Pharmaceutical Research, Vol. 27, No. 2, February 2010 (© 2009)
DOI: 10.1007/s11095-009-0029-6

Research Paper

Pegylated Phospholipids-Based Self-Assembly with Water-Soluble Drugs

Yiguang Wang,[1] Ruiqi Wang,[1] Xiaoyan Lu,[1] Wanliang Lu,[2] Chunling Zhang,[1,4] and Wei Liang[1,3,4]

Received November 9, 2009; accepted December 3, 2009; published online December 22, 2009

Purpose. To investigate the self-assembly of polyethylene glycol (PEG)-phosphatidylethanolamine (PE) conjugate with water-soluble drugs (doxorubicin hydrochloride, vinorelbine tartrate and vincristine sulfate) and give insight into the mechanism of formation and mode of interaction of the drug with PEG-PE as well as the general principles of self-assembly using pegylated lipid micelles.
Methods. One-step self-assembly method to prepare drug-loaded micelles was developed. The micelles were characterized by dynamic light scattering, transmission electron microscopy, encapsulation efficiency, and release study. NMR was used to study molecular assembly of PEG-PE with doxorubicin.
Results. Doxorubicin hydrochloride and vinorelbine tartrate were entrapped into micelles with high efficiency of >99.0% at molar ratios of 1:1 and 2:1 of PEG-PE to drugs, respectively. Drug loading did not measurably perturb either the geometry or the size. It was found that electrostatic interaction and hydrophobic forces are responsible for the intercalation of drugs into PEG-PE micelles. NMR data revealed that the anthracycline ring of doxorubicin was inserted between PE phospholipids, and its amino sugar located in the outer shell of micelle between PEG chains.
Conclusion. Based on our results, the structure and self-assembly mechanism of water-soluble drugs encapsulated in PEG-PE micelles were proposed.

KEY WORDS: doxorubicin; interaction; micelles; pegylated phospholipids; self-assembly.

INTRODUCTION

Polymeric micelles composed of amphiphilic block copolymers have presented special interests because this carrier has some advantages, including easy preparation, high stability, high drug-loading capacity, biodegradability, controllable drug-release profiles, long systemic circulation time, and enhanced accumulation in tumor via the enhanced permeability and retention (EPR) effect (1). Polymeric micelles are currently used as nanoscale carriers for poorly soluble drugs (2,3). A few micellar formulations are currently in clinical trials in different countries (4–7). Paclitaxel micellar injection (Genexol-PM) formulated with poly (ethylene glycol)-*b*-poly (d, l-lactide) has been available commercially in South Korea (8).

Poly (ethylene glycol)-phosphatidylethanolamine (PEG-PE) conjugate, a block copolymer, has been widely used in stealth liposome formulations (9). In the past decade, it has been reported that micelles comprised of PEG-PE can not only effectively increase solubility of poorly soluble drugs but also are stable and long-circulating in bloodstreams (10). A few poorly soluble drugs, such as paclitaxel and tamosifen, have been loaded into PEG-PE micelles and evaluated *in vitro* and *in vivo* (11–13). However, only a few studies have reported water-soluble drugs loaded in PEG-PE micelles.

Anticancer drugs, such as anthracyclines, vinca alkaloids and their derivates, are widely used for the treatment of many malignancies (14,15). But the clinical uses of these drugs are limited due to their serious dose-limiting toxicities, such as cardio-toxicity, marrow depression and neurotoxicity (16–18). Therefore, tumor-targeted drug delivery systems have gained special interest. We have reported the nanoassemblies of PEG-PE and doxorubicin increasing cytotoxicity *in vitro* and enhancing antitumor activity *in vivo* with low systemic toxicity (19). Recently, we have used a one-step self-assembly procedure to load water-soluble drugs, such as doxorubicin hydrochloride, epirubicin hydrochloride, vinorelbine tartrate and vincristine sulfate, into PEG-PE micelles in aqueous medium. We found that doxorubicin- and vinorelbine-loaded PEG-PE micelles had a very high drug-loading efficiency (> 99%) and superior antitumor activities to free drug both *in vitro* and *in vivo,* with a high stability in plasma and sustained release profile.

