A Polymer–Drug Conjugate for Doxorubicin: Synthesis and Biological Evaluation of Pluronic F127-Doxorubicin Amide Conjugates

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ABSTRACT: Higher molecular weight of the polymer carrier is the basis for enhanced accumulation of the pro-drug in a solid tumor tissue due to a tumor-related phenomenon described as the enhanced permeability and retention (EPR) effect. The anticancer drug doxorubicin was covalently bound to F127 through amide group susceptible to lysosomal hydrolysis. The *in vitro* and *in vivo* properties of F127-DOX amide conjugates were studied. F127-DOX amide conjugates (M_w: 13,400) were stable in neutral circumstance and showed the potency and mechanism of action of the released drug. In the *in vivo* experiment, results showed that F127-DOX amide conjugates prolonged blood circulation and lowered *in vivo* toxicity than corresponding DOX, which illustrated the importance of molecular weight. The results demonstrated that F127-DOX amide conjugates may be very promising and clinically suitable candidates for anticancer therapy. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 124: 4953–4960, 2012

Key words: enhanced permeability and retention (EPR) effect; block copolymers; doxorubicin; drug delivery systems; biomaterials

INTRODUCTION

Conventionally cancer chemotherapy drugs can cause several adverse side effects on normal tissues, leading to high-systemic toxicity, which prevented the use of high drug doses that are required for effective killing of cancer cells. Doxorubicin (DOX), an anthracycline antibiotic, is a highly potent and widely used chemotherapeutic agent.[1] However, its short half-life[2,3] and serious cytotoxicities in normal tissues, such as gastrointestinal toxicity, heart failure, and myelosuppression, limit the maximum tolerated dose. Therefore, DOX is a good candidate for research to enhance effectiveness and limit nonspecific toxicity.

Polymer–drug conjugation is one of the major strategies for drug modifications to overcome such adverse effects of cancer chemotherapy drugs. Higher molecular weight of the polymer carrier, which prevents the drug from fast blood clearance and elimination from organism, is the basis for enhanced accumulation of the polymer–drug conjugation in a solid tumor tissue due to a tumor-related phenomenon described as the enhanced permeability and retention (EPR) effect.[4–6] Based on EPR effect in humans polymer–drug conjugations are designed to leak from the blood vessels and accumulate at the around of tumor cells, then enter tumor cells by endocytosis before releasing their drug payloads.[7] Polymer-drug conjugates may reduce drug toxicity, eliminate undesirable interactions, and prolong circulation time.[8–11]

Pluronic triblock copolymers consist of hydrophilic ethylene oxide (EO) and hydrophobic propylene oxide (PO) blocks, arranged in a basic structure of PEO-PPO-PEO. Pluronic are listed in the United States and British Pharmacopoeia under the name "poloxamers" as excipients and are widely used in a variety of clinical applications.[12,13] Recently, Pluronic copolymers have been found to have a broad spectrum of activities in cancer cells. The mechanisms may be as follows: (a) Pluronic copolymers deplete ATP that deprives the MDR cells of the energy source and simultaneous Pgp ATPase activity is inhibited, resulting in a potent inhibition of P-gp drug efflux pump.[14–17] (b) Pluronic copolymers promote generation of reactive oxygen species (ROS) and simultaneously inhibit the glutathione/glutathione S-transferase (GSH/GST) detoxification.[15] (c) Pluronic copolymers attenuate drug sequestration in acidic vesicles, which

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may increase drug bioavailability within the cancer cell.[18] Or (d) Pluronic copolymers decrease membrane potential in mitochondria of MDR cells, promote release of cytochrome C and overall enhance proapoptotic signaling.[19,20] Hence, Pluronic copolymers may be better candidates to be utilized in polymer–drug conjugates.

In previous research, F127 was mainly used in hydrogels or nanoparticles,[21,22] however, F127-DOX amide conjugates were designed through structure modification of F127 in the bases of activities and EPR effect of Pluronic in the study. We hypothesize that the conjugates would (a) lower the toxicity of DOX and simultaneously decrease the multidrug resistance of tumor cells to DOX, or (b) prolong circulation time of DOX *in vivo*. In this article, F127-DOX amide conjugates were synthesized, and the chemical and biological characters of conjugates were studied *in vitro* and *in vivo*.

MATERIALS AND METHODS

Materials

N′-(ethylcarbonimidoyl)-*N*,*N*-dimethylpropane-1,3-diamine monohydrochloride (EDAC), succinic anhydride (SA), triethylamine (TEA), and tetrahydrofuran (THF) were obtained from Nanjing Biorgchem Co. Pluromic F127 (M_w: 12,600) was purchased from BASF Co and used without further purification. DOX was purchased from Bejing Huafeng United Technology Co. Penicillin–streptomycin solution, MTT, Dulbecco's Modified Eagle Medium (DMEM) and *N*-hydroxysuccinimide (NHS), were obtained from Sigma–Aldrich. All solvents used are HPLC grade, which include dichloromethane (DCM), dimethyl sulfoxide (DMSO, anhydrous) and methanol. All reagent water used in the laboratory was pretreated with the Milli-Q Plus System.

