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Environmental-sensitive micelles based on poly(2-ethyl-2-oxazoline)-*b*-poly(L-lactide) diblock copolymer for application in drug delivery

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Abstract

Anticancer drug doxorubicin (DOX) was physically loaded into the micelles prepared from poly(2-ethyl-2-oxazoline)-*b*-poly(L-lactide) diblock copolymers (PEOz–PLLA). PEOz–PLLA consists of hydrophilic segment PEOz and hydrophobic segment PLLA showed pH-sensitivity in the aqueous solution. The DOX-loaded micelle exhibited a narrow size distribution with a mean diameter around 170 nm. The micellar structure can preserve hydrophobic drug DOX under the physiological condition (pH 7.4) and selectively release DOX by sensing the intracellular pH change in late endosomes and secondary lysosomes (pH 4–5). At 37 °C, the cumulated released rate of DOX from micelles was about 65% at pH 5.0 in the initial 24 h. Additionally, polymeric micelles had low cytotoxicity in human normal fibroblast HFW cells for 72 h by using MTT assay. Moreover, DOX-loaded micelles could slowly and efficiency decrease cell viability of non-small-cell lung carcinoma CL3 cells. Taken together, PEOz-*b*-PLLA diblock polymeric micelles may act as useful drug carriers for cancer therapy.

Keywords: Poly(2-ethyl-2-oxazoline) (PEOz); Poly(L-lactide) (PLLA); Diblock copolymers; Polymeric micelle; Drug delivery

1. Introduction

Block copolymers that consist of hydrophilic and hydrophobic segments are self-assembly formed micelles in aqueous solution. The hydrophobic core of micelles provides a loading space for encapsulating water-insoluble drugs, whereas the hydrophilic shell composed of tasseled chains stabilizes the micelle structure. Additionally, the nano-scaled polymer micelles exhibit a long circulation time and reduce the uptake and caption by the mononuclear phagocyte system (MPS) (Kwon and Kataoka, 1995; Kwon and Okano, 1996) and the reticular epithelial system (RES) (Hruby et al., 2005). The stability and efficiency of anticancer drugs are shown to be improved after incorporating them into polymeric micelles (Jones and Leroux, 1999; Shuai et al., 2004) Furthermore, the controlled release of drugs in response to environmental changes can be achieved by using stimuli-sensitive copolymers as drug-loaded micelles (van Nostrum, 2004; Husseini et al., 2000; Lo et al., 2005).

Notably, the accumulation of micelles in solid tumors that eventually led to the complete regression was achieved by the prolonged circulation in blood as well as the enhanced permeability and retention (EPR) effect (Marin et al., 2001; Liu and Wang, 2004; Liu et al., 2004; Meyer et al., 2001). Polymeric micelles were taken up to cells via endocytosis process. The pH value of endosomal vesicles was gradually decreased from 7.4 to 5 because protons were pumped into the vesicles (Chung et al., 2000). The hydrolysis or deformation of the micellar structure was then promoted due to the decrease of the pH in acidic organelles.

PEOz has been shown to be a pH-sensitive, low cytotoxic polymer with favorable pK_a value near neutral pH (Wang and

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Hsiue, 2002, 2003). PLLA was an extensively investigated biodegradable polymer in the field of drug delivery (Molina et al., 2001). In our previous research, the PLLA–PEOz–PLLA micelles prepared by ultrasonic agitation have been shown to release drugs by pH change (Wang et al., 2005). In this study, the micelles based on poly(2-ethyl-2-oxazoline)-*b*-poly(L-lactide) (PEOz–PLLA) were successfully prepared by dialysis method and were expected to have relatively small size and stable structure. The anti-cancer drug, doxorubicin, was incorporated into the micelles. The in vitro drug release, cell viability and growth inhibition of the PEOz–PLLA micelles were also studied.

