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Molecular mechanism study of chemosensitization of doxorubicin-resistant human myelogenous leukemia cells induced by a composite polymer micelle

Min Han[a], Yuan-Yuan Diao[a,b], Hong-Liang Jiang[c], Xiao-Ying Ying[a], Da-Wei Chen[b], Wen-Quan Liang[a], Jian-Qing Gao[a,*]

[a] Institute of Pharmaceutics, College of Pharmaceutical Sciences, Zhejiang University, Hangzhou 310058, PR China
[b] School of Pharmacy, Shenyang Pharmaceutical University, Shenyang 110016, PR China
[c] Department of Polymer Science and Engineering, Zhejiang University, Hangzhou 310058, PR China

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ABSTRACT

The present study was aimed to overcome the multidrug resistance (MDR) of tumor cells which accounts for the failure of clinical chemotherapy. A novel doxorubicin (DOX)-loaded composite micelle consisting of polyethylene glycol (PEG)–polycaprolactone (PCL)/Pluronic P105 has been developed and was proved to inhibit the drug resistance of human myelogenous leukemia (K562/ADR) cells. The modulation mechanism that DOX-loaded the composite micelle inhibited MDR was for the first time investigated at cell levels. Results indicated that the cytotoxicity in K562/ADR cells treated by DOX-loaded PEG–PCL/P105 composite micelle was about 4 times higher than DOX solution at 12 μg/mL of DOX. Confocal images showed that the DOX-loaded composite micelles gradually entered into cytoplasm and nucleus, and stayed in intracellular much longer than DOX solution. All the micelles (PEG–PCL micelle, P105 micelle and PEG–PCL/P105 composite micelle) did not change Pgp expression on the surface of K562/ADR cells. However, further study revealed that micelle containing of P105 (P105 or PEG–PCL/P105 composite micelle) significantly decreased ATP level, and consequently restricted the activity of Pgp by down-regulation of mitochondrial membrane potential. On the other hand, the PEG–PCL micelle had no effect on both mitochondrial membrane potential and ATP level of the K562/ADR cells, but its access to K562/ADR cells through endocytic pathway avoided the recognition of Pgp. The PEG–PCL/P105 composite micelle was designed based on the combination of P105-mediated down regulation of mitochondrial membrane potential the malignant cells and PEG–PCL-mediated internalization effect. Therefore, the novel composite micelle is a promising drug delivery system for anticancer drug to overcome MDR.

1. Introduction

As one of the leading causes of morbidity and accounting for a quarter of all deaths in the world, cancer has attracted unparalleled focuses and incessant medical efforts, among which chemotherapy is a method most widely applied in cancer treatment. However, multidrug resistance (MDR) often limits the use of chemotherapy (Aroui et al., 2009; Lukyanova et al., 2009), and one of the most frequent phenotype of MDR is the overexpression of p-glycoprotein (Pgp), a 170 kDa transmembrane protein, which functions as an ATP-dependent drug efflux pump (Zacherl et al., 1994). And an increased expression of this protein would lower the accumulation of the drug in the cell and consequently weaken the drug effect on MDR cells.

Nanocarriers such as liposome, nanoparticle, microemulsion and dendrimer have been studied to reverse the MDR of the tumor cells (Bansal et al., 2009). Especially micelles, which are developed from amphiphilic block copolymers, have gained significant attention from medical fields for their various advantages in drug delivery applications (Gou et al., 2009). One approach for micelles reversing MDR is to administrate antineoplastic agents concurrent with MDR modulatory agents such as verapamil and SDZ PSC-833, etc. (Jabr-Milane et al., 2008; Zacherl et al., 1994). While another approach that has received newly concern is the employment of the surfactants and copolymers. The nonionic polyethoxylated surfactants, such as Cremophor, Tween 80, Solutol HS15, and Pluronic family composed of poly(ethylene oxide)–(polypropylene oxide)–poly(ethylene oxide) triblock copolymers have also been employed to modulate MDR (Bogman et al., 2003; Chong et al., 1993; Coon et al., 1991). It is suggested in the relevant reports that the capability of such surfactants as lipids and nonionic detergents in modulating MDR in cancer cells may be attributed to the inhibition of Pgp by the polymers, the membrane fluidity enhanced by the

* Corresponding author. Tel.: +86 571 88208437; fax: +86 571 88208437.
E-mail address: gaojianqing1029@yahoo.com.cn (J.-Q. Gao).

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degradation product of the polymers, or the drug uptake in tumor cells increased by endocytosis of the drug carriers. Among the surfactants stated, micelles composed of Pluronic polymers, especially Pluronic 85 (P85), has also been studied for its remarkable effect on MDR reversion (Alakhov et al., 1996; Kabanov et al., 2002; Minko et al., 2005).

And yet the simple polymer micelle may not present satisfactory effect while investigations are seldom carried out from the perspective of modulation mechanism. In our previous report, we have first discovered the combination of PEG–PLGA and Pluronic P105 prominently improved the cytotoxicity and the intracellular accumulation of loaded DOX in a drug-resistant tumor cell line, K562/ADR (Diao et al., 2010) and further revealed the potentiality of the composite micelles in reversing MDR of the tumor cells. In this study, we used two kinds of polymer PEG–PCL and P105 as drug carriers to overcome MDR, and examined the mechanism of the sensitization effects. The cytotoxicity and the distribution of the composite PEG–PCL/P105 micelle in K562/ADR cells have also been probed by MTT assay and confocal laser scanning microscopy. Moreover, the study detected the effect of the composite micelle on the Pgp expression and investigated the Pgp activity related influencing factors, such as intracellular ATP level and mitochondrial membrane potential.