Synthesis of F127-DOX amide conjugates

DOX (0.29g, 0.5 mmol), succinic anhydride (0.10 g, 1 mmol), and TEA (0.101 g, 1 mmol) were mixed in CH_2Cl_2 and stirred at 40°C for 8 h. The mixed solution was removed by a rotary evaporator. The resulting mixture was dissolved into 5-mL distilled water and stirred for 30 min in ice bath. The precipitant was removed by filtering to remove excessive succinic anhydride and the red filtrate was collected and dried in vacuum to obtain succinoylated DOX reagent.[23] The succinoylated DOX (30 mg, 44 μmol) with EDAC (16.9 mg, 88 μmol) was dissolved in 2-mL PBS (pH 7.2) and stirred at room temperature for 30 min. The mixture was dropwised into the F127 solution (60 mg, 5 μmol) and reacted at room temperature in the dark for 24 h. The total reaction volume was 10 mL. The product was dialyzed using MWCO 3000 membrane in distilled water for 24 h to remove excess succinoylated DOX. The resulting solution was freeze-dried to get the red powder. The chemical structure of conjugations was characterized by ^{1}H-NMR and FTIR, and the Mw of conjugations was characterized by GPC. The whole synthetic scheme was shown in Figure 1.

Gelation temperature

The gelation temperatures of F127 and F127-DOX amide conjugates were determined by the "Visual Tube Inversion Method," as previously described.[24] Briefly, glass vials with a diameter of 13 mm containing 1 g of sample were placed in a water bath. The temperature of the bath was slowly increased at 0.5°C/min; the temperature at which the sample solution stopped flowing on tilting was noted as the gelation temperature (t_1). Similarly, samples were placed in a hot bath, which was slowly cooled; the temperature at which the gel started flowing was noted as the gel melting temperature (t_2). Three measurements of t_1 and t_2 were made on each sample; each sample was prepared and analyzed in duplicate. The critical gelation temperature was then calculated as the mean $\pm$ SD of the measured t_1 and t_2 values.

In vitro release of F127-DOX amide conjugates and plasma stability

The rate of DOX release from the conjugate was investigated in buffers at pH 3.0, 5.0, and 7.2, respectively. Plasma stability was investigated in mice serum solution, pH 7.2 at 37°C .The conjugation solution of 1 mg DOX equiv./mL was placed in a dialysis bag (MWCO: 3000) and incubated in 20 mL of the PBS solution with gentle shaking. The incubated solution was collected at designated time points and equal volume of fresh medium was compensated. Analysis of the DOX was accomplished by C18 RP-HPLC (Symmetry C18 column, 50 × 2.1 mm^2, 5 μm) with an isocratic mobile phase consisting of 0.1% acetic acid and methanol (30 : 70, v/v). The flow rate was set at 1 mL/min, the temperature was set at 30°C, and detection was followed at 254 nm.

Cell culture

HepG2 cells were employed as *in vitro* models. The cells were cultured in the DMEM medium supplemented with 10% FBS, 1% penicillin–streptomycin solution, and incubated in SANYO CO_2 incubator at 37°C in humidified environment of 5.0% CO_2.

In vitro cell uptake of F127-DOX amide conjugates

HepG2 cells were seeded at a density of 5 × 10^3 cells/well in 96-well transparent plate.[25–27] When

