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Paclitaxel-loaded Pluronic P123/F127 mixed polymeric micelles: Formulation, optimization and in vitro characterization

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ABSTRACT

The objective of this study was to optimize and characterize a novel polymeric mixed micelle composed of Pluronic P123 and F127 loaded with paclitaxel (PTX). A Doehlert matrix design was utilized to investigate the effect of four variables, namely P123 mass fraction, amount of water, feeding of PTX and hydration temperature on the responses including drug-loading coefficient (DL %), encapsulation ratio (ER %) and the percentage of PTX precipitated from the drug-loaded mixed micelles after 48 h at 37 (PTX precipitated %) for improvement of drug solubilization efficiency and micelle stability. PTX-loaded P123/F127 mixed micelles were prepared by thin-film hydration method. The optimized formulation showed a particle size of about 25 nm with ER % > 90%, and a sustained release behavior compared to Taxol. Micelle formation was confirmed by NMR spectroscopy. The mixed micelles had a low CMC of 0.0059% in water. In addition, micelle stability studies implied that introduction of Pluronic F127 (33 wt%) into P123 micelle system significantly increased the stability of PTX-loaded micelles. More importantly, in vitro cytotoxicity was assessed using human lung adenocarcinoma cell lines SPC-A1 and A-549 and was compared to Taxol and the free drug. The cell viability assay against A-549 cells exhibited the 50% inhibition concentration (IC50) of PTX-loaded P123/F127 mixed micelles (0.1 μg/ml) was much lower than those of Taxol injection (0.4 μg/ml) and the free PTX (1.7 μg/ml). Therefore, PTX-loaded P123/F127 mixed micelles may be considered as an effective anticancer drug delivery system for cancer chemotherapy.

1. Introduction

Paclitaxel (PTX), one of the most successful anticancer drugs, is the first of a new class of microtubule stabilizing agents, and has shown its potency against a broad spectrum of cancers, especially against non-small-cell lung cancer, metastatic breast cancer and refractory ovarian cancer (Spencer and Faulds, 1994; Singla et al., 2002). However, because of the poor aqueous solubility and low therapeutic index of PTX, the clinical application is extremely limited. Presently, the only available commercial preparation of PTX is Taxol, a concentrated solution composed of a 50:50 (v/v) mixture of Cremophor EL (polyoxyl 35 castor oil) and dehydrated alcohol, which is diluted 5–20-fold in normal saline or dextrose solution before administration. Unfortunately, serious side effects, such as hypersensitivity, nephrotoxicity and neurotoxicity as well as effects on endothelial and vascular muscles causing vasodilatation, labored breathing, lethargy and hypotension, attributable to Cremophor EL have been reported (Weiss et al., 1990).

Accordingly, a number of alternative formulations were investigated for solubilization of PTX, including polymeric micelles (Huh et al., 2005, 2008), liposomes (Crosasso et al., 2000; Yang and Choi, 2007), microspheres (Liggins and Burt, 2004; Ma and Song, 2007), nanoparticles (Matsumoto et al., 1999; Zhang and Feng, 2006), PTX–polymer conjugates (Xie et al., 2007) and dendritic polymers (Ooya et al., 2003). One promising nanomedicine-based technology is polymeric micelles, which have been evaluated in several clinical trials as carriers for anticancer drugs (Danson et al., 2004; Mizumura et al., 2002; Matsumura et al., 2004). In particular, doxorubicin loaded mixed micelles composed of Pluronic L61 and F127 have been successfully evaluated in Phase II study in patients with advanced esophageal carcinoma (Kabanov et al., 2003a,b). Polymeric micelles have a core–shell structure which enables the system to incorporate poorly soluble drugs and protect from inactivation in biological media. Due to their small particle size (<100 nm), these systems exhibit many advantages such as targeting ability, long circulation and easy production on effective delivery of drugs (Torchilin, 2001). However, recent developments indicate that by selecting the proper polymers, not only could nanomaterials serve as inert carriers, but also as biological response modifiers (Batrakova and Kabanov, 2008). One representative of such materials is Pluronic block copolymers that are amphiphilic

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synthetic polymers containing hydrophilic poly(ethylene oxide) (PEO) blocks and hydrophobic poly(propylene oxide) (PPO) blocks arranged in triblock structure: PEO–PPO–PEO. These block copolymers were shown to be inhibitors of P-glycoprotein (P-gp) that sensitize multidrug resistant (MDR) tumors to doxorubicin, paclitaxel, vinblastine and other anticancer agents in vitro (Alakhov et al., 1996; Venne et al., 1996) and in vivo (Batrakova et al., 1996; Alakhov et al., 1999). Thus, Pluronic micelles represent a novel type of nanomedicines that can increase solubility, improve circulation time and release drugs at the target sites. In addition, they can also release individual block copolymer molecules, which are shown to inhibit P-gp and to sensitize MDR cells (Kabanov and Alakhov, 2002).

Recently, we have reported that PTX-loaded polymeric micelle prepared with Pluronic P123 or P105 could effectively prolong blood circulation time and modify the biodistribution of PTX in vitro (Han et al., 2006; Wang et al., 2008), and exert higher cytotoxicity against MDR tumor cells (Wang et al., 2007). However, the in vitro micelle stability and drug-loading characteristics of the PTX-loaded Pluronic micelles are still to be improved. In order to overcome the shortcomings of Pluronic micelles described above, mixed micelles composed of P123 (PEO_{20}–PPO_{65}–PEO_{20}) and F127 (PEO_{100}–PPO_{69}–PEO_{100}) were studied. Pluronic F127 was chosen for its biocompatibility, long PEO repulsive property and its approval by FDA. In addition, it is reported that aggregation behavior of binary Pluronics appears to be PPO dependent. Pluronics with similar PPO moieties show cooperative aggregation and those with different PPO moieties show non-cooperative binding (Gaisford et al., 1997; Chaibundit et al., 2007). The evidence outlined above indicates these two block copolymers, which have very close hydrophobic blocks, will comicellize.

The aim of this study was to develop a novel P123/F127 mixed micelle system for PTX, intended to be intravenously administered. To achieve this purpose, Doehlert matrix design (DMD), a more efficient and uniform experimental design than central composite design (CCD) and Box–Behnken design (BBD) (Ferreira et al., 2004), and desirability function approach were employed to evaluate the effects of selected variables and to optimize the formulation parameters (Paterakis et al., 2002). The optimal preparation was further characterized in terms of critical micelle concentration, particle size distribution and morphological observation, in vitro release, micelle stability and drug existence state. Finally, the in vitro antitumoral activity of PTX-load mixed micelles was assessed using human lung adenocarcinoma cell line SPC-A1 and A-549.