In this study, two water-soluble anticancer drugs, doxorubicin hydrochloride and vinorelbine tartrate, both composed of hydrophobic bulk and positive charged groups, (Fig. 1 A and B), were chosen as model drugs, and the interaction of these drugs with PEG-PE (Fig. 1C) in the process of their self-assembly was investigated. The electrostatic and hydrophobic interactions between these drugs and PEG-PE were found to play critical roles in their recognition and high efficient assembly.

[1] Protein & Peptide Pharmaceutical Laboratory, National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing, 100101, China.
[2] School of Pharmaceutical Sciences, Peking University, Beijing, 100083, China.
[3] National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, 15 Datun Road, Beijing, 100101, China.
[4] To whom correspondence should be addressed. (e-mail: zhangcl@moon.ibp.ac.cn; weixx@sun5.ibp.ac.cn)

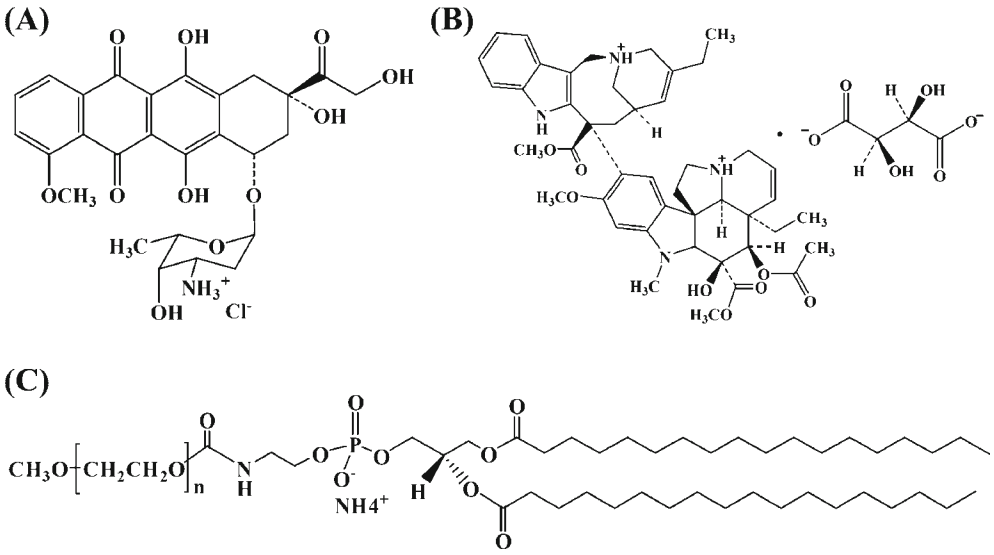

Fig. 1. Chemical structures of (**A**) doxorubicin hydrochloride, (**B**) vinorelbine tartrate, and (C) PEG-PE.

MATERIALS AND METHODS

Materials

Distearoyl-*sn*-glycero-3-phosphoethanolamine-*n*-[methoxy (polyethylene glycol) -2000] (PEG-PE) and 1, 2-dioleoyl-3-trimethylammonium-propane (DOTAP) were purchased from Avanti Polar Lipids (Alabaster, AL). Doxorubicin hydrochloride was kindly provided by Hisun Pharmaceutical Co. Ltd (Zhejiang, China). Vinorelbine tartrate and gemcitabine hydrochloride were obtained from Hansen Pharmaceutical Co. Ltd (Jiangsu, China). Vincristine sulfate was purchased from Hanfang Pharmaceutical Co. Ltd (Guangdong, China). Paclitaxel was obtained from Huiang Biopharmaceutical Co. Ltd (Guangxi, China). Water used throughout was distilled and deionized in a Milli-Q water purification system (Millipore, Bedford, U.K.). All other reagents used were of analytical grade.