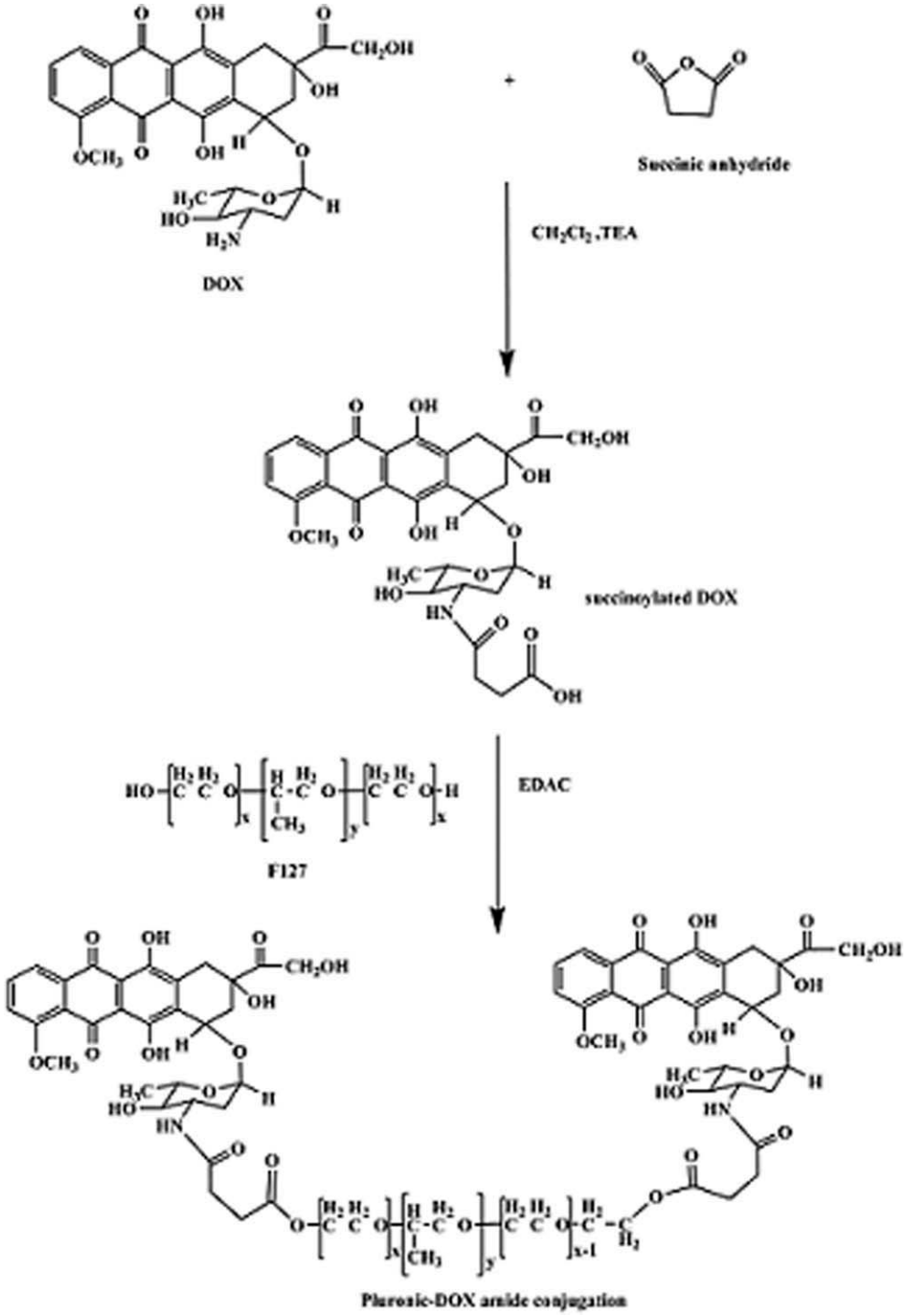

Figure 1 Synthesis of F127-DOX amide conjugates.

cultured 24 h at 37°C, the medium was replaced by 100 μL F127-DOX amide conjugate or free DOX solution in medium at 1 mg DOX equiv./mL for 2 h. The sample wells were washed three times with 50 μL PBS and then 100 μL of PBS was added. Cellular uptake efficiency was studied by flow cytometry. The wells were seeded in blank medium for control. For each sample, six wells were seeded.

In vitro cytotoxicity again HepG2 cells

HepG2 cells were seeded at a density of 5×10^3 cells/well in 96-well transparent plate and incubated for 24 h. The medium was then replaced by the free DOX or F127-DOX amide conjugates at various drug concentrations from 0.002 to 100 μ*M* equivalent in the medium. The cell viability was determined by the MTT assay. For each sample, six