2. Materials and methods

2.1. Materials

Doxorubicin hydrochloride was obtained from Zhejiang Hisun Pharmaceutical Co, Ltd. MPEGs and £-caprolactone were purchased from Sigma–Aldrich. Pluronic P105 was kindly gifted from BASF Corporation (Ludwigshafen, Germany) The Pgp overexpressing human myeloid progenitor cell line K562/ADR and non-Pgp expressing counterpart K562 were generous gifts from the 2nd Affiliated Hospital of School of Medicine, Zhejiang University (Hangzhou, China). Flow cytometric immunophenotyping was used to determine the amount of Pgp expression in K562 and K562/ADR cells. The K562/ADR cells were found to have 3.9-fold more expression of Pgp than the K562 cells. RPMI 1640 medium and fetal bovine serum were obtained from PAA Laboratories GmbH (Austria). Rabbit anti-human Pgp and HRP conjugated goat anti-rabbit secondary antibody were purchased from Bioss Corp. (Beijing, China) and BD Corp. (USA), respectively. JC-1 and ATP Assay Kit were bought from Beyotime (Shanghai, China). MTT was purchased from Shanghai Sangon Biological Engineering Technology & Service Co, Ltd. Dichloromethane (CH_2CL_2), triethylamine (TEA), and other reagents were of analytical grade supplied by Huadong Medical (China).

2.2. Synthesis and characterization of PEG–PCL diblock copolymers

The PEG–PCL diblock copolymers were synthesized by previous reported method with minor modifications (Shuai et al., 2004; Zhang et al., 2006). Briefly, the ring-opening polymerization of £-caprolactone in CH_2CL_2 was induced by using mPEG and calcium ammoniate as the macroinitiator and catalyst, respectively. The precipitates were appeared by adding cold methanol into the CH_2CL_2 solution and collected by filtration. This product was under vacuum dried at 40 °C. The molecular weight of this segments in the diblock copolymer was determined by the intensity of the terminal methoxy proton signal of MPEG at $Q = 3.39$ ppm and the methylene proton signal of PCL at = 2.31 ppm in ^1H NMR spectroscopy. The weight ratio of the PEG/PCL repeated units was calculated to be 7.08 from the integral values of characteristic peaks and with a molecular weight of 19,278 Da. A similar molecular weight was also measured by gel permeation chromatography (GPC).

2.3. Preparation of DOX loaded composite micelle

DOX-loaded polymeric micelles were prepared by solvent evaporation method. Briefly, DOX hydrochloride (3 mg) was dissolved in CH_2CL_2 with triple molar ratio of triethylamine and stirred on a magnetic stirrer at 500 rpm for 4 h at room temperature. PEG–PCL copolymer and Pluronic 105 (with a ratio of 1:5, w/w) were added into DOX solution and dissolved by vortex. The organic solution was then mixed with 20 mL distilled water and followed by vigorous ultrasonication agitation. The solvent was evaporated by vigorous stirring at 10,000 rpm for 1 h and following a slow stirring at 200 rpm overnight. The residual CH_2CL_2 was removed by a rotary evaporator. The resulting micelle solution was concentrated into 5 mL and then was passed through a 0.22 μm syringe filter to eliminate the polymer and DOX aggregates. All procedures were carried out under light protection. The similar method as above was applied to prepared DOX-loaded into PEG–PCL micelle and DOX-loaded P105 micelle.

2.4. Characterization of the micelle

The morphology of the micelles was observed under transmission electron microscopy (TEM; Morgagni 268 D, Fei, Netherlands). Particle size was measured by dynamic light scattering (MALVERN Nano ZS®, Malvern, UK). An analytical high performance liquid chromatograph (HPLC, Agilent 1100 system) was developed to quantify DOX. The unloaded drug was separated by passing through ultra-filtration membrane (MILIPORE, USA) and measured by HPLC. Drug entrapment efficiency was calculated by the entrapped DOX into micelle with respect to total micellar DOX.

2.5. Cytotoxicity assay

K562 cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C and 5% CO_2 humidified atmosphere. K562/ADR cells were cultured in the same medium with the addition of 1 μg/mL DOX. Prior to the cytotoxicity experiments, the cells were cultured for two weeks in drug-free medium, then seeded in 96-well plates at a density of 1×10^4 cells/well and allowed to grow overnight. In the following day, an indicated concentration of various DOX formulations was added into the culture medium. Equal volume of 20 μL sterile water was used as a control. After 48 h of incubation, the cytotoxicity was evaluated by a standard MTT assay and the absorbency was determined by a microplate reader (KHB,ST-360) (λ = 560 nm). All the experiments were repeated for three times and the results were calculated by the following equation:

$$\text{Cytotoxicity}\,\% = \frac{\text{Abs}_{(\text{control group})} - \text{Abs}_{(\text{experiment group})}}{\text{Abs}_{(\text{control group})}} \times 100\%$$