Preparation of Drug-Loaded PEG-PE Micelles

Water-soluble drugs, including doxorubicin hydrochloride, vinorelbine tartrate, vincristine sulfate and gemcitabine hydrochloride, were dissolved and PEG-PE was suspended in double-distilled water, respectively. The resulting drugs and PEG-PE stock solutions were 5 mM, respectively. Then, PEG-PE and drug stock solutions were mixed in a round-bottom flask to obtain the desired PEG-PE:drug ratio. The mixture was incubated at 60°C for 30 min to allow for drug entrapment. After incubation, the resulting solution was filtered using 0.22 μm polyethersulfone (PES) syringe membrane. The filtered solution was stored at 4°C until use. Empty micelles were prepared using an identical procedure without drugs. For preparation of paclitaxel-loaded micelle, paclitaxel powder was suspended in double-distilled water and mixed with PEG-PE stock solution at 1:16 molar ratio of paclitaxel:PEG-PE. The mixture was incubated at 60°C for 30 min with gentle shaking. After incubation, the resulting suspension was filtered using 0.22 μm PES syringe membrane. Drugs in PEG-PE micelles were determined by RP-HPLC method using a C18 column.

Dynamic Light Scattering

Particle size distribution and zeta potential of doxorubicin-loaded micelle (M-Dox, 2:1 molar ratio of PEG-PE:Dox) and vinorelbine-loaded micelle (M-Vino, 4:1 molar ratio of PEG-PE:Vino) were determined by dynamic light scattering (DLS) analysis using Malvern Zetasizer Nano ZS (Malvern Instrument Ltd, Malvern, UK) with 633 nm laser at 25°C and at a scattering angle of 90º. The empty micelles, M-Dox and M-Vino were diluted to the lipid concentration of 1 mg/mL with deionized water, respectively. Particle sizes were expressed as intensity-weighted diameters. The values were reported as the mean ± standard deviation (SD) based on three individual measurements performed in triplicate.

Cryogenic Transmission Electron Microscopy (Cryo-TEM)

The shape and size of the micelles were determined by a Philips Tecnai 20 transmission electron microscope (FEI-Philips, Oregon, USA) at 160 kV. Sample preparation was carried out in a controlled environment vitrification system at 25°C and at saturation to avoid evaporation of water during specimen preparation. 10μL of the solution was placed on a hydrophilized perforated carbon filmed grid. Excess fluid was blotted to form a 100–250 nm thick liquid film, and the grid was immediately plunged into liquid ethane (−184°C), producing a vitrified specimen. The specimens were imaged at −175°C sample temperature in the minimal electron dose mode by CCD camera.

Measurement of Drug Encapsulation Efficiency

The encapsulation efficiency of drugs into micelles was determined by ultrafiltration. Briefly, 500 μL of micellar formulations, including doxorubicin hydrochloride, vinorelbine tartrate, vincristine sulfate and gemcitabine hydrochloride loaded in PEG-PE micelles, were added in amicon ultra

centrifugal filter devices (MWCO 30,000, Millipore) and then centrifuged at 12000 × g for 10 min at 4°C. The concentrations of free drugs in filtrate were quantified by reverse-phase HPLC with a C18 column. For the determination of paclitaxel encapsulation efficiency, the content of paclitaxel in micelles was evaluated by HPLC, and the encapsulation efficiency was calculated by the ratio of incorporated paclitaxel to initial paclitaxel added.

In Vitro Release Study

Drug release was assessed using equilibrium dialysis method. M-Dox (PEG-PE:Dox = 2:1, molar ratio) or M-Vino (PEG-PE:Vino=4:1, molar ratio) solutions were diluted to 1 mg/mL of doxorubicin or vinorelbine with PBS buffer or fresh mouse serum and sealed in a dialysis bag (MWCO 10,000, Pierce). The dialysis bags were placed in 150 mL of PBS buffers (pH 7.4) at 37°C with gentle shaking. Aliquots of release medium were removed at designated time points. The released drugs were quantified by RP-HPLC methods.