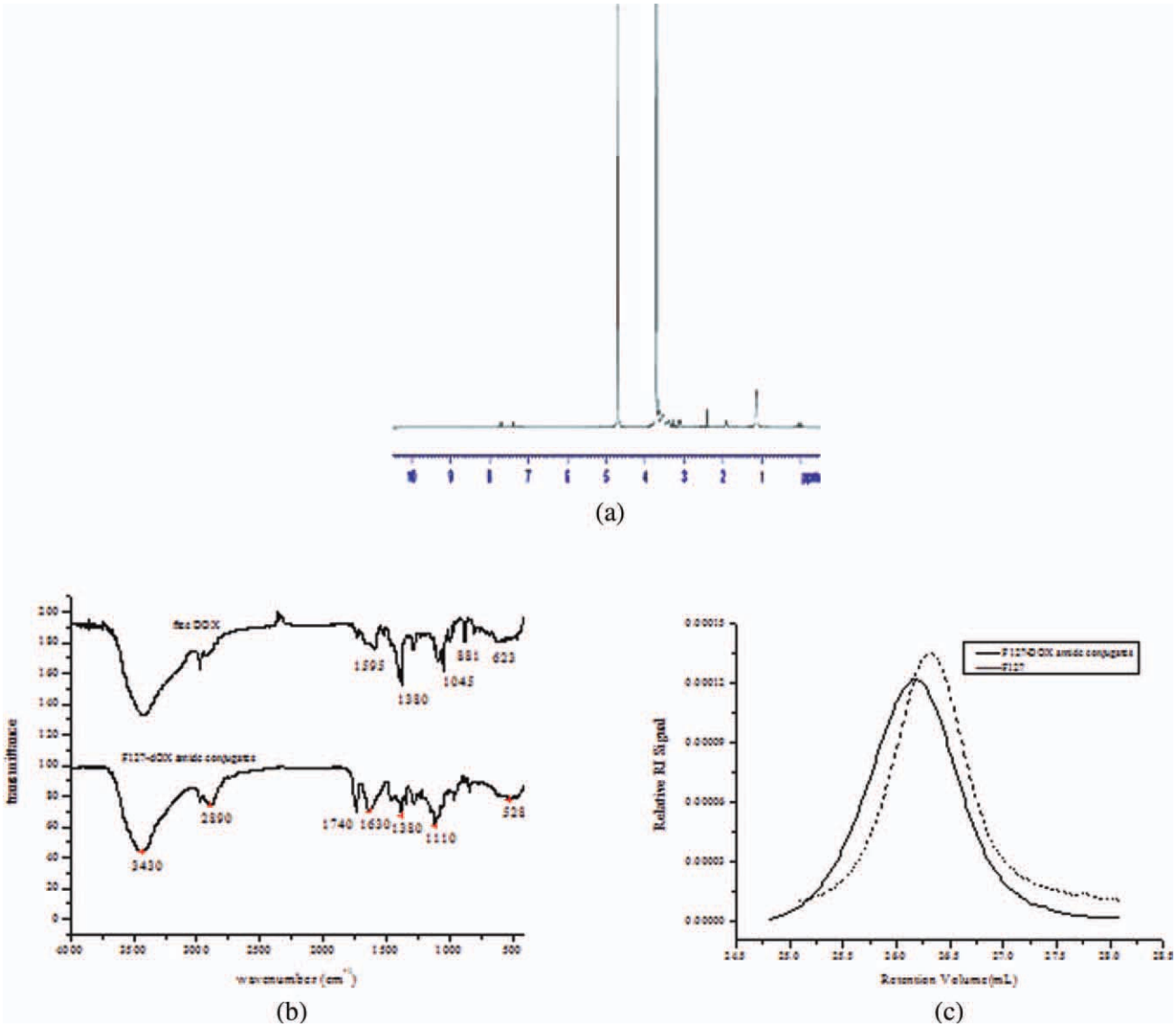

Figure 2 ^{1}H-NMR spectrum (D_2O) (a, b) and FTIR spectra in KBr (c) of F127-DOX amide conjugates. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

wells were seeded. At the designated time, the medium was removed and the wells were washed twice with PBS. Ten percent MTT (5 mg/mL in PBS) in medium was added and the cells were incubated for 3–4 h. After that, the precipitant was dissolved in DMSO and each well was finally analyzed by the microplate reader with absorbance detection at 570 nm. All assays were performed at least three times in quadruplicate to determine the IC50.

Blood clearance in SD rats

Blood clearance studies of F127-DOX amide conjugates were carried out in Sprague-Dawley (SD) rats (about 250 g/SD rat). The mice were intravenously injected with 5 mg DOX equiv./kg. Then, blood samples from tail veins were taken at the following times after injection: 0.5, 1, 2, 4, 6, 8, 10, 12, 24, 36, 48, and 96 h. The blood samples were collected in heparinized tubes. The samples were tested for the total DOX content (free and polymer-bound DOX). The total DOX content was determined after quantitative acid hydrolysis in 1*M* HCl. After incubation for 1 h at 50°C, doxorubicinone (aglycon of DOX) was extracted with ethyl acetate, the organic phase was evaporated to dryness, the solid completely dissolved in methanol and analyzed by LC-MS .The calibration curve was obtained by injection of exact amounts of free DOX into blood. For each sample, three SD rat were included.

In vivo antitumor activity of the conjugates

Antitumor activity of the conjugates was tested on HepG2 tumor models.[28] In the case, 1×10^6 cells were implanted on the right flank of BALB/C nude

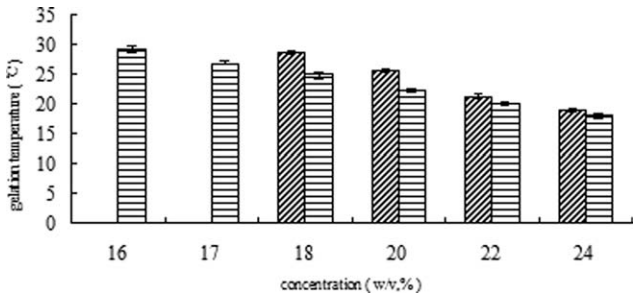

Figure 3 Gelation temperature of aqueous solutions of F127 (▤) and F127-DOX amide conjugates (▨).

mice (22–24 g/BALB/C nude mouse) and the mice were allowed to treat with a single dose of 10 mg DOX equiv./kg of the conjugates when the tumor was growing to 150–200 mm^3. For each sample, 10 animals were included.