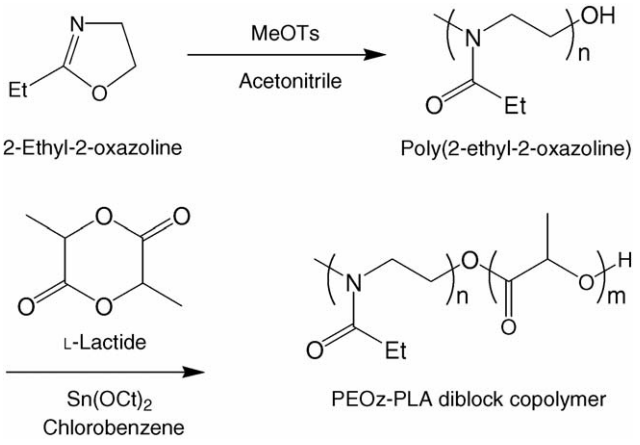
2. Materials and methods

2.1. Materials

L-Lactide (Aldrich) was recrystallized from tetrahydrofuran. 2-Ethyl-2-oxazoline (Aldrich) and methyl *p*-toluenesulfonate (Aldrich) were purified by vacuum distillation over CaH_2. Acetonitrile and benzyl alcohol was dried over CaH_2 and distilled under dry nitrogen. Stannous octoate (Sigma), bovine serum albumin (Sigma), triethylamine (Tedia), dichloromethane (Tedia) and diethyl ether (Tedia) were used as received. Doxorubicin–HCl was provided by TTY Biopharm, Taiwan. 3-(4,5-Dimethylthyazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from ICN Biomedicals Inc. Dulbecco's Modified Eagle's Medium (DMEM), RPMI1640 medium, fetal bovine serum (FBS) and trypsin–EDTA were obtained from Gibco/Life Technologies.

2.2. Synthesis and characterizations of PEOz-b-PLLA diblock copolymers

Monohydroxyl poly(2-ethyl-2-oxazoline) (PEOz-OH) was synthesized by the cationic ring-opening polymerization of 2-ethyl-2-oxazoline at 100 °C for 24 h using methyl *p*-toluenesulfonate (MeOTs) as an initiator (Scheme 1). The crude product was flowed through silica gels and precipitated in diethyl ether. The resulting PEOz-OH was subsequently polymerized with L-lactide in the chlorobenzene at 140 °C for 24 h using stannous octoate as the catalyst. The obtained PEOz–PLLA was purified by precipitation in diethyl ether. The molecular weight and molecular weight distribution were recorded by gel permeation chromatography (GPC, pump: SpectraSystem P100, TSP) with a refractive index detector (RefractoMonitor IV, TSP). *N*,*N*-dimethylformamide (DMF) was the mobile phase at a flow rate of 1.0 mL/min. The GPC columns (Phenogel 5 μ500Å, Phenomenex) were calibrated using poly(methyl methacrylate) standards (Polymer Laboratories). The composition of the copolymer was analyzed by a ^{1}H-NMR using $CDCl_3$ as a solvent. The critical micelle concentration (CMC) of each copolymer was determined by a fluorescence technique using pyrene as a hydrophobic fluorescence probe. A saturated aqueous solution of pyrene (6×10^{-7} mol/L) was used for these experiments. The concentration of copolymer was varied from 0.0001 to 10 mg/mL. Fluorescence intensities of the pyrene entrapped in the micelle core were determined by a fluorescence spectrophotometer (Hitachi F-2500). The excitation wavelength for the emission spectra was 339 nm and excitation spectra were recorded at 390 nm.



Scheme 1. Synthesis route of PEOz–PLLA diblock copolymers.

2.3. Preparation and characterizations of PEOz-b-PLLA micelles

The PEOz–PLLA micelles were prepared by the dialysis method. Ten milligrams of diblock copolymer was dissolved in a 10 mL of DMF and then the polymer solution was stirred at room temperature to solubilize entirely. The solution was dialyzed with dialysis membrane (MWCO 6000–8000 g/mol) against 3 L of Milli-Q water. The fresh water was replaced at every time interval of 6 h for 3 days. The resulting micelles were collected by freeze-drying. ^{1}H-NMR spectra was used to determine the micelle structure as sample dispersed in D_2O. The average diameter and size distribution of micelles (0.2 mg/mL) in phosphate buffered saline (PBS) were determined by dynamic light scattering (Malvern 3000 HS) at 25 °C. The argon laser beam was 633 nm, the scattering angle was 90° and the analysis method was CONTIN. The particle morphology was analyzed by atomic force microscopy (Bioscope AFM). The micelle solution (0.1 mg/mL) was dropped on a silica wafer and dried in the dry box for 2 days before use.