2. Materials and methods

2.1. Materials

Paclitaxel was purchased from Xi'an Sanjiang Bio-Engineering Co. Ltd. (Xi'an, China). Taxol injection (Anzatax Injection Concentrate, 30 mg/5 ml) was produced by FH Faulding & Co. Ltd. trading as David Bull Lab (Melbourne, Australia). Samples of Pluronic P123, F127 and Cremophor EL were kindly supplied by BASF Ltd. (Shanghai, China). 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was purchased from Sigma (St. Louis, MO, USA). Penicillin–streptomycin, RPMI 1640, fetal bovine serum (FBS) and 0.25% (w/v) trypsin–0.03% (w/v) EDTA solution were purchased from Gibco BRL (Gaithersberg, MD, USA). All other solvents were analytical or chromatographic grade.

2.2. HPLC analysis

The analyses of PTX in vitro were determined by RP-HPLC method on a system equipped with a LC-10AT_{VP} pump, a SPD-10A_{VP} UV detector (Shimadzu, Kyoto, Japan) and a HS2000 interface (Hangzhou Empire Science & Tech, Hangzhou, China) operated at 230 nm. A reversed-phase column (Gemini 5 μm C18, 150 mm × 4.6 mm, Phenomenex, California, USA) was used at room temperature. The mobile phase consisting of acetonitrile, and ammonium acetate buffer solution (10 mM, pH 5.0) (50:45, v/v) was freshly prepared for each run and degassed before use. Sample solution was injected at a volume of 20 μl. With a flow rate of 1.0 ml/min for the mobile phase, the typical retention time was around 6 min. The concentrations of PTX were determined by comparing the peak areas with the standard curve.

2.3. Preparation of PTX-loaded mixed micelles

PTX-loaded mixed micelle was prepared by thin-film hydration method (Zhang et al., 1996). Briefly, 1–7 mg of PTX and 270 mg of Pluronic mixture composed of P123 and F127 in several proportions were dissolved in 10 ml acetonitrile in a round-bottom flask. The solvent was evaporated by rotary evaporation at 50 °C for about 1 h to obtain a solid PTX/copolymer matrix. Residual acetonitrile remaining in the film was removed under vacuum overnight at room temperature. Then, the resultant thin film was hydrated with different amount of water, while the hydration temperature was varied according to the experimental design. The mixture was stirred at 700 rpm for 30 min to obtain a micelle solution, which was then filtrated through 0.2 μm filter membrane to remove the unincorporated drug aggregates, followed by lyophilization. The content of PTX was determined by RP-HPLC assay after disruption of the micelles and solubilization of PTX in acetonitrile.

2.4. Experimental design

2.4.1. Doehlert matrix design

The Doehlert matrix design (DMD) describes a spherical experimental domain and it stresses uniformity in space filling. In addition, this design permits study of the factors at a different number of levels (3, 5, 7), thus giving the researcher the possibility to study some factors in greater detail. Before using the design, a number of preliminary experiments were conducted to identify the control factors and their levels. Then a Doehlert matrix was used for optimization of four variables, including P123 mass fraction (X_1), amount of water (X_2), feeding of PTX (X_3) and hydration temperature (X_4). Three responses, including drug-loading coefficient (DL%, Y_1), encapsulation ratio (ER%, Y_2) and the percentage of PTX precipitated from the drug-loaded mixed micelles after 48 h incubation at 37 °C (PTX precipitated %, Y_3), which were calculated by the following equations, were selected since they were generally regarded as significant factors for assessing the qualities of polymeric micelles:

$$\mathrm{DL}\% = \frac{\text{weight of the drug in micelles}}{\text{weight of the feeding polymer and drug}} \times 100 \quad (1)$$

$$\mathrm{ER}\% = \frac{\text{weight of the drug in micelles}}{\text{weight of the feeding drug}} \times 100 \quad (2)$$

$$\text{Precipitated PTX}\% = \frac{\text{original PTX added (mg)} - \text{PTX remained in the supernatant (mg)}}{\text{original PTX added (mg)}} \times 100 \quad (3)$$

A four-factor DMD was undertaken to investigate the main effects and the interactions of the four factors on the three responses. The factors chosen and settings of factor levels were presented in Table 1.The data obtained for the three responses in each

Table 1
Doehlert matrix of the experiments and results for the measured responses and the desirability.

Run	P123 mass fraction (X_1)		Amount of water (X_2)		Amount of PTX (X_3)		Hydration temperature (X_4)		Response			Overall desirability (D)
	Real values (%)	Coded values	Real values (ml)	Coded values	Real values (mg)	Coded values	Real values (°C)	Coded values	DL (%)	ER (%)	PTX precipitated (%)	
1	83.3	1	16	0	4	0	60	0	1.46	99.74	28.29	0.00
2	16.7	−1	16	0	4	0	60	0	0.34	23.25	0.00	<0.0001
3	66.7	0.5	28	0.866	4	0	60	0	0.87	58.94	18.22	0.44
4	33.3	−0.5	4	−0.866	4	0	60	0	0.84	57.39	0.00	0.60
5	66.7	0.5	4	−0.866	4	0	60	0	1.36	93.05	5.87	0.87
6	33.3	−0.5	28	0.866	4	0	60	0	0.55	37.27	0.00	0.35
7	66.7	0.5	20	0.289	7	0.816	60	0	0.82	31.98	9.81	0.35
8	33.3	−0.5	12	−0.289	1	−0.816	60	0	0.37	99.81	0.00	0.37
9	66.7	0.5	12	−0.289	1	−0.816	60	0	0.37	99.29	0.00	0.37
10	50	0	24	0.577	1	−0.816	60	0	0.31	83.94	3.32	0.00
11	33.3	−0.5	12	0.289	7	0.816	60	0	0.54	20.80	0.00	0.00
12	50	0	8	−0.577	7	0.816	60	0	0.70	27.00	5.09	0.28
13	66.7	0.5	20	0.289	5	0.204	70	0.791	1.25	68.22	7.64	0.71
14	33.3	−0.5	12	−0.289	3	−0.204	50	−0.791	0.45	40.87	0.00	0.32
15	66.7	0.5	12	−0.289	3	−0.204	50	−0.791	0.87	79.27	8.00	0.64
16	50	0	24	0.577	3	−0.204	50	−0.791	0.63	56.75	5.10	0.47
17	50	0	16	0	6	0.612	50	−0.791	0.61	27.69	3.76	0.27
18	33.3	−0.5	12	0.289	5	0.204	70	0.791	0.76	41.43	0.00	0.47
19	50	0	8	−0.577	5	0.204	70	0.791	1.03	56.01	5.61	0.61
20	50	0	16	0	2	−0.612	70	0.791	0.73	98.74	2.46	0.69
21	50	0	16	0	4	0	60	0	0.99	67.75	4.66	0.67
22	50	0	16	0	4	0	60	0	1.07	73.20	3.43	0.73
23	50	0	16	0	4	0	60	0	1.03	70.31	3.41	0.70

trial were fitted to the classical non-linear quadratic model. The mathematical model was expressed as follows:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{44} X_4^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 + \beta_{34} X_3 X_4 \quad (4)$$

where X_1–X_4 correspond to the studied factors, Y represents the response associated with each factor level combination, β_0 is an intercept and β_1–β_{34} are the regression coefficients. Data were analyzed by non-linear estimation using STATISTICA 6.0 software.