2.6. Confocal laser scanning microscopy (CLSM) analysis

K562/ADR cells were seeded in 12-well plates at a density of 1×10^4 cells/well. After 24 h of incubation, the culture medium was replaced by 1 mL of phosphate buffered solution (PBS) containing 24 μg/mL DOX or the same concentration of DOX-loaded composite micelle. At 0.5, 2 and 8 h, the cells were rinsed with PBS, and the confocal laser scanning microscopy (CLSM) (Car Zeiss, Shanghai Co. Ltd) with an emission wavelength of 458 nm was applied to investigate accumulation of DOX in the K562/ADR cells. In another

independent study, after 6 h of incubation with a DOX solution or DOX micelle, the medium was removed and the RPMI 1640 medium was added back. Then CLSM was applied to investigate efflux of DOX from K562/ADR cells at 0.5, 2 and 3 h by observing the fluorescence intensity of DOX in cells.

2.7. Pgp protein determination

Pgp detection was performed by color flow cytometry on a Becton Dickinson FACScan, and data was analyzed by Becton Dickinson CELL Quest software. K562/ADR cells were exposed to various DOX formulations (solution, PEG–PCL micelles, P105 micelle and PEG–PCL/P105 composite micelle) and their corresponding drug-free formulations. After 12 h of incubation, 100 μL cell suspension was withdrawn and mixed with the Pgp-FITC antibody (10 μg/mL) on the ice for 30 min. The unbound antibody was removed by centrifugation. The cells were kept from light exposure and washed by 1 mL of PBS twice and finally re-suspended in 500 μL of cold PBS for immediate flow cytometric analysis (Leith et al., 1995; Yang et al., 2002).

In addition, western blot analysis was also performed to detect expression of Pgp. K562/ADR cells were seeded into 6-well plates at a density of 5×10^5/well with 2 mL growth medium and allowed to attach for 24 h. After DOX treatment which is similar with that of Pgp test in flow cytometry. Protein was extracted from the cells and separated on a 4–15% SDS-PAGE gradient gel, and then transferred onto nitrocellulose membrane. Blots were incubated with Rabbit anti-Pgp antibody (1:200, v/v) (Bioss Corp., Beijing, China) and rabbit anti β-actin antibody at 4 °C overnight. After incubated with HRP conjugated goat anti-rabbit secondary antibody monoclonal antibody for 1 h at room temperature, the membrane was exposed to Kodark film using ECL Plus reagent, and the protein bands were determined.

2.8. ATP assay

Cells grew in 24-well plates until confluent, then exposed to fresh culture medium containing different kinds of DOX-loaded micelles, drug-free micelles, and DOX solution at 24 μg/mL of DOX for 2 h. After washing ice-cold PBS, the cells were solubilized in a Triton X-100 solution (1%) and the cell lysates was quickly collected for ATP quantification by a luciferin/luciferase assay. PBS was used as a control.

2.9. Analysis of mitochondrial membrane potential by JC-1 staining

The probe 5,5′, 6,6′-tetrachloro-1,1′, 3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) can freely permeate cells and undergoes reversible transformation from a monomer into an aggregate form when bound with high mitochondrial membrane potential. The JC-1 aggregate form is distinguished from the mono-meric form by taking on bright red orange (590 nm) fluorescence in reaction to 488 nm excitation while the monomer on a background of green fluorescence (525 nm).

K562/ADR cells were planted at a density of 1×10^6 cell/mL in a 6-well plate and incubated overnight. Three drug-free micelles (PEG–PCL, P105, composite micelle) were then added. PBS was used as a control. Cells were harvested after 2 h of incubation and stained with 1 mL of JC-1 (10 μg/mL) stock solution. After an additional 20 min of incubation, cells were harvested by centrifugation at 1000 rpm for 5 min and washed by double volume of PBS twice. The cells were finally re-suspended in a 0.5 mL of PBS and immediately analyzed by the flow cytometry. All the procedures were kept from light prior to flow cytometry analysis. Results were calculated as the ratio of cells with red orange fluorescence and green fluorescence intensity in channel number (CN) on a log scale.

2.10. Statistical analysis

Values are expressed as mean ± S.D. Statistical comparisons were separately preformed by using two-tailed Student's *t*-test and one-way ANOVA among over three groups, with $P < 0.05$ being considered significant.

3. Results

3.1. Properties of DOX-loaded micelle

Under transmission electron microscopic observation, the PEG–PCL/P105 composite micelle was spherical with a narrow and uniform particle size distribution. The particle size of the PEG–PCL/P105 composite copolymers micelles was around 20 nm as refined by the mean diameter (number distribution). The entrapment efficiency that doxorubicin was encapsulated in the hydrophobic segment of the copolymer was 56.7 ± 1.8%. The critical micelle concentration (CMC), which is the concentration of surfactants above which micelles form and almost all additional surfactants added to the system go to micelles, for the PEG–PCL (EG/CL = 7.08) micelle and Pluronic 105 micelle were 0.98 μg/mL and 0.92 μg/mL, respectively, as determined by fluorescence intensity ratio (I338/I333) using pyrene as a fluorescent probe.