Preparations of Drug-Loaded Micelles at Different Ratios of Lipid to Drug

Doxorubicin-loaded PEG-PE micelles were prepared at different molar ratios of PEG-PE to doxorubicin ranging from 1:0.5 to 1:4 using the identical method described above. The encapsulation efficiency for each preparation was evaluated by ultrafiltration method. Accordingly, the same concentration of free doxorubicin solution as doxorubicin in micelles was prepared and ultrafiltrated with ultra centrifugal filter units for the evaluation of drug adsorption. The effect of the ratio of lipid to drug on the encapsulation efficiency was evaluated.

Addition of DOTAP into PEG-PE Micelles for Drug Encapsulation

PEG-PE/DOTAP mixed micelles were prepared at 1:0, 1:0.5, and 1:1 molar ratios of PEG-PE:DOTAP using film hydration method. Then, the mixed micelle solution was incubated with doxorubicin solution at 1:0.5 and 1:1 molar ratios of PEG-PE:Dox. The encapsulation efficiency of doxorubicin and zeta potentials of PEG-PE micelles were determined using method described above. The correlation between drug loading efficiency and zeta potential was evaluated.

Preparation of Drug-Loaded PEG-PE Micelles in Water Containing Ca^{2+}

PEG-PE stock solutions (5 mM) were incubated with different concentrations of Ca^{2+} solutions overnight. Then, equal volumes of doxorubicin solutions (2.5 mM) and Ca^{2+} containing PEG-PE solutions were mixed and incubated at 60°C. The drug encapsulation efficiency was determined by ultrafiltration. The zeta potentials of the drug-free micelles were analyzed by DLS method. The effect of Ca^{2+} on zeta potential and the correlation between drug loading efficiency and zeta potential was also evaluated.

Preparation of Drug-Loaded PEG-PE Micelles with Different Incubation Temperatures and Times

The drug loading efficiency in micelles as functions of time and temperature was further examined. To analyze the effect of time on assembly, equal volumes of PEG-PE (5 mM) and drug (5 mM) stock solutions were mixed in a round-bottom flask and incubated at 60°C, and aliquots of mixture were withdrawn at designated time points for the evaluation of drug encapsulation efficiency. For the effect of temperature, equal volumes of PEG-PE (5 mM) and drug (5 mM) stock solutions were mixed in different vials, and the mixtures were incubated at different temperatures ranging from 4°C to 60°C for 30 min.

NMR Spectroscopy

PEG-PE (10 mM) and Dox (5 mM) stock solutions were prepared by dissolving the weighed materials in deuterium oxide. Samples for ^{1}H-NMR were prepared by mixing the PEG-PE and Dox stock solutions (PEG-PE:Dox = 2:1, molar ratio) and allowing for assembling at 60°C for 30 min. To investigate interaction of doxorubicin and PEG-PE, PEG-PE solution was used to titrate directly doxorubicin solution (molar ratios of Dox:PEG-PE from 50: 0 to 50: 10).

All NMR spectra were acquired at 298 K with Bruker DPX 400 spectrometer equipped with a Z-gradient BBO probe. The 1D ^{1}H NMR and 2D ^{1}H-^{1}H NOESY experiments were performed on the samples containing 2.5 mM doxorubicin in D_2O. The 1D ^{1}H and ^{31}P NMR spectra of PEG-PE were obtained for the samples containing 5 mM PEG-PE in D_2O. The 2D ^{1}H-^{1}H NOESY experiment was run on the doxorubicin and PEG-PE mixture at the NOE mixing time of 500 ms. Triphenyl phosphate (TPP) in acetone-d6 was used as an external standard for chemical shifts of ^{1}H resonances and ^{31}P resonance as well.