2.4.2. Desirability function

All the three responses in this study should be evaluated in the optimization of PTX-loaded mixed micelles. However, it is almost impossible to optimize all the conditions simultaneously because they do not coincide with each other and conflict may occur between them. Thus, the multi-criteria problem can be treated as single criterion problem by applying the desirability function approach in order to find the best compromising formulation for all responses. The desirability function for the response to be maximized could be described by the following equation:

$$d_1 \text{ or } d_2 = \frac{Y_i - Y_{\min}}{Y_{\max} - Y_{\min}} \quad (5)$$

where d_1 is the individual desirability of DL %; d_2 is the individual desirability of ER %; $Y_{\min}$ and $Y_{\max}$ represent the lowest and the highest value found during the experiment, respectively; Y_i indicates the experimental result. On the contrary, for a response to be minimized, the desirability function is defined as

$$d_3 = \frac{Y_{\max} - Y_i}{Y_{\max} - Y_{\min}} \quad (6)$$

where d_3 is the individual desirability of PTX precipitated %. The overall desirability value (D), a global desirability function, is calculated by the following expression:

$$D = (d_1 d_2 d_3)^{1/3} \quad (7)$$

2.5. Characterization of PTX-loaded mixed micelles

2.5.1. Critical micelle concentration (CMC) determination

In order to determine the CMC of Pluronic block copolymers in DI water, iodine UV spectroscopy method was used as previously reported (Gaisford et al., 1995, 1997). The KI/I_2 standard solution was prepared by dissolving 0.5 g of iodine and 1 g of potassium iodide in 50 ml DI water. Thirty-seven samples of polymer solution with concentrations ranging from 0.00001% to 0.1% were prepared. To each of the P123/F127 binary mixture (2:1, w/w) solutions, 25 μl KI/I_2 standard preparation was added. The mixtures were incubated for 12 h in a dark room at room temperature before measurement. The UV absorbance value of varying polymer concentrations at 366 nm was measured using UV–vis spectrometer (Shimadzu UV-2401, UV-VIS recording spectrophotometer, Japan). Experiments were performed in triplicate. The absorption intensity was plotted against the logarithm of polymer mass concentration. The CMC values correspond to the concentration of the polymer at which the sharp increase in absorbance is observed.

2.5.2. Particle size distribution and surface morphology

Particle mean size and size distribution were measured by the light scattering method using a Nicomp Zeta Potential/Particle Sizer (model 380XLS, Nicomp™, Santa Barbara, CA, USA). The analyses were performed with 5 mW He–Ne laser (632.8 nm) at a scattering angle of 90° at 25 °C. Each freeze-dried or freshly prepared sample was diluted to the appropriate concentration using DI water to avoid multi-scattering phenomena and was placed into a quartz cuvette. The reported experimental result of each sample was expressed as a mean size ± SD for three separate experiments.

The morphology of the P123/F127 mixed micelles was studied by transmission electron microscopy (TEM, Philips CM 120, Netherlands) after negative staining with phosphotungstic acid solution (2%, w/v).

2.5.3. ^1H NMR characterization

To show the drug-loading characteristics of the P123/F127 mixed micelles, ^{1}H NMR spectra were recorded on a Varian 400 MHz spectrometer (Varian, Palo Alto, CA, USA) in deuterated

water (D_2O) or deuterated chloroform ($CDCl_3$) at room temperature.

2.5.4. In vitro release of PTX from micelles

In order to create pseudo-sink conditions, the in vitro release behaviors of PTX from P123 micelles (prepared without addition of F127) and P123/F127 mixed micelles were monitored in an aqueous medium containing 1 M sodium salicylate by dialysis method (Han et al., 2006). One millimeter of PTX-loaded micelle solution (containing 0.1 mg PTX) was introduced into a dialysis bag (MWCO = 5000 Da, Greenbird Inc., Shanghai, China) and the end-sealed dialysis bag was submerged fully into 50 ml of 1 M sodium salicylate solution at 37 °C with stirring at 100 rpm for 24 h. At appropriate time intervals (0, 15, 30 min and 1, 2, 4, 6, 8, 10, 12, 24 h), 0.5 ml aliquots were withdrawn and replaced with an equal volume of fresh medium. The concentration of PTX in samples was determined by HPLC as described above with correction for the volume replacement. PTX release from stock solution and Taxol injection were also conducted under the same conditions as controls.

2.5.5. Micelle stability

To test the storage stability, lyophilized drug-loaded micelles of the optimal formulation were stored at 25 °C for 6 months, and the samples were monitored for time-dependent changes in particle size and drug content during the storage period.

In order to assess the effect of F127 on the physical stability of the micelles, PTX-loaded P123/F127 mixed micelle solution was incubated in a temperature controlled oven at 37 °C with shaking at a speed of 100 rpm for a few days. A solution of PTX-loaded Pluronic P123 micelle prepared without addition of F127 was conducted under the same condition, as control. At predetermined time intervals, each sample was filtered through a 0.2 μm filter membrane followed by dilution with acetonitrile. The concentration of PTX remaining in solution was measured using HPLC.

The effect of dilution on the micelles was studied by incubating the micelles in PBS (pH 7.4) at 10-fold dilution at 37 °C for 48 h. Following the incubation, the samples were filtered through 0.2 μm membrane filter and analyzed for the presence of PTX in solution using the same method described above.

2.6. In vitro cytotoxicity assay

Human lung adenocarcinoma cell line SPC-A1 and A-549 were obtained from Cell Resource Center of China Science Academy and were cultured at 37 °C with 5% CO_2 under fully humidified conditions. The cells were cultured in RPMI 1640 medium, supplemented with 10% FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin sulfate.

