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Evaluation of the Anti-Tumor Effects of Paclitaxel-Encapsulated pH-Sensitive Micelles

Jong Kwon Han, Min Sang Kim, and Doo Sung Lee*

Department of Polymer Science and Engineering, Sungkyunkwan University, Gyeonggi 440-746, Korea

Yoo-Shin Kim and Rang-Woon Park

Department of Biochemistry and Advanced Medical Technology Cluster for Diagnosis and Prediction, School of Medicine, Kyungpook National University, Daegu 700-422, Korea

Kwangmeyung Kim and Ick Chan Kwon

Biomedical Research Center, Korea Institute of Science and Technology, Seoul 136-719, Korea

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Abstract: We evaluated the efficacy of pH-sensitive micelles, formed by methoxy poly(ethylene glycol)-*b*-poly(β-amino ester) (PEG-PAE), as carriers for paclitaxel (PTX), a drug currently used to treat various cancers. PTX was successful encapsulated by a film hydration method. Micelles encapsulated more than 70% of the PTX and the size of the PTX-encapsulated micelles (PTX-PM) was less than 150 nm. In vitro experiments indicated that the micelles were unstable below pH 6.5. After encapsulation of PTX within the micelles, dynamic light scattering (DLS) studies indicated that low pH had a similar demicellization effect. An in vitro release study indicated that PTX was slowly released at pH 7.4 (normal body conditions) but rapidly released under weakly acidic conditions (pH 6.0). We demonstrated the safety of micelles from in vitro cytotoxicity tests on HeLa cells and the in vivo anti-tumor activity of PTX-PM in B16F10 tumor-bearing mice. We concluded that these pH-sensitive micelles have potential as carriers for anti-cancer drugs.

Keywords: paclitaxel, pH sensitive, micelle.

Introduction

Polymeric micelles that are synthesized from amphiphilic block copolymers, a colloidal carrier system, have many advantages for use in tumor-targeting drug delivery.[1-5] First, mononuclear phagocytosis is minimized by their nanometer size range.[6] Second, water-insoluble drugs can be encapsulated within their hydrophobic cores. Third, hydrogen bonding of the hydrophilic block in an aqueous milieu and the tight formation of a core-shell structure allows them to remain in circulation and be less vulnerable to clearance by the reticuloendothelial system (RES) and renal filtration.[7] Finally, drug encapsulated polymeric micelles can accumulate in tumors via an enhanced permeation and retention (EPR) effect.[8]

Paclitaxel (PTX) is a highly hydrophobic mitotic inhibitor that blocks microtubule breakdown during cell division. It is currently used to treat primary epithelial ovarian cancer, breast cancer, colon, head, non-small cell lung cancer, and AIDS related Kaposi's sarcoma.[9-11] Because of its poor water solubility, PTX is currently formulated as a solution with 50:50 (v/v) in Cremophor EL (polyethoxylated castor oil) and absolute alcohol.[10,11] Unfortunately, Cremophor EL (CrEL) causes significant adverse effects including hypersensitive reactions, neurotoxicity, and nephrotoxicity.[9,12] This problem could be avoided by the use of polymeric micelles (e.g., PEG-PDLLA,[13] pHPMAmDL-*b*-PEG,[14] HGC[15]) as PTX carriers. Stimuli-responsive polymeric micelles can target tumors. In particular, because the extracellular pH of tumor cells (~6.0-7.2) is lower than that of normal cells (7.4),[17] pH-sensitive micelles can enhance the therapeutic effects and reduce adverse effects of encapsulated anticancer drugs. In study of Bae *et al.*,[16] micelles composed of poly(*L*-lactic acid)-*b*-poly(ethylene glycol)-*b*-poly(*L*-histidine)-biotin could induce an active uptake by tumor cells at low pH, because biotin conjugated to poly(*L*-histidine) sprang out from hydrophobic core by ionization of poly(*L*-histidine).