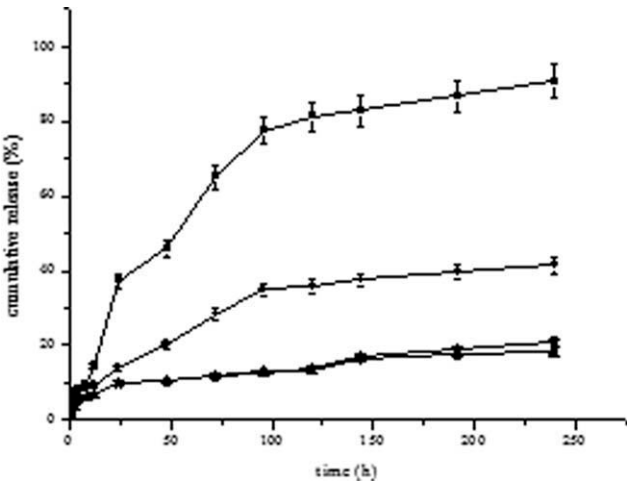

Figure 4 Release of DOX from F127-DOX amide conjugates in buffers. pH 3 (■), pH 5 (●), pH 7.2 (▲), and pH 7.2 with mice serum (□).

RESULTS AND DISCUSSION

Synthesis of F127-DOX amide conjugates

As reported, amide linkers are mainly used in antibody-drug conjugates, and the half life of amide linkers *in vivo* are 6–10 days.[29–32] Hence, F127-DOX amide conjugates were synthesized in the study. The chemical structure of conjugations was characterized by ^{1}H-NMR and FT IR. Major features of ^{1}H-NMR of F127-DOX amide conjugates are: ^{1}H-NMR (D_2O) δ 3.71(C**H**$_2$—O in PEO); δ 3.56(C**H**$_2$CH$_2$—O in PEO); δ 3.41 (C**H**$_2$CH(CH$_3$)—O in PPO); δ 3.50(CH$_2$C**H**(CH$_3$)—O in PPO); δ 1.14(CH$_2$CH(C**H**$_3$)—O in PPO); δ 7.7 (C**H** in DOX); δ 7.4 (C**H** in DOX). FTIR spectra of F127-DOX