Cells were seeded at the density of 5×10^3 cells per well in 96-well plates. After 24 h of incubation at 37 °C with 5% CO_2, the growth medium was replaced with 200 μl medium containing either respective drug samples-free drug dissolved in DMSO, PTX-loaded P123/F127 mixed micelles and Taxol injection with various concentration or three different excipient—Pluronic P123, F127 and Cremophor EL with concentration ranging from 0.1 to 1000 μg/ml. After 72 h incubation, cell survival was then measured using tetrazolium salt MTT assay. 180 μl of fresh growth medium and 20 μl of MTT (5 mg/ml) solution were added to each well. The plate was incubated for an additional 4 h, and then 200 μl of DMSO was added to each well to dissolve any purple formazan crystals formed. The plates were vigorously shaken before measuring the relative color intensity. The absorbance at 570 nm of each well was measured by a microplate reader (Tecan Safire2, Switzerland).

3. Results and discussion

3.1. Doehlert matrix design

In general, micelle formation is essentially a reversible aggregation process induced by limited aqueous solubility of the Pluronic. It therefore displays considerable dependency on a number of factors. The nature of the Pluronic such as EO/PO ratio and molecular weight, environmental features such as temperature, concentration and ionic strength and the compatibility between the core of Pluronic micelles and PTX molecules are all important determinants (Wang et al., 2007; Chiappetta and Sosnik, 2007). Thus in order to evaluate the effects of variations in factor levels, a 23-run Doehlert matrix was applied. In this trial, the total amount of the feeding Pluronic was kept constant while the composition of mixture was varied. Pluronic P123 is a relatively hydrophobic Pluronic with long PO chains and short EO chains. It can usually form cylindrical aggregates in aqueous medium, which exhibit a higher solubilization capacity than spherical micelles formed by a hydrophilic Pluronic. Compared to P123, F127 with a high ratio of EO/PO has a well-known colloidal steric stabilization effect. It is easy to observe that the length of hydrophobic moieties of the selected P123 (PPO: 69) and F127 (PPO: 64) are very close. Hence,

Table 2
A summary of each factor effect and its *p*-value for response Y_1 (DL %), Y_2 (ER %), Y_3 (PTX precipitated %) and D (overall desirability value).

Factor	Y_1		Y_2		Y_3		D	
	Factor effect	*p*-Value	Factor effect	*p*-Value	Factor effect	*p*-Value	Factor effect	*p*-Value
Intercept	1.032	<0.0001***	70.418	<0.0001***	3.832	0.0726	0.699	<0.0001***
X_1	0.425	0.0002***	28.616	0.0003***	10.610	<0.0001***	0.126	0.0499*
X_2	−0.152	0.0454*	−11.785	0.0385*	2.421	0.1304	−0.176	0.0124*
X_3	0.195	0.0159*	−42.373	<0.0001***	2.055	0.1906	−0.055	0.3485
X_4	0.189	0.0182*	9.457	0.0823	−0.182	0.9020	0.123	0.0544
X_1^2	−0.133	0.3397	−8.924	0.3857	10.311	0.0079**	−0.699	0.0002***
X_2^2	−0.126	0.3618	−8.698	0.3973	−0.518	0.8641	0.054	0.6421
X_3^2	−0.710	0.0004***	−10.531	0.2873	−3.646	0.2269	−0.548	0.0009***
X_4^2	−0.193	0.1413	−13.232	0.1710	−0.847	0.7577	−0.047	0.6568
X_1X_2	−0.116	0.5045	−8.076	0.5300	7.129	0.0908	−0.108	0.4655
X_1X_3	0.217	0.2739	10.033	0.4869	3.486	0.4253	0.253	0.1483
X_1X_4	0.027	0.8900	−6.970	0.6387	−3.735	0.4111	−0.077	0.6519
X_2X_3	0.032	0.8680	10.701	0.4592	−6.305	0.1671	0.452	0.0213*
X_2X_4	0.004	0.9829	−1.118	0.9396	−4.434	0.3333	0.152	0.3825
X_3X_4	−0.048	0.8098	−10.373	0.4887	−2.422	0.5896	−0.031	0.8535
F-value	52.47		59.63		9.45		24.96	
R^2	0.9294	–	0.9421	–	0.9152	–	0.9194	–

* Significant effects of factors on individual response: $p < 0.05$.
** Significant effects of factors on individual response: $p < 0.01$.
*** Significant effects of factors on individual response: $p < 0.001$.

proper selection of the ratio of the two Pluronic copolymers was critical for the formation of drug-loaded mixed micelles. Response data for all experimental runs of DMD were presented in Table 1. All the responses observed from 23 runs were fitted to a second-order polynomial model. For the estimation of the significance and validity of the model, the analysis of variance (ANOVA) was applied, using a 5% significance level (Table 2).

From Table 2, it could be concluded that the main effects of four factors on DL % (Y_1) were found statistically significant ($p < 0.05$). The effect of P123 mass fraction (X_1) on DL % in the formulation was highly significant ($p < 0.001$), whereas the other variables played a less role. An increase in X_1 led to a positive effect on response DL %. The results in Table 1 showed that the highest DL % was obtained at the high level of X_1, indicating that P123 has a much better solubilization capacity than F127. In addition, the amount of PTX (X_3) had a positive effect on DL %, which was consistent with the results shown by Wang et al. (2007). It suggested that the hydrophobic interaction between drug and Pluronic copolymers exceeded that between PTX molecules due to the high solubilization potential of P123 as the amount of PTX increased. As shown in Table 2, both amount of water (X_2) and hydration temperature (X_4) had an important effect on DL %. It could be explained that the concentrated micelle solution composed of P123 and F127 of certain proportion could form soft gel at high temperature which was quite favorable for drug loading (Chaibundit et al., 2007). Furthermore, at temperature above 50 °C, solid Pluronic is in its molten state and will be an excellent solvent for water insoluble PTX. In this study, although loading level is not very high, the solubility of PTX has already been successfully increased to many times its intrinsic water solubility limit. For instance, at a loading level of 1.46%, the effective concentration of PTX was 249.4 μg/ml, which was already 830 times higher than its intrinsic solubility of 0.3 μg/ml.