In our previous papers, we reported on the synthesis of a

*Corresponding Author. E-mail: dslee@skku.edu

pH-sensitive block copolymer composed of a hydrophilic methoxy polyethylene glycol (PEG) shell and a hydrophobic poly(β-amino ester) (PAE) core.[18,19] In order to modulate the pH sensitivity of the polymeric micelle, we controlled the molecular weight of the PAE block[18] and used various bisacylate esters of the PAE block.[19] As a result, we obtained polymeric micelles that had pH-sensitive micellization-demicellization behavior under diverse pH conditions. We also evaluated the suitability of these polymeric micelles for cancer chemotherapy. *In vitro* and *in vivo* experiments performed with doxorubicin encapsulated PEG-PAE micelles indicated micelle destabilization below pH 7.0 in B16F10 cells and in B16F10 tumor-bearing mice.[20]

In this paper, we describe the preparation of PTX-encapsulated polymeric micelles from a PEG-PAE block copolymer that are unstable below pH 6.5 and report on the suitability of this PTX carrier as an anticancer agent based on *in vitro* and *in vivo* experiments.

Experimental

Materials. Paclitaxel (Genexol®) was obtained from Samyang Genex Co (Korea). PEG-PAE (Scheme I) was synthesized according to a previously described method.[19]

Drug Encapsulation. PTX was encapsulated into PEG-PAE micelles by a solvent casting method[21] as follows: PTX (5, 10, or 20 mg) was dissolved in 1 mL of ethanol and the solution was added dropwise to a stirred solution containing 50 mg of PEG-PAE in 2 mL of chloroform. After evaporation of the organic solvent using a rotary evaporator, a thin film formed at the bottom of the flask. This film was dispersed by adding 10 mL of PBS buffer (pH 7.4) and shaking for 1 h. To remove the non-entrapped PTX, the mixture was filtered through a 0.45 μm filter. The resulting solutions contained PTX at a concentration of 0.5, 1, 2, and 5 mg/mL. The extent of PTX encapsulation within micelles was determined by reverse-phase HPLC, which was performed on the NS-2004 series (Futecs, Korea) and employed a quaternary gradient pump, variable programmable UV/Vis detector, and data management software (Multichro 2000, Futecs, Korea). The detection wavelength was 227 nm[22] and a C-18 reverse-phase column (GROM-SIL ODS-5 ST, 5 mm) was used for the analysis of PTX. The mobile phase was acetonitrile-water (60:40 v/v), the flow rate was 1.0 mL/min, and PTX concentrations were obtained from a calibration curve for PTX dissolved in acetonitrile.

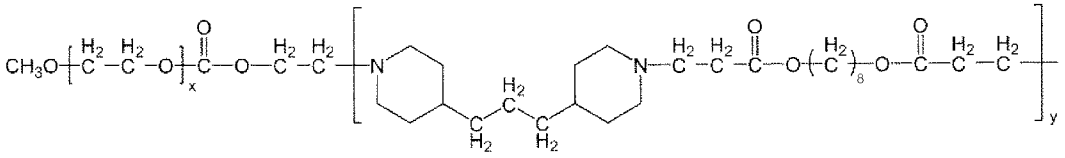
pH Sensitivity. The pH sensitivity of the PEG-PAE micelles in PBS solution was estimated by fluorescence spectroscopy using pyrene as a probe. Fluorescence spectra were recorded by an AMINCOOWMAN® Series 2 luminescence spectrometer (SLM-AMINCO, USA) at room temperature. The pyrene solution in tetrahydrofuran (THF) was poured into the PBS buffer solution, and the THF was then removed by a rotary evaporator (40 °C for 30 min). The final concentration of pyrene in the buffer solution was 1.0×10^{-6} M. Excitation spectra were recorded from 310 to 350 nm with an emission wavelength of 392 nm. The pH sensitivity of micellization-demicellization was determined from the ratio of I_{337} to I_{334} at different pH levels.

The size of PEG-PAE micelles and the amount of encapsulated PTX was measured by dynamic light scattering (DLS) using a Malvern PCS100 spectrogoniometer and a Brookhaven BI-9000AT digital autocorrelator and a helium-neon laser (633 nm). The angle of the detector was maintained at 90° and the temperature at 25 °C. The polymer was passed through a 1.2 μm filter and the concentration was maintained at 5 mg/mL.