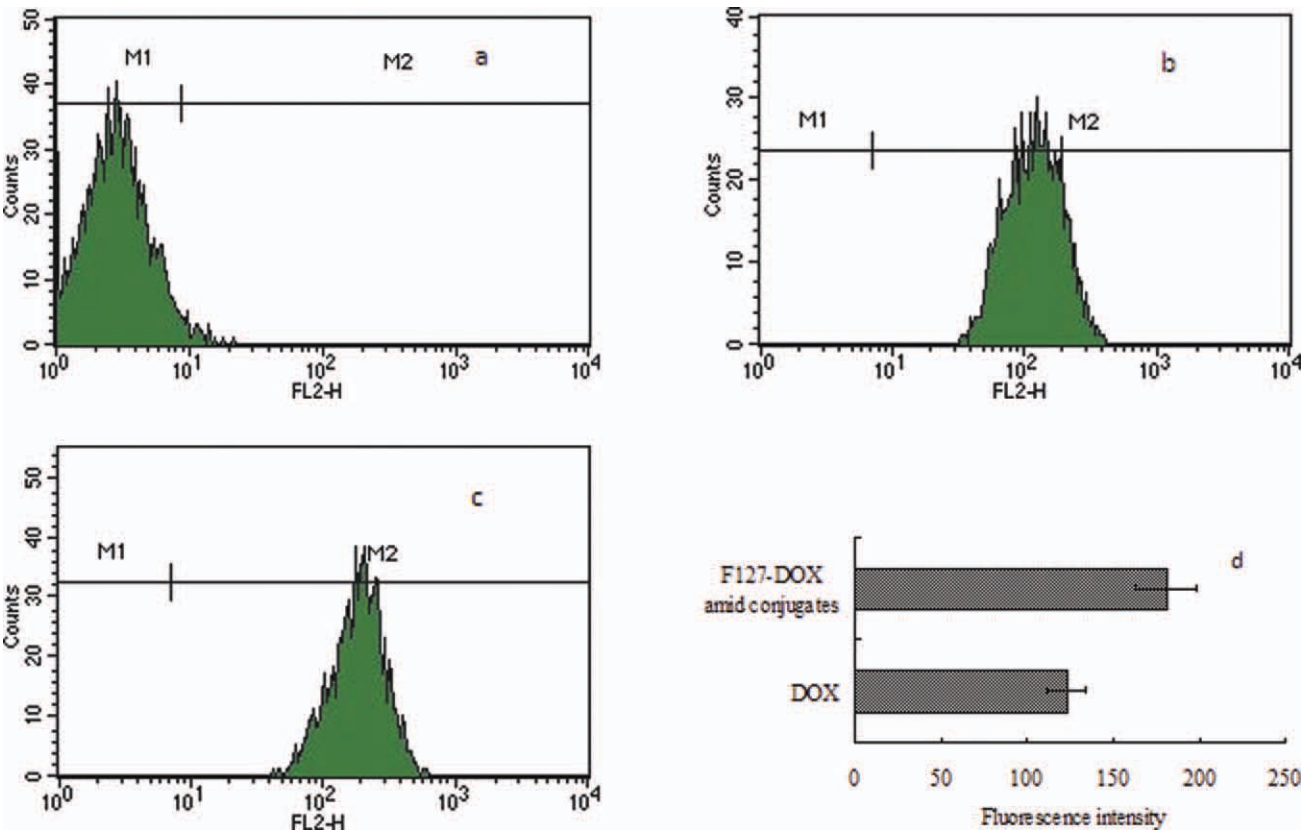

Figure 5 Intracellular uptake of DOX and F127-DOX amide conjugates in HepG2. (a) control; (b) DOX; (c) F127-DOX amide conjugates; and (d) fluorescence intensity of free DOX and F127-DOX amide conjugates. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

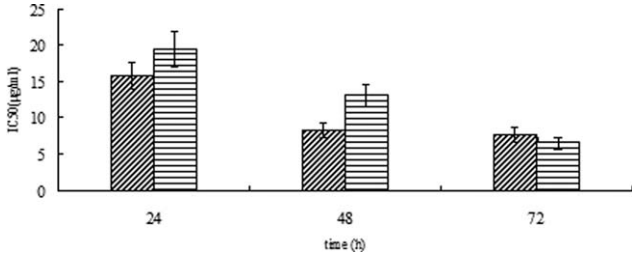

Figure 6 *In vitro* cytotoxicity of DOX (▨) and F127-DOX amide conjugates (▤).

amide conjugates was shown in Figure 2. The band around 1740 cm^{-1} is assigned to the stretching vibration of C═O bonds of ester bond, the band around 1630 cm^{-1} is assigned to the stretching vibration of C═O bonds of amide bond, and the band around 1110 cm^{-1} is assigned to the stretching vibration of C—O—C bonds of Pluronic. The spectra are consistent with successful coupling of DOX with F127. M_w of F127 and F127-DOX amide conjugates was 12,150 and 13,400, respectively, measured by GPC, which indicated that the molar ratio of DOX and F127 was 2 : 1 in F127-DOX amide conjugates.

Gelation temperature of F127 and F127-DOX amide conjugates

Gelation temperature of F127 and F127-DOX amide conjugates was shown in Figure 3. When the concentration of F127 was above 16%, the aqueous solutions of F127 could form gels, and the gelation temperature showed concentration-dependent, which meant that the higher the concentration was, the lower the gelation temperature was. Meanwhile, when the concentration of F127-DOX amide conjugates was above 18%, the aqueous solutions of F127-DOX amide conjugates could form gels, and similar concentration-dependent was found for F127-DOX amide conjugates. The recommended dosage of free DOX in adults is 60–90 mg/m^2 body area, and dosage of F127-DOX amide conjugates in adults could be 0.712–1.068 g/m^2 body area. When the dosage of conjugates was dissolved in 10-mL physiological saline, the concentration of F127-DOX amid conjugates was about 7.12–10.68%, which was below 18%. Hence, when injected by DOX equiv. in clinical application the aqueous solutions of F127-DOX amide conjugates could effectively avoid forming gels at body temperature.

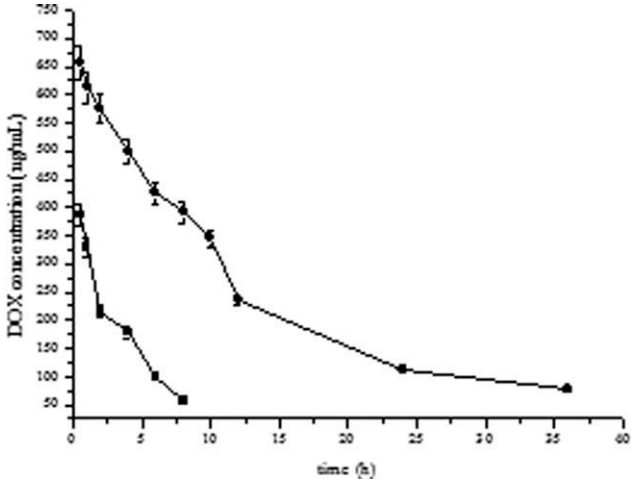

Figure 7 Blood clearance of free DOX (■) and F127-DOX amide conjugates (●) in SD mice.