The ER % values of the 23 batches showed a wide variation ranging from a minimum of 20.8% to a maximum of 99.8% for PTX-loaded mixed micelles, indicating that the response was strongly dependent on the selected variables (Table 1). It was evident from ANOVA that ER % was significantly influenced by X_1, X_2 and X_3. X_1 had a positive coefficient, while the other two factors had negative coefficients. The probable reasons for this might be the good drug-loading efficiency of P123 at high level of X_1 and formation of soft gel by the two Pluronic copolymers at low level of X_2. PTX precipitated % (Y_3) is also a critical parameter to evaluate physical stability of the micelles. The results indicated that PTX precipitated % was significantly influenced by X_1, whereas other variables were of low significance. It could be observed from Table 1 that more PTX precipitation was generated from increasing X_1. Therefore, it was assumed that at high level of X_1, more PTX could be encapsulated into the inner core of the mixed micelles. However, the micelle stability was weakened by excessive stacking of cylindrical aggregates formed by P123. Consequently, it was necessary to determine the proper fraction of P123 in order to yield a relatively stable PTX-loaded mixed micelle system.

3.2. Optimization by desirability function

An overall desirability function dependent on all the investigated variables was used to predict the ranges of variables where the optimum formulation may occur. In our study, a further optimization process was undertaken with desirability function to optimize the three responses simultaneously. The responses: DL % (Y_1), ER % (Y_2) and PTX precipitated % (Y_3) were transformed into the desirability scale d_1, d_2 and d_3, respectively. Among them, Y_1 and Y_2 had to be maximized, while Y_3 had to be minimized. The desirable ranges are from 0 to 1 (least to most desirable). The overall desirability function was calculated by Eqs. (5)–(7) and the results were shown in Table 1. It indicated that the highest values of D (0.87) could be obtained with formulation 5, which was considered the batch fulfilling all the constraints favorable for preparation of PTX-loaded mixed micelles. To confirm the validity of the calculated optimal factors and predicted responses, a fresh batch of the optimized formulation was prepared and evaluated in triplicate. The observed and predicted response values were presented in Table 3. It could be seen that there was a reasonable agreement between the predicted and observed experimental values. For this reason, it could be concluded that the equations in Table 2 describe adequately the influence of the selected factors on the responses in this study.

Table 3
The observed and predicted response values for the optimized formulation.

Factor	Optimized level	
X_1: P123 mass fraction (%)	66.7	
X_2: Amount of water (ml)	4	
X_3: Amount of PTX (mg)	4	
X_4: Hydration temperature (°C)	60	
Response	**Expected**	**Observed**
Y_1: DL (%)	1.30	1.37
Y_2: ER (%)	89.67	93.8
Y_3: PTX precipitated (%)	6.14	5.94
Overall desirability (D)	0.83	0.88

3.3. CMC determination

CMC of the micelle-forming compound influences its in vitro and in vivo stability and low CMC values of Pluronic P123/F127 binary mixture underlay high stability of P123/F127 mixed micelles in solutions upon dilution. In this study, the formation of micelles was monitored by using iodine as a hydrophobic probe. Solubilized I_2 prefers to participate in the hydrophobic microenvironment of Pluronic copolymers, causing the conversion of I_3^- to I_2 from the excess KI in the solution, in order to maintain the saturated aqueous concentration of I_2. The absorption intensity of I_2 has been plotted as a function of polymer concentration in Fig. 1 and the CMC of P123/F127 binary mixture was determined to be ca. 0.0059%. This is a reasonably low concentration, suggesting their high stability and ability to maintain integrity even upon extreme dilution in body. Furthermore, the CMC values of P123 and F127 were measured, respectively, by the same method. The respective CMC values of P123 and F127 were shown to be ca. 0.0068% and 0.0021%, which were compatible with previous reported CMC values of Pluronic block copolymers, determined using pyrene probe method (Kabanov et al., 2003a,b). The finding of lower CMC for Pluronic F127 (PPO length: 65) than P123 (PPO length: 69) showed that CMC values were influenced not only by hydrophobic PPO length, but also by the length of hydrophilic PEO chain, as observed

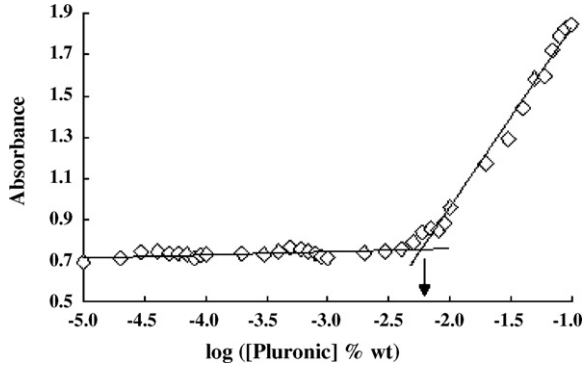

Fig. 1. Plot of UV intensity of I_2 vs. concentrations of Pluronic P123/F127 mixed micelles in DI water.

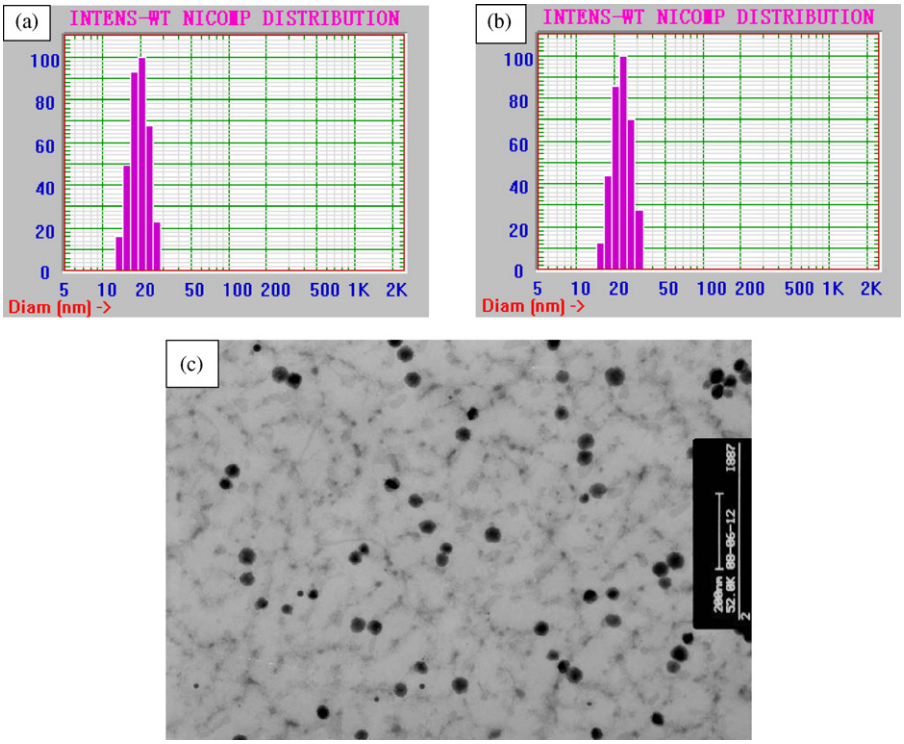

Fig. 2. Micelle size and size distribution of empty P123/F127 mixed micelles (a); PTX-loaded P123/F127 mixed micelles (b); TEM image of PTX-loaded P123/F127 mixed micelles (c).

by other authors (Sezgin et al., 2006). In addition, we observed that the CMC of P123/F127 mixture was lower than that of pure P123. This may be due to the hydrophobic interactions between the two polymer PPO chains.