DOX-loaded PEOz–PLLA micelles were prepared by the dialysis method similar to that of blank micelles. The block copolymer (10 mg) was dissolved in 10 mL of DMF, and the doxorubicin–HCl was subsequently added to the block copolymer solution. The solution was added with the equal mole of triethylamine to remove the HCl ion form doxorubicin–HCl. The solution was dialyzed with dialysis membrane (MWCO 6000–8000 g/mol) against 3 L of Milli-Q water. The fresh water was replaced at every time interval of 6 h for 3 days. The resulting micelles were collected by freeze-drying. The amount of loaded DOX was measured at 485 nm by dissolving micelles in dimethylsulfoxide (DMSO) using a UV/Vis spectrophotometer (Lambda 2S, Perkin-Elmer). Drug loading efficiency was determined from the ratio of the weight of the DOX in micelles to that of the micelles loaded with DOX.

2.4. Determination of micelles deformation

The micelle deformation was determined using a fluorescence technique. Pyrene was dissolved in acetone and then dilute by phosphate buffer solution (pH 7.4, $I = 0.1$). Pyrene in buffer solution (6×10^{-7} M) was added in dry micelle to prepare a micelle solution (5 mg/mL). This stock solution was left in the dark for 24 h. The fluorescence spectra were recorded by a fluorescence spectrophotometer (Hitachi F-2500). Fluorescence excitation was carrier out at 340 nm. Emission spectra range employed was 350–600 nm. The emission and excitation slit widths were set at 2.5 and 5 nm, respectively. The intensity ratio (I_1/I_3) of the first band (374 nm) to the third band (385 nm) was calculated and analyzed.

2.5. Stability of PEOz-b-PLLA micelles

The lyophilized blank/DOX-loaded micelles were dissolved in the PBS containing 5 wt.% of bovine serum albumin (BSA). The hydrodynamic diameter of micelles were determined using dynamic light scattering at 37 °C. The concentration of blank/DOX-loaded micelles in BSA solution was 1 mg/mL. The analysis method was Mono modal.

2.6. In vitro drug release from DOX-loaded micelles

In each experiment, 1 mg/mL of DOX-loaded micelle was incubated at 37 °C in either succinate buffer ($I = 0.1$) at pH 5.0 or phosphate buffer solution ($I = 0.1$) at pH 7.4. The DOX released from micelles was isolated from micellar media using the ultrafiltration membrane (MWCO 1000). The UV absorbance was measured at 485 nm in a time-course procedure; the DOX concentration was determined according to the standard curves of the DOX solution at pH 5.0 and pH 7.4. Additionally, drug release of DOX-loaded micelle was also determined in RPMI-1640 medium with 10% fetal calf serum (FCS) at 37 °C as a comparison sample.

2.7. Cell culture and MTT assay

Human normal diploid fibroblast HFW cell line was gifted from Dr. W.-N. Wen (Institute of Biochemistry, National Taiwan University, Taipei). Human CL3 cell line established from a non-small-cell lung carcinoma was provided by Dr. P.-C. Yang (National Taiwan University Hospital, Taipei) (Yang et al., 1992). HFW cells were cultured in DMEM complete media and maintained at 37 °C in a humidified incubator containing 10% CO_2 in air, while CL3 was cultured in RPMI-1640 complete media and cultured in a 5% CO_2 incubator. All the complete media were supplemented with 2.2% sodium bicarbonate, 0.03% L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% FCS.

MTT assay was adapted to examine the cell viability (Chao and Yang, 2001). HFW cells (1×10^4 cells/well) or CL3 cells (2×10^4 cells/well) in exponentially growth were seeded onto a plate with 96 wells for 24 h, respectively. The cells were then exposed to various dosages of copolymers, DOX, or DOX-loaded micelles in complete medium for 24 or 72 h. After treatment, 100 μL medium containing MTT solution (0.5 mg/mL) was added for 2 h. The formazan crystals were dissolved in 100 μL of dimethyl sulfoxide (DMSO). The absorbance of each well was measured using a microplate reader (Stat Fax 2100, Awareness) at a test wavelength of 570 nm and a reference wavelength of 630 nm.

3. Results and discussion

3.1. Synthesis and characterizations of PEOz–PLLA diblock copolymers

First, the PEOz-OH macromonomers were synthesized by cationic ring-opening polymerization. The chemical structure and molecular weight of each copolymer were verified by ^{1}H-NMR (AMX-500, Bruker) and GPC using DMF as an elution solvent. Then, the diblock copolymers consisted of the biodegradable PLLA block and the water-soluble PEOz block were synthesized by ring-opening polymerization from PEOz macromonomer and L-lactide using stannous octoate as a catalyst. Characterizations of a series of PEOz–PLLA diblock copolymers were summarized in Table 1. The composition and molecular weight of PEOz–PLLA were determined by comparing the integral peak area associated with the methyl groups of PEOz (1.09 ppm) and that of PLLA (1.54 ppm).

