside KB_{v200} cells was significantly enhanced by attaching a tumor-targeting ligand, folate, to the micellar delivery vehicles and then using these FG020326-loaded targeting micelles to pretreat the cells. Rhodamine 123 efflux appeared unhindered when a large amount of free folate was introduced to compete with the targeting micelles containing FG020326, as evidenced in Figure 7(b). Apparently, targeting micelles transport FG020326 more efficiently than their nontargeting counterparts. Finally, the Rhodamine 123 fluorescence intensity detected by flow cytometry when using FG020326-loaded folate-PEG3k-PCL1k micelles rather than any other micelle formula, including the FG020326-

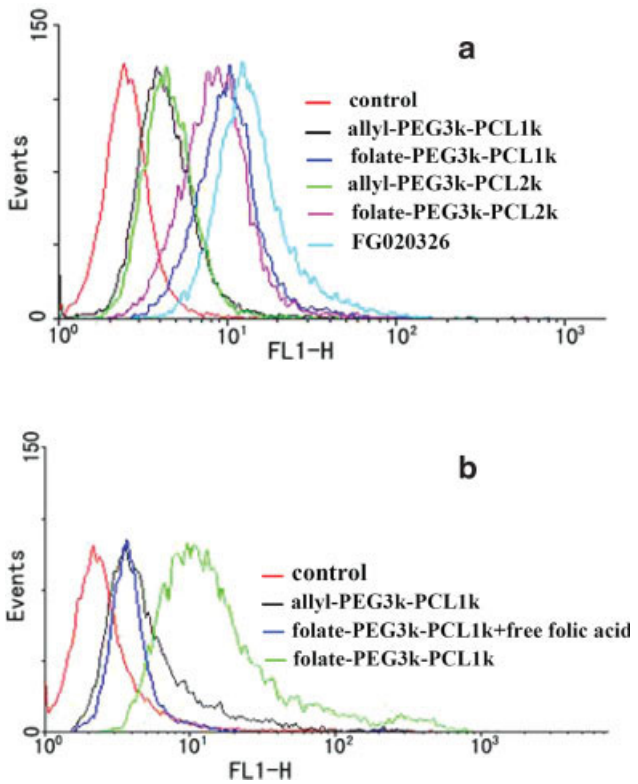


Figure 7. (a) Effect of FG020326 and FG020326-loaded polymeric micelles on Rhodamine 123 efflux in KBv200 cells, incubated at the same FG020326 concentration 2 μmol L^{-1}. (b) Flow cytometry histogram of micelle uptake in KB_{v200} cells implies that cell uptake of targeting micelle is inhibited by the presence of free folate ligands (10 m*M*) in solution. Fluorescence height represents the Rhodamine 123 amounts remained in KB_{v200} cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

loaded folate-PEG3k-PCL2k micelles, approached the fluorescence intensity detected when using free FG020326 [Fig. 7(a)]. These results, which are in agreement with the cytotoxicity data discussed in the previous section, indicate once again that, in addition to the folate-induced targeting effect, micelle composition is also decisive and thus must be properly adjusted to achieve the highest FG020326 delivery efficiency.

CONCLUSION

Biodegradable diblock copolymers, folate-PEG-PCL, of different molecular weights and compositions were successfully synthesized by multistep reactions. These copolymers self-assembled into tumor-targeted core-shell micelle structures smaller than 100 nm in diameter with a hydrophobic PCL core capable of encapsulating FG020326, a potent MDR modulator, at a DLC of up to 6.4 wt %. The targeting micelles can deliver FG020326 into the human MDR KB_{v200} cells more efficiently than their nontargeting counterparts. FG020326 delivered by the folate-encoded micelles demonstrates strong MDR reversal activity, comparable to that of free FG020326 in the KB_{v200} cells. Although preliminary, our studies provide the first evidence of effectively delivering a cancer MDR modulator using biodegradable and targeted polymeric micelles that can recognize molecular signatures on cancer cells surface. Because of their enormous therapeutic potential in drug delivery, such as prolonged blood circulation, EPR effect, and active targeting to tumor cells, these MDR modulator-loaded and folate-encoded micelles may find application in cancer chemotherapy to overcome drug resistance. We are currently conducting animal tests with these folate-functionalized and FG020326-loaded micelles to investigate their *in vivo* antitumor efficacy.

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