3.4. Particle size distribution and surface morphology

In order to achieve longevity during systemic circulation, the micelles must be small enough to evade detection and destruction by the reticulo-endothelial system (RES). In our study, the average micelle size and the unimodal size distribution of both empty and PTX-loaded P123/F127 mixed micelles are illustrated in Fig. 2a and b, indicating a formation of mixed micelles. The mean diameter of blank micelles as well as mixed micelles was close to 20 nm, with an acceptably good polydispersity index (PDI) between 0.14 and 0.23. Loading micelles with PTX did not visibly affect their size and size distribution. Very slight and statistically insignificant average size increase after PTX loading (from 20.1 nm to 23.5 nm) might nevertheless reflect a certain increase in the hydrophobic micelle core size because of solubilization of PTX there. As seen from Fig. 2c, the mixed micelles exhibited spherical shape of moderate uniform particle size and the particle size measured from the TEM images was in good agreement with that measured by the laser scattering technique. By close observation of TEM picture, it was found that bright and dark regions were seen in these mixed micelles. The bright region should be attributed to the PEO block of Pluronic copolymers and the dark region should respond to the hydrophobic core of PPO chains. This core–shell structure of Pluronic micelles plays an important role in providing long circulation times in blood.

3.5. ^1H NMR characterization

The drug entrapment into the inner core of the P123/F127 mixed micelles was confirmed by the analysis of ^1H NMR spectra. Fig. 3 showed the ^1H NMR spectra of PTX in $CDCl_3$ (a), blank mixed micelles in $CDCl_3$ (b), PTX-loaded P123/F127 mixed micelles in $CDCl_3$ (c) and D_2O (d), respectively. In $CDCl_3$, resonance peaks corresponding to the Pluronics and PTX were clearly observed. In contrast, only Pluronics resonance peaks were detected in D_2O, whereas the characteristic peaks of PTX were hardly seen. These results clearly indicated that PTX was successfully entrapped into the hydrophobic PPO inner core of mixed polymeric micelles. This is in agreement with ^1H NMR studies with PTX-loaded PEtOz–PCL micelles in D_2O (Lee et al., 2003).

3.6. In vitro release

The in vitro cumulative release profiles of PTX from different formulations are shown in Fig. 4.The maximum concentration of PTX in the medium was 2.0 μg/ml, while the solubility of PTX in 1 M sodium salicylate medium was 28.1 μg/ml (Cho et al., 2004), thus good sink conditions were respected. In addition, prior to conducting these release assays, PTX release from stock solution and Taxol were investigated as controls. It was found that >80% PTX in the stock solution and nearly 76% of PTX in Taxol injection were released within the first 2 h. This suggested that PTX could freely diffuse through the dialysis membrane. During the same time period, 46.3% and 33.4% of PTX were released from the P123/F127 mixed micelles and P123 micelles, which were much slower than Taxol injection. This result showed that the micelle carrier can not only solubilize the poorly soluble drugs, but also sustain PTX release. As can be seen, the PTX release rate of mixed micelles in this medium was faster than that of P123 micelles. It can be explained that the increase in PEO length by Pluronic F127 enhances distribution of PEO units and water molecules into the core of the micelles, leading to more formation of hydrophilic channels (Kovzlov et al., 2000; Hu et al., 2003).

3.7. Micelle stability

As presented in Fig. 5a, drug content of PTX-loaded P123/F127 mixed micelles did not change in 6 months, showing the high stor-

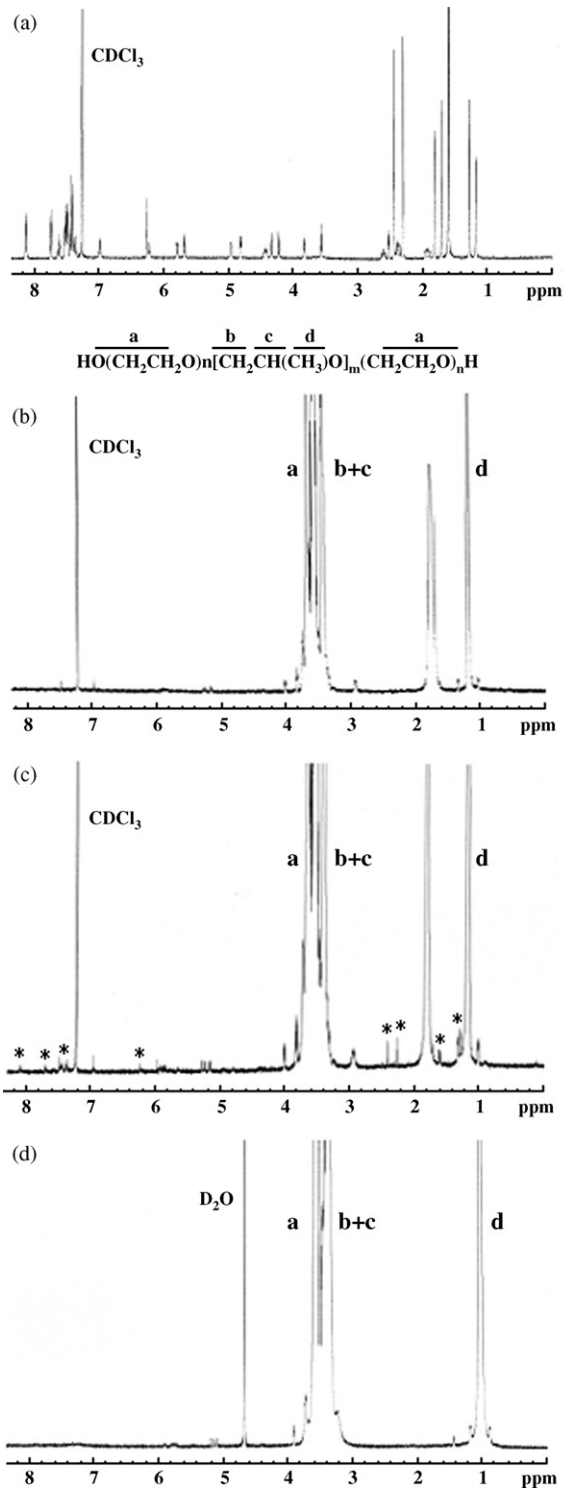

Fig. 3. ^{1}H NMR spectra of PTX in $CDCl_3$ (a); empty P123/F127 mixed micelles in $CDCl_3$ (b); PTX-loaded P123/F127 mixed micelles in $CDCl_3$ (c) and PTX-loaded P123/F127 mixed micelles in D_2O (d). The (small) signals of the PTX-protons are indicated by *.