The CMCs of diblock copolymers were determined by a fluorescence spectrophotometer measurement. Pyrene molecule was used as a hydrophobic probe because of the red shift of the pyrene partitioning changed it's surrounding from aqueous media into hydrophobic structure as micelle occurred. The maximum peak for pyrene, which is at 335.5 nm in water, was shown to shift to 337.5 nm upon addition of block copolymer. The CMC of each copolymer was determined by taking a mid-point of the copolymer concentration at which the relative excitation fluorescence intensity ratio of $I_{337.5}/I_{335.5}$. The result summarizes in Table 2.

Table 1
Characterizations of the PEOz–PLLA diblock copolymers

Polymer code	LLA/EOz[a]	M_w[b]	M_n[b]	Polydispersity index
PEOz-5K	–	6248	5680	1.10
PEOz-10k	–	12150	9000	1.35
AB-5k0.2	0.19	8247	6760	1.19
AB-5k0.4	0.35	9278	7668	1.21
AB-10K0.2	0.18	13800	10620	1.30

[a] Estimated by ^{1}H-NMR.
[b] Estimated by GPC.

Table 2
Characterizations of the blank micelles and DOX-loaded micelles

Polymer code	CMC (mg/L)	Size of blank micelles (nm)	Size of DOX-loaded micelles (nm)	Drug loading efficiency (%)
AB-5k0.2	0.045	121	170	27.4
AB-5k0.4	0.037	147	185	28.5
AB-10K0.2	0.030	164	210	31.2

It observes that the copolymer with longer PLLA chain had lower CMC value. Comparing with our previously study, the CMCs from diblock copolymers were smaller than that from PLLA–PEOz–PLLA triblock copolymers, perhaps because the triblock copolymers are the entropic penalty for blending the PEOz segment to produce the flower micelles. Micelles from diblock copolymers have a lower critical micelle concentration (CMC) than those from triblock copolymer, which may exhibited a relative stability in circulation.

3.2. Preparation and characterizations of PEOz–PLLA micelles

Ultrasonic agitation was used to prepare PLLA–PEOz–PLLA micelles in our previous study. However, one problem is that the ultrasonic agitation has its congenital deficiency of micellar sizes. The average diameters of PEOz–PLLA micelles were ranged from 170 to 260 nm by ultrasonic agitation (data not shown). Thus, the PEOz–PLLA micelles were prepared by a dialysis method against distilled water using a cellulose membrane bag in this study. The micelle sizes were listed in Table 2. Comparing with triblock copolymer system, PEOz–PLLA diblock chain in a micelle can only be found in a dangling topology, whereas the triblock chain can form a loop, a bridge, or a dangling chain (Balsara et al., 1991; Nguyen-Misra and Mattice, 1995; Terreau et al., 2003; Ma et al., 2003). The PEOz–PLLA micelles were easily prepared by dialysis and exhibited small diameters. To evaluate the micellar structure, the freeze-dried micelles were dispersed in the D_2O and then measured by using Bruker AM-400 NMR spectrometer. Fig. 1 shows the ^{1}H-NMR spectra of AB-5k0.2 diblock copolymers and micelles. Due to the limited mobility and slow relaxation of PLLA chains in the core of the micelles, the intensity of proton peak ($\delta = 1.25$ ppm) originated from PLLA was dramatically reduced (Fig. 1b). This result provides one of the evidences that PEOz–PLLA diblock copolymers form micelles with core-shell structure in aqueous solution.

The shape of PEOz–PLLA micelles was determined by atomic force microscopy (AFM). Fig. 2 shows an AFM image of PEOz–PLLA micelles (AB-5k0.2) spread on the silica wafer. It demonstrates that micelles have a uniform size and are spherical. The diameter was around 100 nm, which was slightly small than the hydrodynamic diameter obtained from the DLS experiment. This observation may be because the micelles will shrink and collapse more upon drying.