3.2. Cytotoxicity of different DOX formulations in K562/ADR cells

Cytotoxicities of DOX solution, the blank composite micelle and three DOX-loaded micelles in K562/ADR cells were evaluated. As shown in Fig. 1, DOX-loaded PEG–PCL micelles did not show notable improvement of cytotoxicity compared with the DOX solution. The cytotoxicity was significantly enhanced with the treatment of DOX-loaded P105 micelle. More interestingly, it was observed that the composite PEG–PCL/P105 micelle generated the highest cytotoxicity among the three kinds of micelles at DOX concentration above 12 μg/mL, suggesting a synergistic effect was achieved by PEG–PCL and P105 polymers. No cytotoxicity was observed for the blank composite micelle, indicating the cytotoxicity was caused by DOX rather than the carrier.

3.3. Distribution of DOX solution and DOX-loaded PEG–PCL/P105 composite micelle K562/ADR cells

The distribution of DOX solution and DOX-loaded composite micelle in K562/ADR cells was studied by confocal laser scanning microscopy (CLSM, Fig. 2). Strong fluorescence was observed in the cells after 0.5 h of treatment with DOX solution. Then, the fluorescence intense became weak after 2 h of treatment and almost disappeared at 8 h of incubation. By contrast, weaker fluorescence were observed after 0.5 h of incubation for the cells treated with DOX-loaded PEG–PCL/P105 micelle, however, the fluorescence intense turned stronger in the cells, especially in the nucleus after 2 h of incubation. The strong fluorescence intense was still observed after 8 h of incubation, indicating plenty of DOX were retained in the cells.

CLSM images of K562/ADR cells after the removal of DOX solution or DOX-loaded composite micelles following incubation were compared in Fig. 3. As shown in Fig. 3A–C, no visible fluorescence was observed at 0.5, 2 and 3 h for the DOX solution group, indicating that most of drug was pumped out by the cells. Whereas, K562/ADR cells treated with DOX-loaded composite

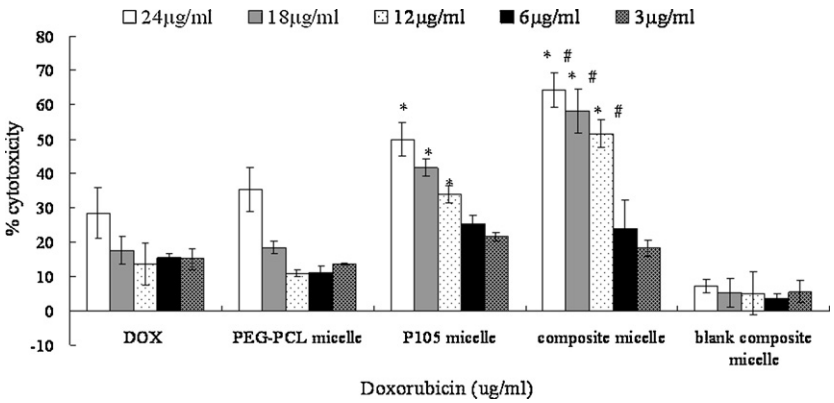


Fig. 1. Cytotoxicity assay in K562/ADR cells. The cells were incubated with DOX-loaded micelle and DOX solution at indicated concentrations for 72 h. Error bars represent the standard deviations ($n = 3$). * represents cytotoxicity of micelles is significantly higher than that of DOX solution ($P < 0.05$), and # represents cytotoxicity of composite micelle is significantly higher than that of P105 micelle ($P < 0.05$).

micelles emitted conspicuous fluorescence at 0.5 h (Fig. 3D), and the fluorescence was still detectable up to 2 h (Fig. 3E) and 3 h (Fig. 3F), which suggested that less DOX was pumped out by K562/ADR cells due to the encapsulation of DOX in micelle.

3.4. Expression of Pgp in tumor cells by various DOX formulations

The effect of micelles on Pgp expression was detected by flow cytometry (Fig. 4A) and western blot analysis (Fig. 4 B). Both of these two analyses showed no significant difference among various treatments after cells were exposed to solution, PEG–PCL, P105 and composite micelles with or without DOX loaded. Thus, micelles did not show the suppression effect on the expression of Pgp, which suggests that the sensitization of PEG–PCL, P105 and PEG–PCL/P105 composite micelle did not directly participate in the modulation of Pgp expression on K562/ADR cells.

3.5. Intracellular ATP level in tumor cells induced various placebos and DOX formulations

Effect of various DOX formulations (solution or different micelles) and placebos (unloaded drug) on the intracellular ATP level was explored (Fig. 5). The cells treated with PBS were used as a control and the ATP level was normalized as 100%. The ATP levels of the cells increased to about 155% after treated with DOX solution. There was no significant APT level change in the cells treated with DOX-loaded or drug-free PEG–PCL micelles. The ATP levels of the cells reduced to approximate 30% of normal level when treated with single P105 micelle placebo, but it was remarkably recovered to 120% of normal level (4 folds) when treated with DOX-loaded P105 micelle. For the cells treated with PEG–PCL/P105 composite micelle placebo or DOX-loaded PEG–PCL/P105 composite micelle, the ATP level dropped to the same level at about 55% of normal level.