***In vitro* Release Experiments.** The *in vitro* release of PTX from PTX-PM was evaluated by a dialysis method. PTX-PM solutions (2 mL) were introduced into each preswollen dialysis membrane bag (Maxi GeBAflex-tube, cut-off 12-14 kDa). The membrane bags were immersed in 500 mL PBS buffer solution at pH 6.0, 6.5 or 7.4. The bags were then maintained in a shaking water bath (20 strokes/min) at 37 °C. At predetermined time intervals, 30 mL of the solution was removed and replaced with an equal volume of fresh medium. The PTX concentrations in different samples were measured by HPLC, as described above.

Cytotoxicity Test. The biocompatibility of pH-sensitive polymeric micelles was evaluated by determination of cell cytotoxicity. HeLa cells were seeded in a 96-well plate (5×10^3 cells per well) and incubated for 1 day. PTX free PEG-PAE micelles and PTX-PM at 0.5 mg/mL of PTX were prepared with PBS buffer solutions. Stock solutions were further diluted with PBS buffer to give a polymer concentration of 0.1 to 100 μg/mL. To evaluate their cytotoxicity, 10 μL of each different polymer formulation and 100 μL of culture medium (DMEM+10% FBS) were added to the cells. The cells were incubated for 24 h at 37 °C in a humidified atmosphere with 5% CO_2. After incubation, 10 μL of CCK-8 solution was added and the cells were incubated again for 2 h. Finally, the number of viable cells was determined using an ELISA colorimetric assay.

***In vivo* Anti Tumor Activity.** The *in vivo* anti-tumor



Scheme I. Structure of PEG-PAE.

activity of PTX-PM was evaluated in B16F10 tumor-bearing mice. Male C57BL/6 mice (7-weeks old, Institute of Medical Science, Tokyo) were used. Subcutaneous tumors were established by inoculating 1.0×10^6 B16F10 cells into the backs of mice. Seven days after subcutaneous inoculation, when the tumor volume was 50 to 100 mm^3, tumor-bearing mice (3 per group) were injected in the tail vein with saline (control), CrEL-PTX (4, 8, or 16 mg PTX/kg), or PTX-PM (4, 8, or 16 mg PTX/kg). Samples were injected once every 3 days for 16 days. The body weight of the mice was recorded and the tumor size was calculated by $a\times b^2/2$, where a is the largest diameter and b is the smallest diameter. To compare tumor volume, we took pictures 10 days after drug administration.

Results and Discussion

Drug Encapsulation. Table I shows the results of size and encapsulation efficiency of PTX-PM with 0.5, 1, and 2 mg/mL of PTX. PEG-PAE micelles without PTX were larger than PTX-PM at 0.5 mg/mL of PTX due to hydrophobic interactions between PTX and PAE. For 0.5 to 2 mg/mL PTX, the more PTX that was encapsulated, the larger the micelles. The sizes at 0.5, 1, and 2 mg/mL of PTX were 101.7 and 110.8 and 140.1 nm respectively and the loading efficiencies of the micelles at 0.5, 1 and 2 mg/mL PTX were 70.6, 74.8, and 79.5% respectively. We measured the size distribution of PTX-PM at 0.5 mg/mL PTX by DLS (Figure 1). DLS of the micelles indicated a homogeneous distribution.

Table I. Effect of PTX Concentration on Encapsulation Efficiency and Micelle Size

PEG-PAE Concentration (mg/mL)	PTX Concentration (mg/mL)	Encapsulation Efficiency	Size (nm)
5	0	-	70.0 ± 10.8
	0.5	70.6 ± 1.3	101.7 ± 0.7
	1	74.8 ± 0.5	110.8 ± 7.9
	2	79.5 ± 0.7	140.1 ± 20.6

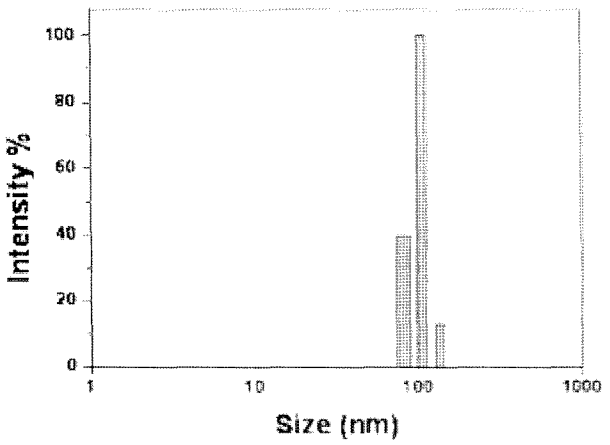


Figure 1. Size distribution of PTX-PM with PTX (0.5 mg/mL) determined by DLS.