3.3. Micelle deformation

Pyrene was widely used as a hydrophobic probe for micelle formation determination (Bae et al., 2005a,b; Lo et al., 2005; Wang et al., 2005). The ratio I_1/I_3, as defined to be the intensities of the first over the third vibrational band can then be used as an index of environmental polarity. Higher ratio value indicates more polar surrounding around the pyrene probe. Fig. 3 shows the polarity change, diameter change and zeta-potential of micelle (AB-5k0.2) as a function of pH. The ratio I_1/I_3 shows more hydrophobicity as micelles dispersed in the pH 7.4 solution than in the pH 5.0 solution, indicating that pyrene molecule changed its surrounding from the micellar inner core to buffer solution because of structural deformation to expose pyrene. Dynamic light scattering was used to observe the aggregation

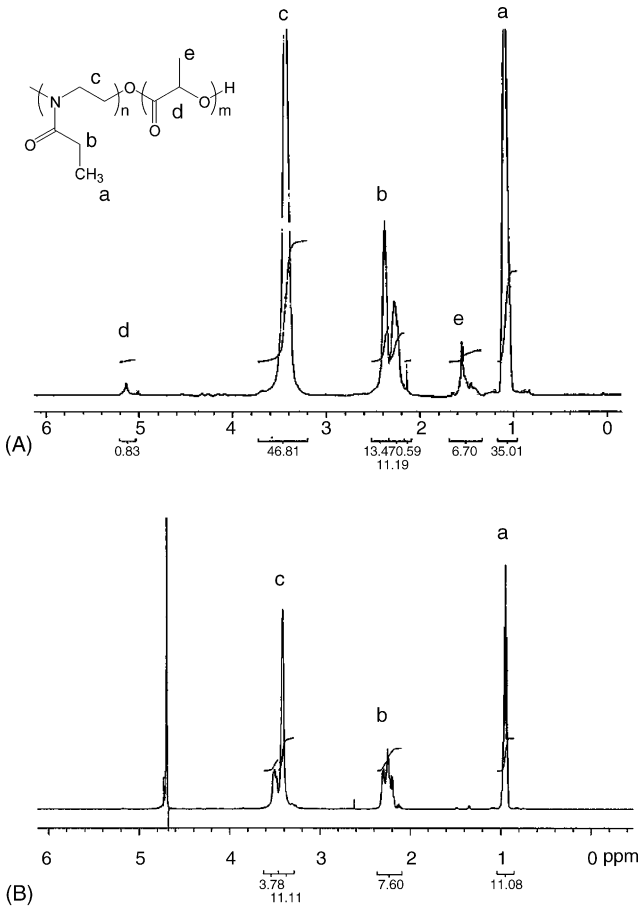


Fig. 1. ^{1}H-NMR spectra of (A) PEOz–PLLA diblock copolymer in the $CDCl_3$ and (B) PEOz–PLLA micelles in the D_2O.

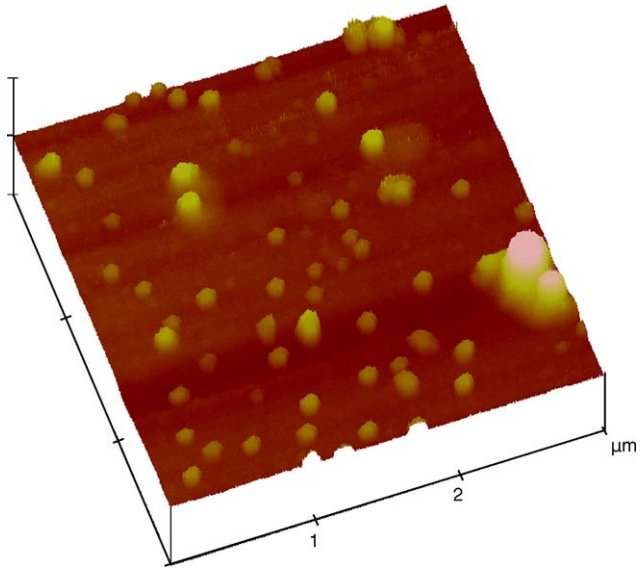


Fig. 2. AFM image of micelles (AB-5k0.2).

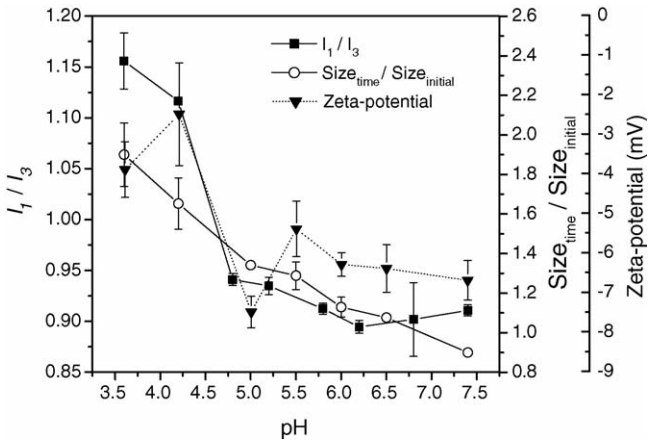


Fig. 3. The polarity change, diameter change and zeta-potential of micelle (AB-5k0.2) as a function of pH.