RESULTS

Drugs Intercalated into PEG-PE Micelles

The average diameter of empty PEG-PE micelles as determined by DLS was 15.8±0.4 nm, consistent with previous studies (20). The size of doxorubicin-loaded micelles and vinorelbine-loaded micelles was similar, with diameters of 14.8±1.2 nm and 14.6±0.9 nm, respectively. The particle sizes of both drug-loaded micelles were similar to that of empty micelles (Figs. 2 and 3), implying that the encapsulation of doxorubicin or vinorelbine into PEG-PE micelles did not disrupt the structure of micelles. The morphology of these micelles characterized by Cryo-TEM was spherical with a uniform size distribution (Fig. 3).

Doxorubicin and vinorelbine incorporated into PEG-PE micelles showed a high encapsulation efficiency: 99.2% for doxorubicin at 1:1 molar ratio and 99.4% for vinorelbine at 1:2 molar ratio of drug to PEG-PE. Drug loaded in PEG-PE micelles also demonstrated slow release, and the released drugs up to 24 h were 36.7% and 16.5% for doxorubicin and vinorelbine, respectively. These sustained-release profile and high encapsulation efficiency could be related to the molecular structures of both PEG-PE and drugs; PEG-PE is a

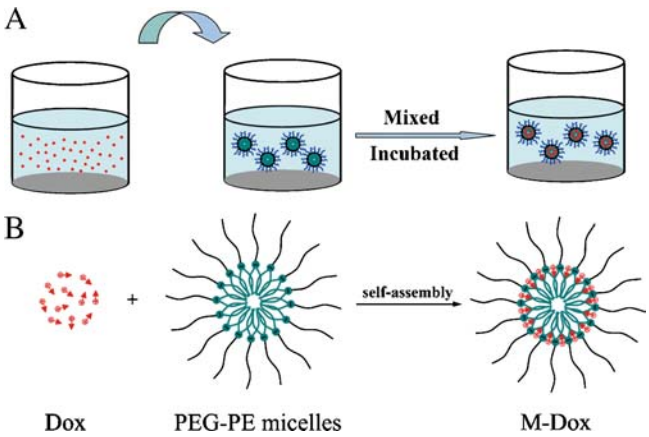

Fig. 2. Schematic illustration of the self-assembly of water-soluble drugs, such as doxorubicin hydrochloride, and PEG-PE in double-distilled water. (**A**) One-step self-assembly procedure. (**B**) Detailed structure of nanoassemblies of PEG-PE with water-soluble drugs.

conjugate of PE with PEG through the amino group of PE, and thus, PEG-PE remains a phosphate group with negative charge at physiologic pH, in which an electrostatic interaction between PEG-PE and doxorubicin and vinorelbine should occur. Furthermore, the sustained release manner and encapsulation efficiency of these two drugs encapsulated in PEG-PE micelles are a consequence of the hydrophobic interaction between two hydrocarbon chains of PE and hydrophobic doxorubicin and vinorelbine. Thereby, we propose a self-assembly procedure for water-soluble drugs like doxorubicin or vinorelbine loaded in PEG-PE micelles (Fig. 2). This procedure is very simple for preparing the drug carriers with high drug encapsulation efficiency and controlled drug release in aqueous solution and without organic solvents.

Negative Charge of PEG-PE Plays an Important Role to Trigger the Assembly of Drugs and PEG-PE