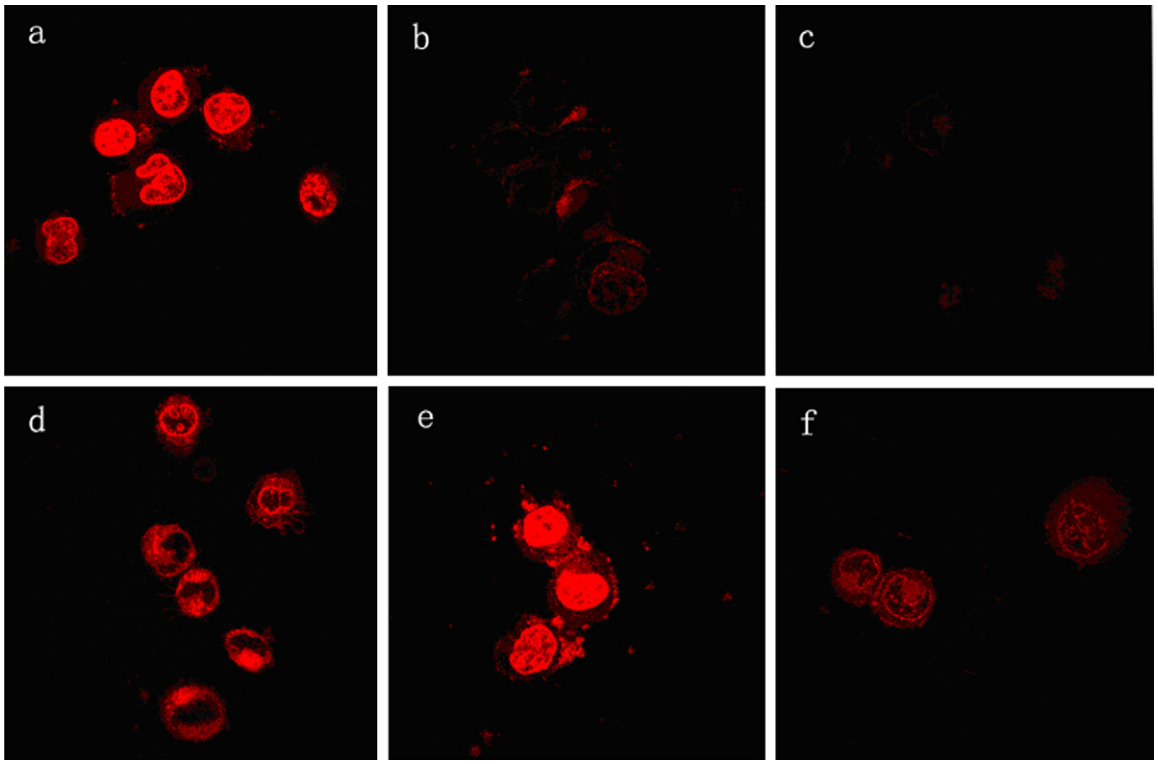


Fig. 2. Confocal Laser Scanning Microscope images of K562/ADR cells that were treated with DOX solution (top row) and DOX micelle (bottom row) for 0.5 (a, d), 2 (b, e), 8 h (c, f), respectively.