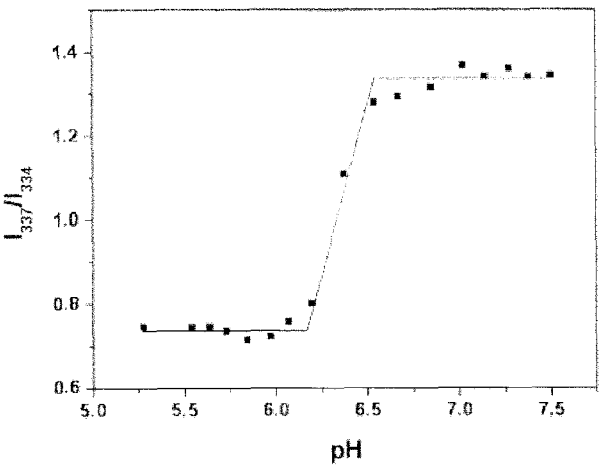


Figure 2. pH sensitivity of demicellization of PEG-PAE micelles without PTX, based on pyrene excitation spectra.

pH Sensitivity. We used fluorescence spectroscopy to measure the pH sensitivity of PEG-PAE micelles. The I_{337}/I_{334} ratio was ~0.75 at pH 5.25-6.18 and was ~1.3 at pH 6.53-7.5 (Figure 2). We interpret these results as being due to the complete ionization of the tertiary amine of the PAE block below pH 6.18 and its complete deionization above pH 6.53. Fluorescence spectra also indicated that the micellization-demicellization transition appeared at pH 6.18-6.53 (Figure 3). Taken together, these results indicate that PEG-PAE micelles may be useful for targeting tumors, which are more acidic than the surrounding millieu.

We used DLS to measure the pH sensitivity of PM and PTX-PM with PTX of 0.5 mg/mL (Figure 3). The results show that micelle size was similar at pH 7.48-6.57, but increased steadily at pH 6.57-5.95. As above, we interpret these results as being due to the ionization of the tertiary amine of the PAE block at low pH and its deionization at high pH.

***In vitro* Release.** Next, we investigated the *in vitro* release of PTX from PTX-PM by dialysis. There is a clear pH effect of PTX release from micelles (Figure 4). At pH 7.4 (where micelles are intact), PTX was released very slowly. In contrast, at pH 6.0 (where demicellization occurs), PTX was released very rapidly. After 3 days, 13.47% of the PTX was released at pH 7.4, 23.11% was released at pH 6.5, and 45.02% was released at pH 6.0. Aggregated PTX remained in dialysis membrane bags at pH 6.0 because of its poor solubility in aqueous solutions.

Cytotoxicity Test. We evaluated the *in vitro* biocompatibility of micelles by studying their effect on HeLa cells at polymer concentrations from 0.1 to 100 μg/mL. For com-