age stability of this formulation in lyophilized form. But it was found that the particle size of mixed micelles reconstituted after freeze-drying was increased from 22.6 nm to 32.3 nm during the storage period. This might be due to the slight aggregation of hydrophobic micelle core within this period. To elucidate protective effects of F127 on the in vitro physical stability of mixed micelles, P123/F127 mixed micelle and P123 micelle solutions were investigated at 37 °C for 5 days. It was observed that the percent PTX remaining in the

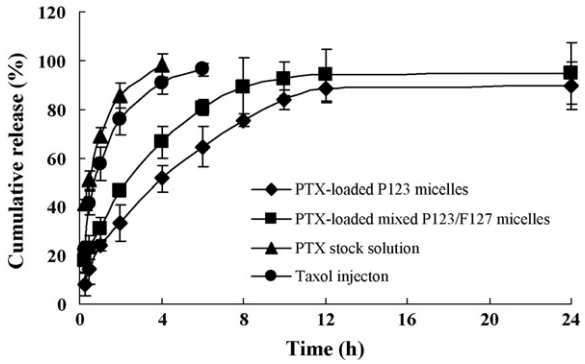

Fig. 4. Release profiles of PTX from Pluronic P123 micelles and P123/F127 mixed micelles in 1 M sodium salicylate medium at 37 °. Each point represents average ± SD (n = 3).

sample solutions for mixed micelle and P123 micelle after incubation at 37 °C for 48 h was 93.1% and 72.6%, respectively (Fig. 5b). This meant that the P123/F127 mixture was much more kinetically stable in aqueous environment than P123 micelles, and insertion of a certain percentage of F127 into a P123 micelle system could

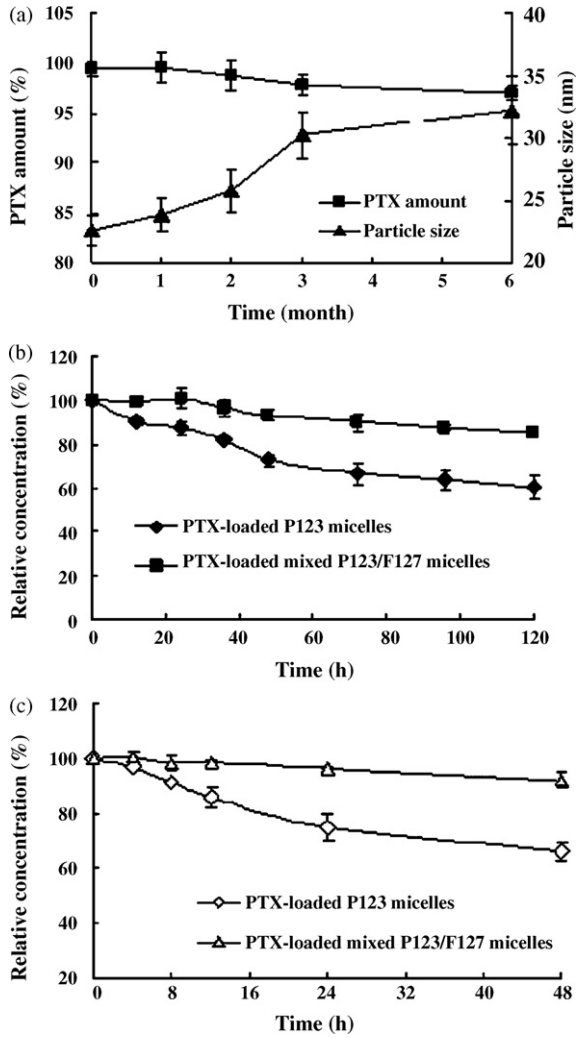

Fig. 5. PTX amount and particle size of lyophilized mixed micelles as functions of time (a); kinetic stability of PTX-loaded P123 micelles and P123/F127 mixed micelles in aqueous medium (b); the stability of drug-loaded P123 micelles and P123/F127 mixed micelles upon 10 times dilution and incubation with PBS (pH 7.4) at 37 °C (c). Each point represents average ± SD (n = 3).

increase the micelle stability. This was thought to be due to the stabilization effect of long PEO chains of hydrophilic F127 blended with P123 in mixed micelles which might prevent the stacking of cylindrical aggregates formed by the long PPO chains of P123 (Oh et al., 2004). Indeed, previous works had reported the steric stabilization of lipid vesicles by incorporation of F127 into the lipid membrane (Szleifer et al., 1998; Kostarelos et al., 1999). But in this experiment, the PTX remaining after 120 h incubation at 37 °C decreased to 85.2%, suggesting that the stabilization effect seemed to be governed by kinetic factors. Although the mixed micelles are kinetically stabilized, they will finally separate since they are thermodynamically unstable (Oh et al., 2004). In case of stability of PTX solubilized in two different micelles upon dilution in PBS (pH 7.4), similar kinetic profiles (Fig. 5c) were observed. P123/F127 mixed micelles were able to retain >90% of PTX when diluted 10 times with PBS and incubated for 48 h, while nearly 35% of PTX was precipitated out of P123 micelles. The concentration of Pluronic P123 was still above CMC in 10-fold diluted solution; therefore, the precipitation of PTX could not have resulted from micelle dissociation. Instead, it reflected the tendency for drug-loaded P123 micelle to form cylindrical aggregation and eventually revert to the phase separated state. These results indicated that the hydrophilic long PEO-shell of the micelles formed by F127 had a protective effect on the micelle dispersion. However, the mixed micelles were still in a dynamic state and presented only temporary stability.