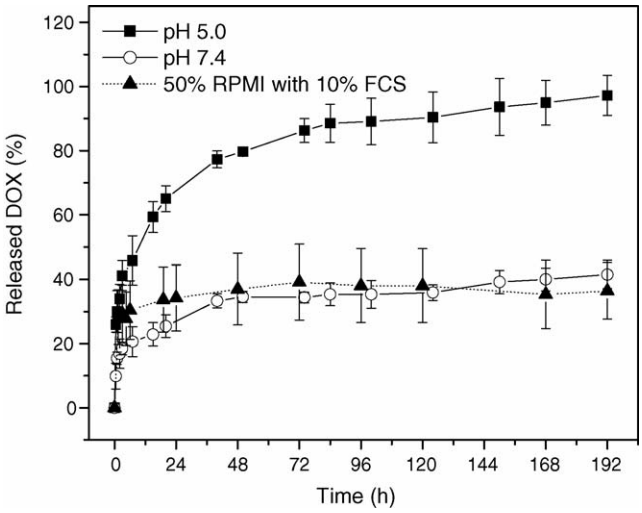


Fig. 4. In vitro release of the DOX-loaded micelles (AB-5k0.2).

behavior of micelles when their structure changed. It revealed a considerable increase in micelle size as the solution pH was below 7.4. This observation speculated on the PEOz, a tertiary amide absorbed proton inducing intra/intermolecular interactions between micelles; so that, large aggregates were formed. These results suggest that the aggregation and collapsed of outer shells of micelles caused micelle deformation.

The outside surface of micelles was investigated by measuring the zeta-potential (Fig. 3). The zeta-potential of micelles exhibited a slightly negative charge under neutral conditions. But it was dramatically decreased at pH 5.0, whereas the I_1/I_3 began increasing. The decrease of zeta-potential is perhaps because of the structure deformation causing hydrophobic PLLA exposure. As the pH declined below 4.5, the zeta-potential increased, I_1/I_3 increased, and the aggregations of micelles were formed. That is perhaps because the hydrogen-bonded complexes formed between the PEOzs, indicating that the outer shells changed their conformation inducing the micelle deformation. It is noted that the positive charges of zeta-potential are a bit expected for PEOzs (such as the triblock copolymer system of our previous reported) if the polymers absorbed protons under acidic surroundings. However, the value of zeta-potential only increased to −4 mV. The reason is probably because that the great exposure PLA inner core directly affected the zeta-potential of micelles; so that, zeta-potential displayed unexpected results.

3.4. Drug loading and drug release

The PEOz–PLLA micelles were incorporated with the anticancer drug DOX (free base). The drug loading efficiency and micelle size were shown in Table 2. The loading efficiency was very close to that of PLLA–PEOz–PLLA triblock copolymer system, but the micelle size was small because of the difference in preparation. Here, the DOX-loaded micelle (AB-5k0.2) with small size was chosen for the in vitro release experiment. As shown in Fig. 4, the pH value considerably affected the release of DOX from the micelles. The release of DOX was effectively suppressed at pH 7.4. Beyond the initial burst (20 wt.%), the release profile reached a plateau, suggesting the good stability of DOX-loaded micelles under physiological conditions. Additionally, the release rate of DOX of micelles in 50% RPMI medium was faster than that in pH 7.4 buffer solutions in the initial 24 h, but the total cumulated release of DOX was closed to that in pH 7.4 buffer solutions after 48 h. The slow release rate of DOX from micelles in circulation as well as the high incorporation efficiency into carrier is considered as beneficial factors concerning the activity of DOX (Horowitz et al., 1992). The relative stability of the DOX-loaded micelles at pH 7.4 is advantageous so that free DOX will not cause the unwanted side effects and heart cytotoxicity during blood circulation. In contrast, micelles exhibited a separation into a burst release followed by a second phase of release in pH 5.0 buffer solution. Over 65 wt.% of DOX was released from micelles at 24 h due to micelle structure broken. The release of DOX approached to 100% after 8 days of sustained release. The release of DOX from micelles at pH 5.0 is somewhat faster than that reported for our previous triblock copolymer system and other micellar systems (Soppimath et al., 2005; Yoo et al., 2002). Overall these results indicate that the DOX-loaded micelles from PEOz–PLLA diblock copolymers have characteristics ideal for DOX release behavior in acidic surroundings, as compared with PLLA–PEOz–PLLA triblock copolymers.