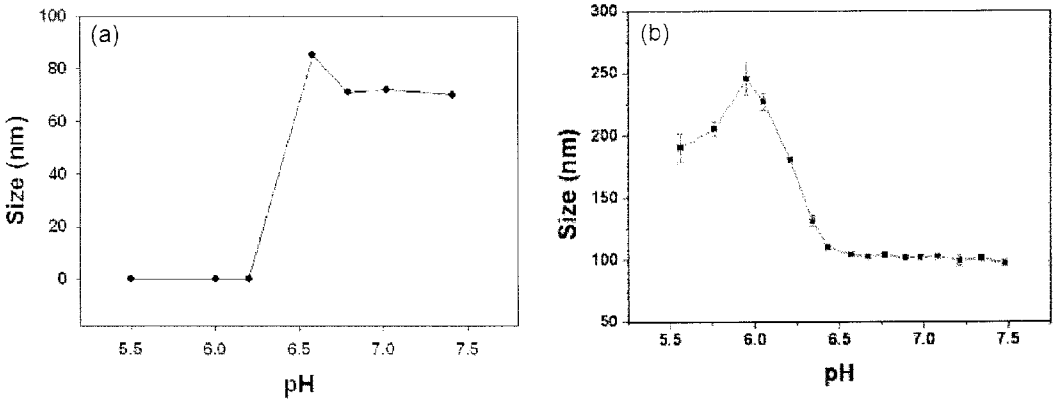


Figure 3. pH sensitivity of (a) PM [19] and (b) PTX-PM size, as determined by DLS.

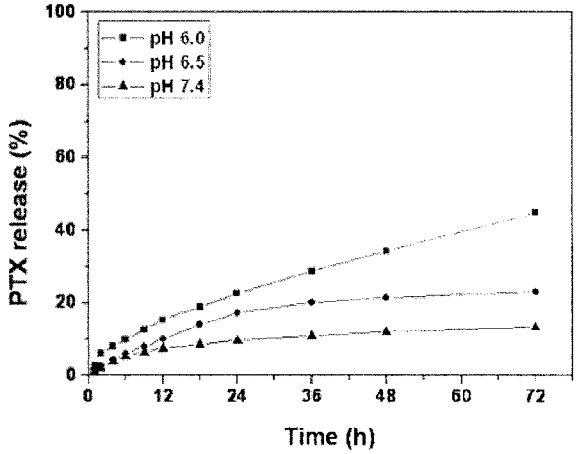


Figure 4. pH sensitivity of *in vitro* release of PTX from PTX-PM at 37 °C (■, pH 6.0; ●, pH 6.5; ▲, pH 7.4).

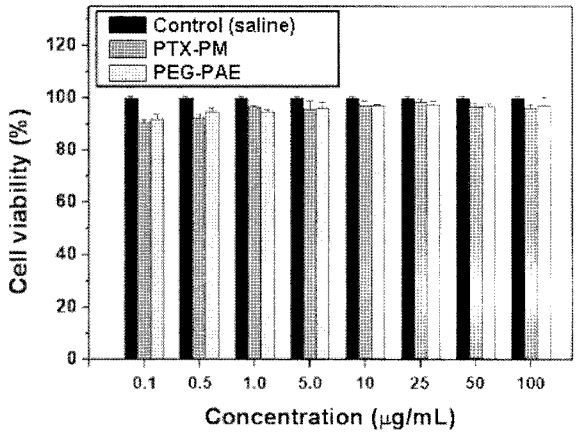


Figure 5. Effect of concentration on *in vitro* cytotoxicity of PTX-PM and PEG-PAE.

parison, we also determined the cytotoxicity of PEG-PAE micelles (Figure 5). PEG-PAE micelles had no significant toxicity, even at 100 μg/mL. Thus, there is no direct interaction between PTX and cells when PTX is encapsulated within the core of polymeric micelles. The outer shell (composed of PEG) presumably reduces the interaction between micelles and HeLa cells by forming a "stealth" surface.

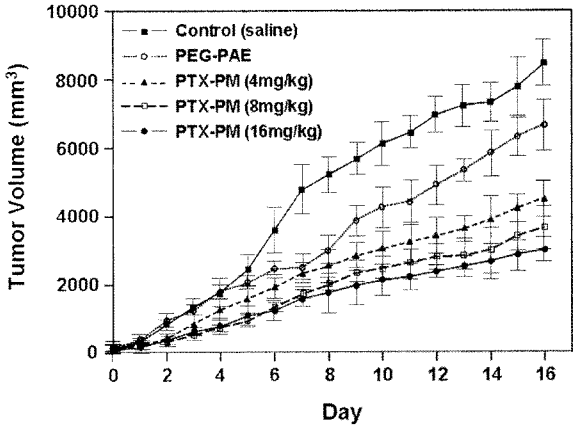


Figure 6. Effect of PTX concentration on tumor growth in B16F10 tumor-bearing mice (■, saline; ○, PEG-PAE; ▲, 4 mg/kg PTX-PM; □, 8 mg/kg PTX-PM, ●, 16 mg/kg PTX-PM).