Release of DOX from the conjugates and plasma stability

The rate of DOX release from F127-DOX amide conjugates *in vitro* was investigated in buffers at pH 3.0, 5.0, and 7.2 at 37°C. As shown in Figure 4, dramatic initial burst was not found in the DOX release study. During the pH ranging from 3.0 to 7.2, pH-dependence, which meant that the lower the pH value was, the faster the drug released, was found. The drug from F127-DOX amide conjugates released about 90% at pH 3.0 within 10 days, whereas the DOX release from F127-DOX amide conjugates at pH 5.0 was much slower, implying 41.55% in the same period .While only a small amount of drug from the conjugates was released at pH 7.2 over the observed period. Such results indicated that F127-DOX amide conjugates was stable in neutral circumstance.

The plasma stability of F127-DOX amide conjugates was characterized indirectly in the DOX release experiment. Serum from mice, was mixed with PBS (pH 7.2) with the ratio of $v/v = 1/9$. About 20% of the drug released from the conjugates was detected during the period .The result of release of DOX indicated that the F127-DOX amide

TABLE I
Pharmacokinetic Parameters of DOX and F127-DOX Amide Conjugates (mean ± SD, $n = 3$)

Compound	Parameters			
	k (h^{-1})	$t_{1/2}$ (h)	AUC (ng h/mL)	CL (L/h)
DOX	0.243 ± 0.007	2.85 ± 0.25	1706.79 ± 17.77	1.465 ± 0.056
F127-DOX amid conjugates	0.061 ± 0.002	10.98 ± 0.57	9930.43 ± 32.14	0.252 ± 0.014

conjugates could be stable in human plasma and the conjugate would have the potency and mechanism of action of the released drug.

Uptake and cytotoxicity of F127-DOX amide conjugates *in vitro*

The intrinsic DOX fluorescence (red) was used to analyze the extent of cellular DOX uptake by flow cytometry to assess endocytosis. As shown in Figure 5, when cultured at 37°C, F127-DOX amide conjugates were taken up by HepG2 cells. The conjugates incubated with HepG2 cells emitted higher fluorescence intensity ($P < 0.05$) compared with free DOX.

The cytotoxicity of F127-DOX amide conjugates was investigated and compared with that of free DOX using HepG2 cells. Cell cultures were used as a blank control. After incubated 24, 48, and 72 h, respectively, cells inhibition was analyzed using the MTT assay (shown in Fig. 6). F127-DOX amide conjugates had lower cytotoxicity ($P < 0.05$) and higher IC50 compared with free DOX when incubated for 24 and 48 h, respectively. For F127-DOX amide conjugates, time-dependence was found, which meant that the longer the incubated time was, the lower the IC50 was. It was surprised that IC50 of F127-DOX amide conjugates was lower than IC50 of DOX after incubated 72 h. This may be concerned with the multidrug resistance of HepG2.[33]

Pluronic with intermediate length of PO block (from 30 to 60 units) and HLB < 20 are most effective at inhibiting P-gp efflux. HLB of F127 is 18–22 and inhibition of P-gp drug efflux pump is limited. When conjugated with DOX, HLB of F127 is below 20 definitely.[34] When taken up, DOX was released by enzymatic hydrolysis in tumor cells[35] and F127 depleted ATP,[34] resulting in more retention time of DOX in tumor cells. In the study, we found that F127-DOX amid conjugates emitted higher fluorescence intensity compared with free DOX and IC50 of F127-DOX amide conjugates was lower than IC50 of DOX after incubated 72 h. The phenomenon showed that F127-DOX amide conjugates may overcome the multidrug resistance of HepG2 *in vitro* experiment to some extent.

Drug level in plasma

An important factor leading to improved efficacy of polymer conjugates is the increased plasma residence time. Thus, it is necessary to evaluate whether F127-DOX amide conjugates could circulate in the body for a longer time than free DOX. After injecting DOX and F127-DOX amide conjugates in blood all pharmacokinetic parameters were calculated. As shown in Figure 7, the plasma level of DOX reached a nadir within 8 h, whereas the plasma levels of F127-DOX amide conjugates declined more slowly within 36 h. The pharmacokinetic parameters were listed in Table I. The clearance value (CL) (0.2524 L/h) of F127-DOX amide conjugates was apparently lower than the 1.4653 L/h of DOX, implying that