3.5. Stability of the micelles

In order to understand the interaction between PEOz–PLLA micelles and albumin, the blank micelles and DOX-loaded micelles were dispersed in phosphate buffered saline (PBS) in the presence of 5 wt.% of BSA. The micelle size was monitored by DLS at different incubation times. The analytic method was Mono modal. As shown in Fig. 5, the hydrodynamic diameter of micelles was increased in two stages. Both blank micelles and DOX-loaded micelles were relatively stable at the first 30 h while significant aggregations were formed thereafter. The result indicated that micelles with or without DOX incorporation showed similar profiles. However, serum-binding proteins such as albumin have been shown to have different interactions with loading

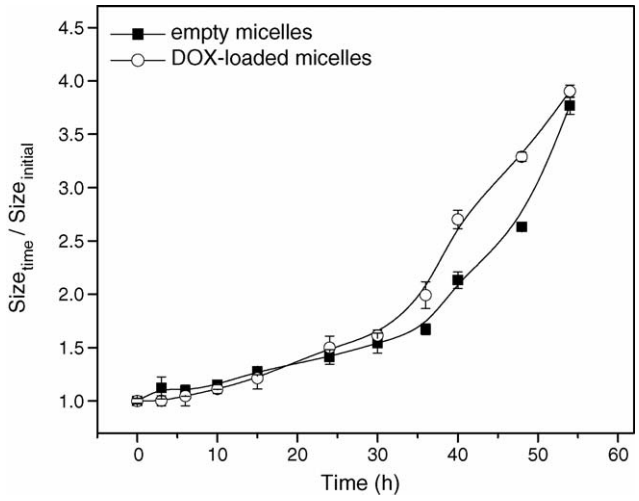


Fig. 5. The change of micelle size as a function of time in the PBS containing 5 wt.% of BSA.

drugs (Opanasopit et al., 2005). The stability of micelles may be enhanced due to the aggregation of drug binding to the hydrophobic inner core of the micelles.

3.6. Cell viability of PEOz –PLLA micelles and DOX-loaded micelles

To explore whether PEOz–PLLA micelles have cytotoxicity, the cell viability is assessed by using MTT assay on human normal fibroblast HFW cells. From our result (Fig. 6), the cell viability was above 80% for human normal fibroblast cells (HFW) under PEOz-*b*-PLLA micelles (0.05–1 mg/mL) treatment for 72 h incubation, indicating that the PEOz–PLLA micelles has good biocompatibility. However, PEOz–PLA micelles exhibited high cytotoxic to human non-small-cell lung carcinoma (CL3), perhaps because that CL3 cells were more sensitive to PEOz–PLA.

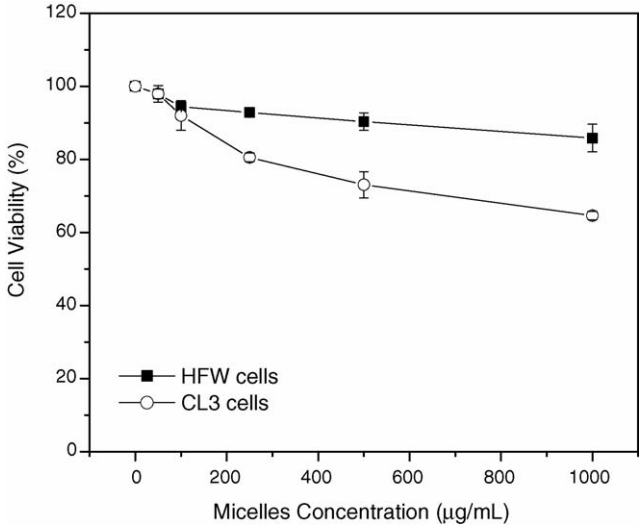


Fig. 6. Cell viability of empty micelles in human normal diploid fibroblast HFW cells and human non-small lung carcinoma CL3 cells.