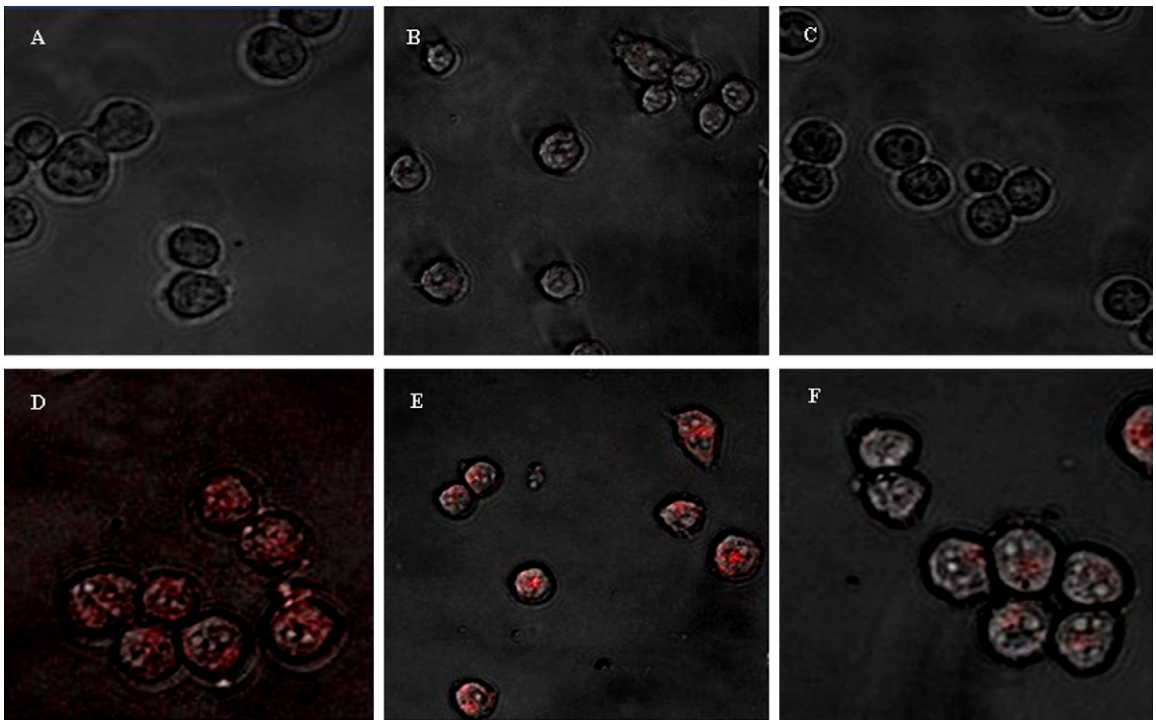


Fig. 3. Confocal Laser Scanning Microscope images of K562/ADR cells that were cultured in RPMI 1640 medium at 0.5 (A, D), 2 (B, E) and 3 h (C, F) after treated with DOX solution (top row) and DOX micelle (bottom row) for 6 h.

3.6. Composite micelles decreased the mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\Psi m$) in K562/ADR cells treated with drug-free micelles (PEG–PCL, P105 and PEG–PCL/P105) or PBS was measured. In contrast to PBS (70.11/25.41 = 2.76), the PEG–PCL micelle did not affect the $\Delta\Psi m$ (R1/R2 = 65.21/28.36 = 2.30) while the micelles containing P105 was significantly decreased. The values of $\Delta\Psi m$ treated by P105 micelle and the PEG–PCL/P105 composite micelle were 1.22 and 1.40, respectively (Fig. 6).

A

The effect of different preparation on Pgp expression in K562/MDR cells (n=3)

Preparation	P- gp expression (RFU)
Solution*	36.95±10.90
Solution	30.07±11.37
PEG-PCL*	38.28±12.66
PEG-PCL	42.80±8.48
P105*	44.46±14.53
P105	41.95±15.28
Composite micelle (PEG-PCL/P105) *	44.42±14.17
Composite micelle (PEG-PCL/P105)	33.27±8.99

Notation: * means doxorubicin loaded

B

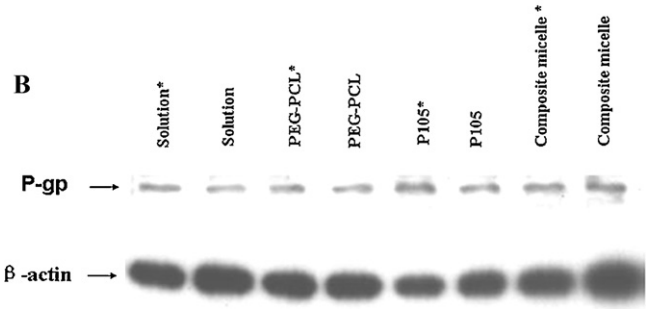


Fig. 4. P-glycoprotein expression using flow cytometry (A) and western blot analysis (B) after the cells was exposed to DOX solution or DOX micelles.

4. Discussion

In contrast to the commercial product of Doxil, in which DOX is encapsulated into a stealth liposomes to provide a longer half-life in circulation, more selectivity of tumor targeting and less cardiotoxicity than DOX injection (O'Brien et al., 2004), polymeric micelle system offers other unique advantages such as incorporating hydrophobic drugs into the inner core phase without losing its targeting capability, having very uniform particle size distribution by selecting the appropriate chemical structure and chain length of polymers, and modifying drug release in a very wide range from minutes to days by modulating physical characteristics of polymers (Inoue et al.). In our preliminary studies, we have prepared several DOX nanoparticle drug delivery systems including DOX loaded conventional liposome, DOX loaded polyethyleneimine modified cationic liposome and DOX loaded micelles, The cellular cytotoxicity and DOX intracellular accumulation in K562/ADR cells were compared as well as DOX solution. Results indicated that neither of the two DOX-loaded liposomes was superior to the