3.8. In vitro cytotoxicity

The in vitro cytotoxicity of PTX-loaded P123/F127 mixed micelles was investigated and compared with that of the free drug and Taxol injection using human SPC-A1 and A-549 cells. Fig. 6a showed the drug-entrapped polymeric micelles exhibited similar activity to those observed with Cremophor EL-base commercial formulation and the free PTX in inhibiting the growth of SPC-A1 cells. The IC_{50} values for various formulations were 8.4 ± 1.4 ng/ml for free PTX, 9.9 ± 1.8 ng/ml for Taxol and 8.7 ± 0.4 ng/ml for mixed micelles, respectively. It was important to note that the corresponding block copolymer concentration of polymeric micelles was below the CMC of P123/F127 mixture (0.0059%) at the reported IC_{50} value for drug-loaded mixed micelles. Thus, the PTX was likely present in free form and exhibits therapeutic effects as the free drug. In this study, in vitro biocompatibility of Pluronic P123, F127 and Cremophor EL was also carried out using SPC-A1 and A-549 cells. In the concentration ranges used for various formulations of PTX, the cytotoxicity of PTX-unloaded carriers including P123, F127, and Cremophor EL was negligible, as shown in Fig. 6b and d. However, it was shown that P123 displayed increasing cytotoxicity as the concentration increased and this effect might be partly due to the cytostatic action of Pluronic micelles. Rapoport et al. (2003) also demonstrated that even 48 h incubation with Pluronic P105 micelles did not kill the A-2780 ovarian carcinoma cells but effectively prevented cell proliferation. Additionally, it was shown by others that the cytotoxic effect of Pluronic unimers and micelles on non-cancerous cells was significantly lower than that on cancerous cells (Rapoport et al., 2003; Melik-Nubarov et al., 1999). Although the growth inhibition of Pluronic P123 alone against both of human lung adenocarcinoma cells was significant at 1 mg/ml, Cremophor EL exhibited much higher cytotoxicity than P123 at the identical concentration. The viable SPC-A1 and A-549 cells after 3 days exposure with Cremophor EL were less than 20%, which was similar to the other results tested on HeLa cervix carcinoma cells, KB human epidermoid carcinoma cells and C26 murine colon carcinoma cells (Lee et al., 2003; Danhier et al., 2009; Carstens et al., 2008). As reported previously, Cremophor EL was significantly cytotoxic at concentration above 1 mg/ml (Liebmann et al., 1993, 1994). For A-549 cell line, the IC_{50} values were 1.68 ± 0.59 μg/ml for free PTX, 0.39 ± 0.09 μg/ml for Taxol and 0.10 ± 0.04 μg/ml for P123/F127 mixed micelles, respectively. The PTX-loaded mixed micelles demonstrated a superior cytotoxicity compared to those of free drug and Taxol as shown in Fig. 6c, which could be explained by the hypersensitizing effect of Pluronic copolymer (Batrakova et al., 2003). This might occur since expression of a number of ATP-binding cassette (ABC) efflux pumps such as P-glycoprotein (P-gp), multidrug resistance-associated protein (MRP), breast cancer resistance protein (BCRP) and lung resistance-related protein

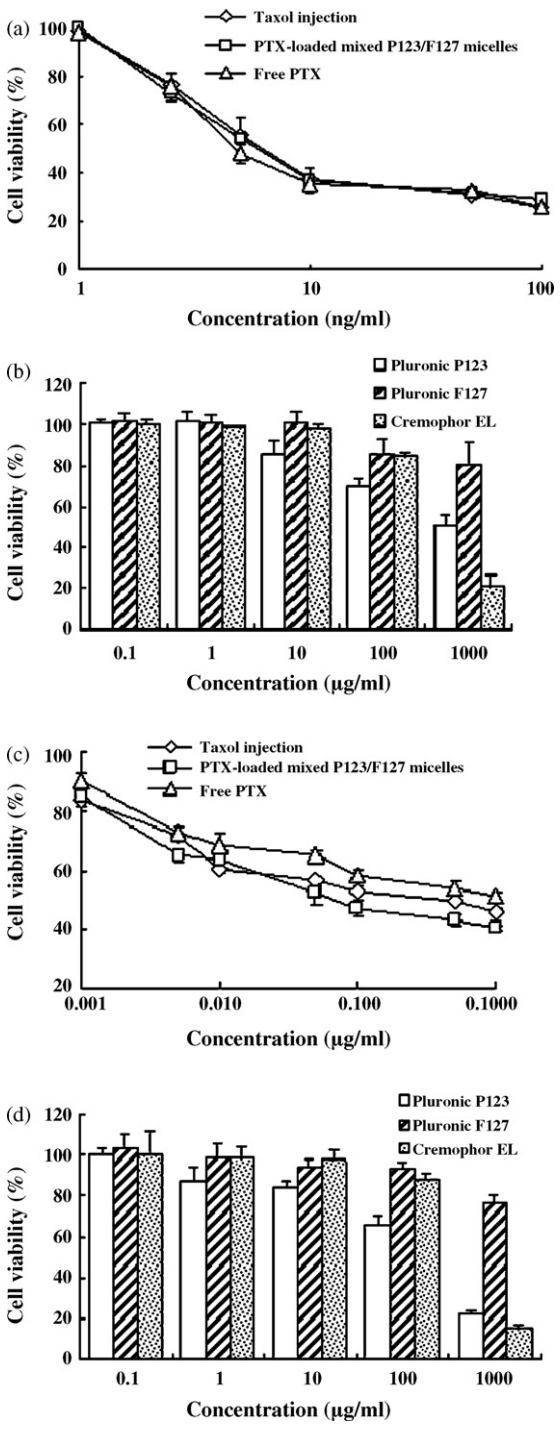

Fig. 6. In vitro cytotoxicy of various formulations of PTX against SPC-A1 and A-549 cells (a and c); viability of SPC-A1 and A-549 cells as a function of varying concentrations of excipients (Pluronic P123, F127 and Cremophor EL) (b and d). Each point represents average ± SD ($n = 3$).

(LRP) were reported in A-549 cells (Kang et al., 2005; Imai et al., 2002; Hira et al., 2008; Meschini et al., 2002). However, further studies are required to examine whether PTX is a substrate of other transporters involved in MDR except P-gp and to understand how Pluronic copolymers mediate the efflux of PTX in A-549 cells.

4. Conclusions

The aim of the study was to design mixed micelles composed of Pluronic P123 and F127 loaded with the poorly soluble anticancer drug PTX. The optimization of PTX-loaded P123/F127 mixed micelles was carried out by Doehlert matrix design combined with desirability function. The effects of P123 mass fraction, amount of water and PTX, and hydration temperature, on the drug-loading coefficient, encapsulation ratio and PTX precipitated % were investigated as well. It was shown that the optimum formulation could display a low CMC, a high entrapment efficiency and micelle stability. PTX release was sustained as a result of encapsulation into the inner core of the micelles. PTX encapsulation by mixed micelles also demonstrated an increased in vitro cytotoxicity compared to Taxol injection and free PTX in A-549 cells. Future studies will focus on reversal of MDR cells by the mixed micelles through the inhibition of P-gp function and in vivo evaluation of PTX-loaded P123/F127 mixed micelles.

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