Previous studies have shown that DOX is used for the therapy of non-small-cell lung carcinoma; however, there are many side effects under DOX treatment (Einhorn et al., 1982; Martoni et al., 1991). To further compare the cytotoxicity of DOX-loaded micelle and free DOX, CL3 cells and HFW cells were assessed for MTT assay. As shown in Fig. 7, exposure of free DOX significantly inhibited the viability of HFW cells and CL3 cells at 24 h. The free DOX concentrations that kill 50% of cells (IC_{50}) were approximately 10 and 0.1 μg/mL for HFW cells and CL3 cells, respectively. Conversely, DOX-loaded micelles treatment showed lower cytotoxicity comparing with free DOX at 24 h and then exhibited cytotoxicity as similar as free DOX at 72 h in both HFW cells and CL3 cells. These data suggest that DOX-loaded micelles could gradually and efficiently release DOX to induce lung cancer cell death. In particular, the HFW cells showed higher cell viability for 1 day treatment. Even if the HFW cells were treated with free DOX or DOX-loaded micelles for 3 days, the cytotoxicities were more unapparent than that for CL3 cells. The higher potency of DOX-loaded micelles for CL3 cells might be due to the higher carrier cytotoxicity and DOX activity.

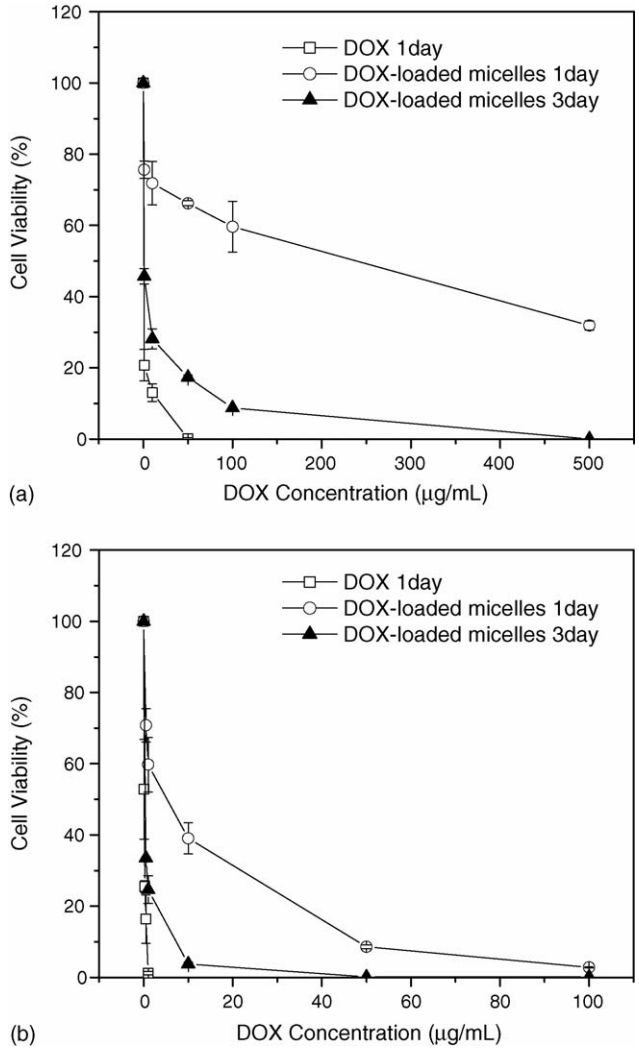


Fig. 7. Cell viability of DOX-loaded micelles in (a) human normal diploid fibroblast HFW cells and (b) human non-small lung carcinoma CL3 cells.

4. Conclusions

PEOz–PLLA block copolymers possessed the advantages of pH-sensitivity and biocompatibility. The drug-released behavior of DOX-loaded micelles showed different profiles in the neutral and acidic buffer solutions. The blank micelles were very low cytotoxic to the HFW normal cells whereas the DOX-loaded micelles exhibited great cytotoxicity to cancer cells after 3 days of coincubation. Comparing with triblock copolymer system, PEOz–PLLA diblock copolymeric micelles involved several advantages, including easily preparation, smaller micelles/DOX-loaded micelles size, well micellar structure and stronger pH-responsive for drug release. Taken together, these DOX-loaded PEOz–PLLA micelles that have small sizes and fast DOX release (under low pH condition) have a great potential for the application of intravenous injection and intracellular drug delivery, and may be useful for non-small-cell lung carcinoma therapy to decrease side effects of free DOX.

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