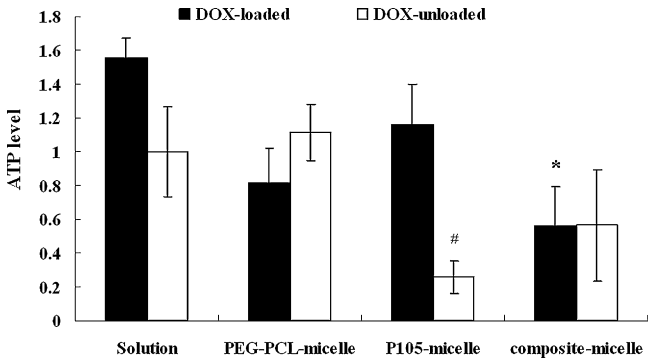
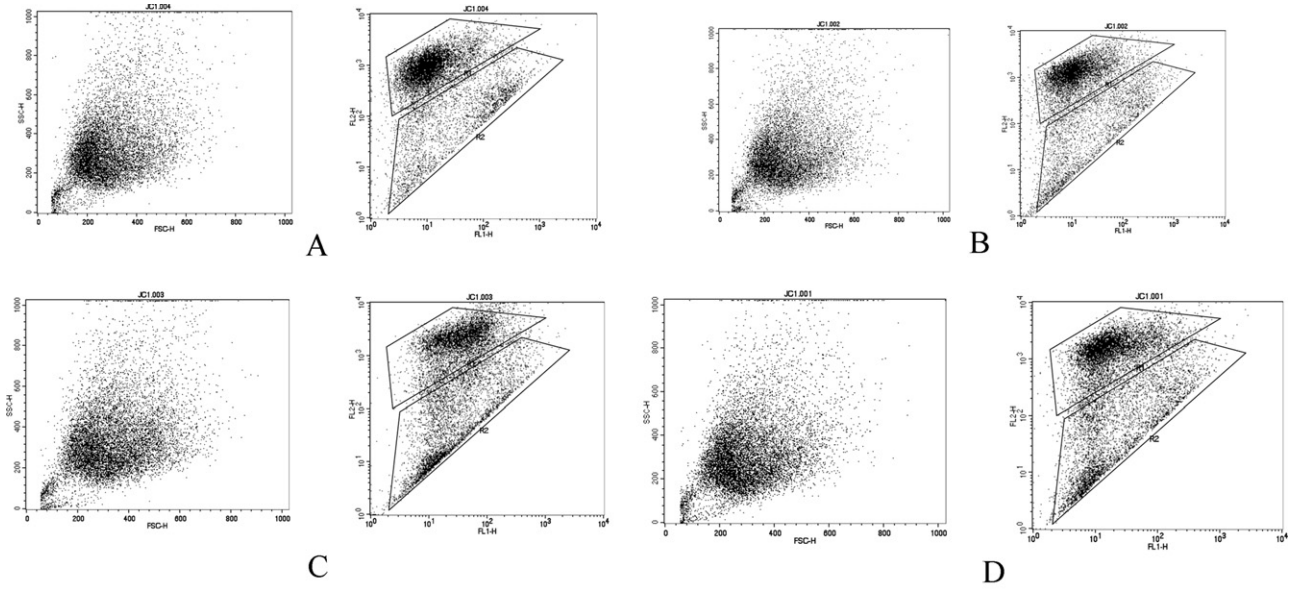


Fig. 5. Influence of various micelles on the ATP levels of K562/ADR cells after 2 h incubation ($n = 3$), * and # represent APT level of micelles were significant lower than that of DOX solution and DOX-unloaded solution, respectively ($P < 0.05$).



Sample	R1	R2	R1/R2
A	70.11	25.41	2.76
B	65.21	28.36	2.30
C	51.45	42.32	1.22
D	55.59	39.57	1.40

Fig. 6. Effect of micelles on mitochondrial membrane potential. K562/ADR cells were exposed to PBS solution (A), PEG–PCL micelle (B), P105 micelle (C) and PEG–PCL/P105 composite micelle (D) for 2 h. Cells were stained with 1 mL of JC-1 (10 μg/mL) stock solution and incubated for 20 min before flow cytometry analysis. R1 represent the percentage of cells with a high $\Delta\Psi$ (red orange fluorescence), R2 represent the percentage of cells with a low $\Delta\Psi$ (green fluorescence).

DOX solution. Among DOX micelles, the DOX-loaded P105 micelle displayed an enhancement in cellular cytotoxicity and intracellular accumulation. This difference between liposome and micelle may be attributed to the effects of micellar polymer on the K562/ADR cells, which will be discussed below.

This study has put its emphasis on an innovative construction of DOX loaded PEG–PCL/P105 composite micelle to overcome MDR. The PEG–PCL/P105 composite micelle was prepared by solvent evaporation method. This method produced a relative small particle size distribution, which was due to the presence of low molecular weight and high hydrophilicity of P105. The relative low entrapment efficiency ($56.7 \pm 1.8\%$) of DOX in the composite micelle might caused by the leakage of DOX into the dispersion medium during preparation.

It has been reported that the micelles enhance the cytotoxicity of anti-tumor drug and reverse MDR. For example, Wang et al. (2007) noticed that paclitaxel-loaded P105 micelle and P105/L101 micelle could overcome MDR in MCF-7/ADR tumor cells. They speculated that this phenomenon might be related to by the drastic sensitization of tumor cells caused by the interaction between Pluronic and MDR cancer cells (Kabanov et al., 2002). Our previous study also proved that the PEG–PLGA and P105 composite micelle loaded with DOX substantially increased the cytotoxicity in the K562/ADR cells but not in DOX sensitive K562, where the physical mixture of polymer and DOX showed the same cytotoxicity as the DOX solution (unpublished results). However, so far the molecular mechanism of sensitization in tumor cell is still unclear. In this study, we observed that DOX-loaded PEG–PCL/P105 composite micelle generated greater cytotoxicity in K562/ADR cells than DOX solution or DOX-loaded PEG–PCL or P105 single micelle. To elucidate these results, we further studied the sensitization mechanism by the combination of CLSM observation, Pgp expression determination, ATP assay analysis and mitochondrial membrane potential ($\Delta\Psi$m) in the following experiments.