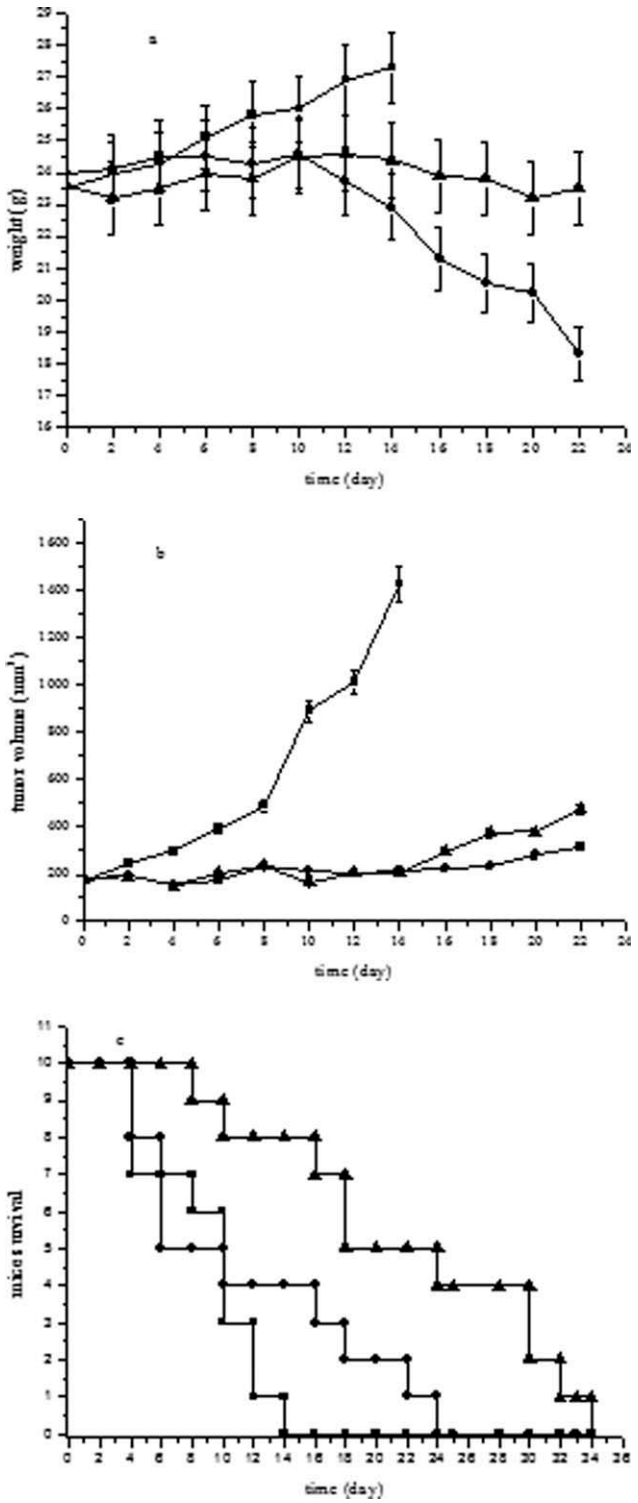

Figure 8 *In vivo* therapeutic efficacy of the conjugates in immunocompromised mice with subcutaneous human tumor xenografts. Treated with control (saline) (■), DOX (●), and F127-DOX amide conjugates (▲). (a) Body weight changes in animals; (b) tumor volume in animals; and (c) survival in animals.

F127-DOX amide conjugates were eliminated much more slowly from the body than DOX. These results suggested F127-DOX amide conjugates could provide prolonged serum level of DOX. Also similar results were confirmed by $t_{1/2}$.

Generally, slow elimination promotes better access of drugs to remote targets. From calculation of the area under curve (AUC), it is clear that accumulation of F127-DOX amide conjugates was approximately six times higher than accumulation of free DOX. F127-DOX amide conjugates could be passively accumulated in the tumor tissue due to EPR effect.

Evaluation of *in vivo* anticancer activity of F127–DOX amide conjugates

The potential of F127–DOX amide conjugates was tested on HepG2 tumor models. After treated with F127-DOX amide conjugates, no signs of acute toxicity [weight decrease, shown in Fig. 8(a)] were observed when compared with group of control (saline) and free DOX respectively. While, there was no apparent difference in tumor growth inhibition between free DOX and F127-DOX amide conjugates during the period experiment [shown in Fig. 8(b)]. Survival days of mice after treatment with F127-DOX amide conjugates were apparently prolonged compared with free DOX [shown in Fig. 8(c)].

After treatment of mice bearing HepG2, F127-DOX amide conjugates showed advanced lower toxicity than free DOX .On the contrary, no apparent differences in the anticancer activity of F127-DOX amide conjugates and free DOX were quite evident that demonstrated an important role of higher molecular weight of the drug carrier in lowering the toxicity of cancer chemotherapy drugs.

CONCLUSIONS

The anticancer drug DOX was covalently bound to F127 through amide group susceptible to lysosomal hydrolysis. F127–DOX amide conjugates were fairly stable in buffers at pH 7.2. Meanwhile, F127-DOX amide conjugates showed apparently prolonged blood clearance, lower toxicity, and preserved antitumor activity when compared with free DOX *in vivo*. High-molecular weight F127–DOX amid conjugates showed potential of a new formulation of DOX are very promising and clinically suitable candidates for anticancer therapy.

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