***In vivo* Anti Tumor Activity.** We evaluated the *in vivo* anti-tumor activity of PTX-PM by use of B16F10 tumor-bearing mice (Figure 6). Tumor growth rates of mice treated with saline (control) increased rapidly. Mice treated with CrEL-PTX almost died due to strong toxicity of CrEL (data not shown). Tumor volume increased more slowly in mice treated with PTX-PM. After 16 days, mice treated with PTX-PM micelles at PTX concentrations of 4, 8, and 16 mg PTX/kg exhibited 47.07%, 57.13%, and 64.74% reduction in tumor volume (relative to the saline control). Photographs of these mice after 10 days of tumor growth (Figure 7) clearly show that mice treated with 16 mg PTX/kg had the smallest tumor volumes.

The body weight of mice treated with saline increased rapidly (Figure 8). Because of the rapid growth of these tumors and the non-toxic effects of PTX-PM, the body weight of mice treated with PTX-PM increased very slowly. PTX-PM at 16 mg PTX/kg exhibited the largest effect.

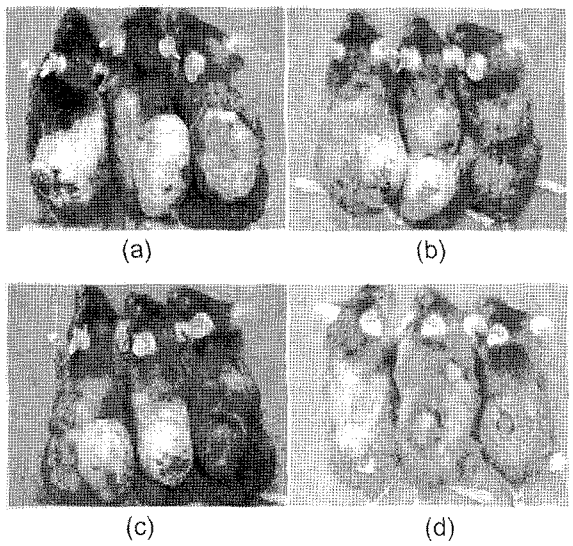


Figure 7. Tumor size 10 days after initiation of drug therapy in mice treated with (a) saline, (b) 4 mg/kg PTX-PM, (c) 8 mg/kg PTX-PM, and (d) 16 mg/kg PTX-PM.

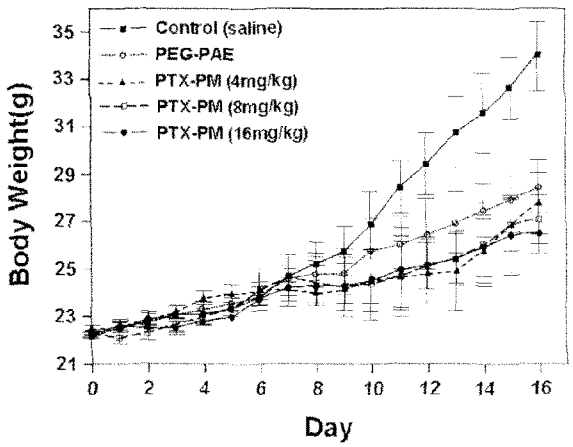


Figure 8. Effect of PTX concentration on body weight change of mice (■, saline; ▲, 4 mg/kg PTX-PM; □, 8 mg/kg PTX-PM, ●, 16 mg/kg PTX-PM).

Conclusions

We successfully synthesized pH sensitive polymeric micelles of PEG-PAE block copolymers and efficiently encapsulated PTX into these micelles. PEG-PAE micelles and PTX-PEG-PAE micelles undergo micellization-demicellization at ~pH 6.0-6.5. An *in vitro* release study indicated that PTX was rapidly released from PTX-PM at a lower pH. An *in vitro* cytotoxicity test indicated that PEG-PAE and PTX-PM have low toxicity and good biocompatibility. PTX-PMs exhibited anti-tumor activity in mice and had lower toxicity than CrEL-PTX. We conclude that pH-sensitive PEG-PAE block copolymers may be useful as hydrophobic drug carriers for cancer therapy.

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