First, we applied CLSM to study the efflux of DOX from the K562/ADR cells. It was commonly accepted that the migration of small molecular drug enter into the cells was mediated by a passive diffusion. As shown in Fig. 2, DOX solution quickly entered the nucleus of the K562/ADR cells within 0.5 h, then diminished in the cells and at 2 h and almost disappeared after 8 h, indicating the rapid efflux of DOX in K562/ADR cells. Unlike DOX solution, uptake of DOX-loaded PEG–PCL/P105 composite micelle was through endocytosis. The micelle slowly entered the cells, but could avoid of being pumped out and continued to accumulated around the cell nucleus. A high concentration of DOX in K562/ADR cells was reached at 2 or 8 h incubation. Similar results were also obtained by Shuai et al. (2004). Further investigation was performed to compare the efflux behavior of DOX solution and DOX-loaded composite micelle in K562/ADR cells. The results showed that DOX solution was soon pumped out by the cells while micelle inhibited DOX efflux from cells and retained in the cells.

Multidrug resistance (MDR) was commonly known as one of the bottlenecks in anti-cancer therapy, P-glycoprotein (Pgp) is one of the key ingredients in MDR process and plays a role of drug efflux pumps. Drug loaded into nanoparticles have been reported in several studies to inhibit MDR1 genes encoding drug efflux pumps, and down-regulating expression of the Pgp. In the present study, K562/ADR cells were detected to identification of Pgp overexpression. However, no significant difference in Pgp expression was observed in K562/ADR cells treated with micelles detected by both flow cytometry and western blot. This finding indicated that micelle enhanced cytotoxicity and accumulation of DOX in K562/ADR through other resistance mechanisms rather than inhibition of Pgp expression.

And some factors affecting activity of Pgp were further investigated in present study, such as membrane fluidization and ATP depletion influenced by composite micelles.

As an ATP dependent multidrug-efflux pump, Pgp involved in energy metabolism must be associated to mitochondria membranes (Jezek and Plecita-Hlavata, 2009). The bulk of ATP in most mammalian cells comes from mitochondrial oxidative phosphorylation (Campanella et al., 2009). Central to mitochondrial function is an electrochemical proton gradient across the mitochondrial inner membrane which establishes an electrical membrane potential (MP). A high MP is needed to maintain the normal activity of the ATP metabolism. The outward pumping of protons across the inner mitochondrial membrane produces a proton gradient that drives the conversion of ADP to ATP and is reflected by the MP (Milgram et al., 1990). The results of the MP determination and ATP assay in this study showed blank P105 micelle caused the reduction of MP and induced the decrease of ATP level, which resulted in the inhibition of Pgp-mediated drug efflux and the enhancement of the drug accumulation in the cells (Batrakova et al., 2003)

It was expected that the DOX solution entered the cells accompanying with an ATP dependent process since ATP level in K562/ADR cells was increased to 155% of normal level when treated with DOX solution (standardized by PBS treatment). By this way, DOX could not be avoiding of the recognition of Pgp pump and most DOX would eventually be pumped out from the cells. When K562/ADR cells were treated with the blank P105 micelle, the ATP level went down to about 30% of normal level, indicating the function of APT level inhibition by P105. However, the ATP level in cells treated with DOX-loaded P105 micelle was recovered to 120% of normal level. This could be explained that there existed a lot of free DOX in P105 micelle and a similar process that involved with Pgp pump recognition and DOX pumped out from the cells happened in the same way as in DOX solution. It was not surprised that most DOX was free in P105 micelle because P105 was relative high hydrophilic and low molecular weight. Therefore, encapsulation of DOX in P105 micelle was difficult and the entrapment efficiency was pretty low.

In contrast, the ATP level in the cells exposed to DOX-loaded PEG–PCL micelles is somewhat lower than that in the blank PEG–PCL micelles. And it is logical to infer that DOX transported by the micelles enters the cells via internalization. Allen et al. (1999) also investigated the cellular internalization of polycaprolactone-b-poly (ethylene oxide) (PCL20-b-PEO44) copolymer micelles in PC12 cells, and the internalization process of the micelle was found to fulfill the basic criteria of endocytotic uptake. Intracellular entry of DOX-loaded micelles renders the drug inaccessible for Pgp and increases the intracellular accumulation. It is consistent with the previous findings that a series of diblock copolymers based on methoxypolyethylene glycol-block-poly (caprolactone) (MePEG-b-PCL) were examined to promote the cellular accumulation of another Pgp substrate, rhodamine-123 (R-123), in caco-2 cells (Zastre et al., 2002, 2007). The increase in the accumulation of DOX in K562/ADR cells also suggested the potential of micellar DOX to reverse MDR Pgp-mediated multidrug resistance.

Therefore, it clinches on the synergistic effect of the internalization resulted from PEG–PCL and the inhibition of Pgp aroused by P105 to overcome MDR caused by composite micelles.

5. Conclusions

Prepared by the application of Pluronic 105(P105) and polyethyleneglycol–polycaprolactone (PEG–PCL), DOX-loaded composite micelles demonstrate in this study an enhanced cytotoxicity in K562/ADR cells. While further studies reveal that P105 could reduce mitochondrion membering potential, which results in a depletion of ATP, and further affected the normal activity of the Pgp. Besides, PEG–PCL enveloping DOX in the hydrophobic core may avoid the efflux effection of the Pgp. In this sense, the composite micelles, by exhibiting the synergistic effect might be a promising nanocarrier to overcome or reverse MDR in drug-resistant tumor cells.

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