To determine the effect of ratio of drug to PEG-PE on drug encapsulation efficiency, we changed molar ratios of doxorubicin to PEG-PE ranging from 0.5:1 to 4:1. Up to 99.2% drug encapsulation efficiency was obtained at the 1:1 molar ratio of drug to PEG-PE. Decreasing molar ratio of drug to PEG-PE at 0.5:1, drug encapsulation efficiency (99.8%) was almost the same as 1:1, indicating that doxorubicin was completely loaded into the PEG-PE micelles (Table I). However, increasing the ratio of drug to PEG-PE from 1:1 to 4:1, the drug encapsulation efficiencies linearly decrease. We calculated the relationship between the experimentally determined encapsulation efficiency (EE_d) and predicted drug encapsulation efficiency (EE_p), which was based on the presumption that the interaction of doxorubicin and PEG-PE would occur at a molar ratio of 1:1, by linear regression. The least squares regression equation is $EE_d = 0.9917 \times EE_p + 0.2803$, and the correlation coefficient is 0.9994 (Table I). After removing unloading drug, ratios of drug to PEG-PE in micelles using the different initial ratios of PEG-PE to drug (1:1 to 1:4) were 1:1, indicating that to encapsulate one molecule of doxorubicin in a micelle, at least one molecule of PEG-PE is needed.

To determine whether electrostatic interaction of PEG-PE with doxorubicin plays an important role in doxorubicin loaded into PEG-PE micelles, DOTAP, one kind of cationic lipid, was incorporated into PEG-PE micelles to neutralize negative charges of PEG-PE micelles. The zeta potentials of PEG-PE micelles in the absence of DOTAP was −25.9 mV; addition of DOTAP to PEG-PE to form the micelles at molar ratio of 1:1 markedly decreased the zeta potentials of micelles to −1.2 mV (Table II). Interestingly, DOTAP incorporated into the PEG-PE micelles (1:1, molar ratio) significantly decreased the amount of doxorubicin loaded into these micelles; only 16.8% drug was encapsulated in the micelles at the 1:1 molar ratio of drug to PEG-PE (Table II). However, decreasing the ratio of DOTAP to PEG-PE to 0.5:1 in the micelles with the −5.9 mV zeta potential, the capacity of PEG-PE encapsulated doxorubicin was partially rescued (54.0% drug encapsulation efficiency, Table II). Vinorelbine loaded in PEG-PE micelles had similar results to doxorubicin (data not shown). These data demonstrate that electrostatic interaction between PEG-PE and drug plays an important role for drug loading.

To further verify the importance of electrostatic interaction in the self-assembling process of doxorubicin and PEG-PE, we performed pre-incubation of PEG-PE micelles with Ca^{2+} at different concentrations for 24 h and then mixed with doxorubicin solution. The results are shown in Fig. 4A. Increase of Ca^{2+} concentrations decreased the absolute value of zeta potential of micelles, and correspondingly, decreased the encapsulation efficiency of doxorubicin in the micelles. These results further confirm that negative charge in PEG-PE molecule is essential to recognize the guest molecule for their high effective assembly.

Hydrophobic Block of PEG-PE Provides Stable Reservoir for Drug Loading

As shown in Fig. 1, doxorubicin and vinorelbine have a hydrophobic bulk and small groups with positive charges, while PEG-PE di-block polymer contains the hydrophobic block of PE and the hydrophilic block of PEG with one negative charge. In water, PEG-PE molecules aggregate to form a core-shell micelle; the core is composed of PE with hydrophobic property, which should be suitable for hydrophobic drug loading. Our findings revealed that with neutralization of PEG-PE micelles using DOTAP, 35.7% drug-loading capacity remains, as shown in Table II. Hydrophobic block of PEG-PE could provide stable reservoir for drug like doxorubicin and vinorelbine. To broaden our understanding of what structures of hydrophobic bulk in drugs are necessary for their loading into PEG-PE micelles with high efficiency, we selected several anticancer drugs with different structure-based physicochemical properties. As shown in Fig. 4B, gemcitabine hydrochloride, a nucleoside analog with a high water solubility (>15 mg/mL at pH 2.7–9.0), could not be incorporated into PEG-PE micelles (0.3% encapsulation efficiency). Based on molecular structure of gemcitabine, we can see that the basic group of gemcitabine with strong polarity is both donor and accepter of hydrogen bonds, which can not be stabilized using two aliphatic hydrocarbon chains of PE, resulting in very low encapsulation efficiency. Furthermore, we used a low water-soluble drug (~1 μg/mL),

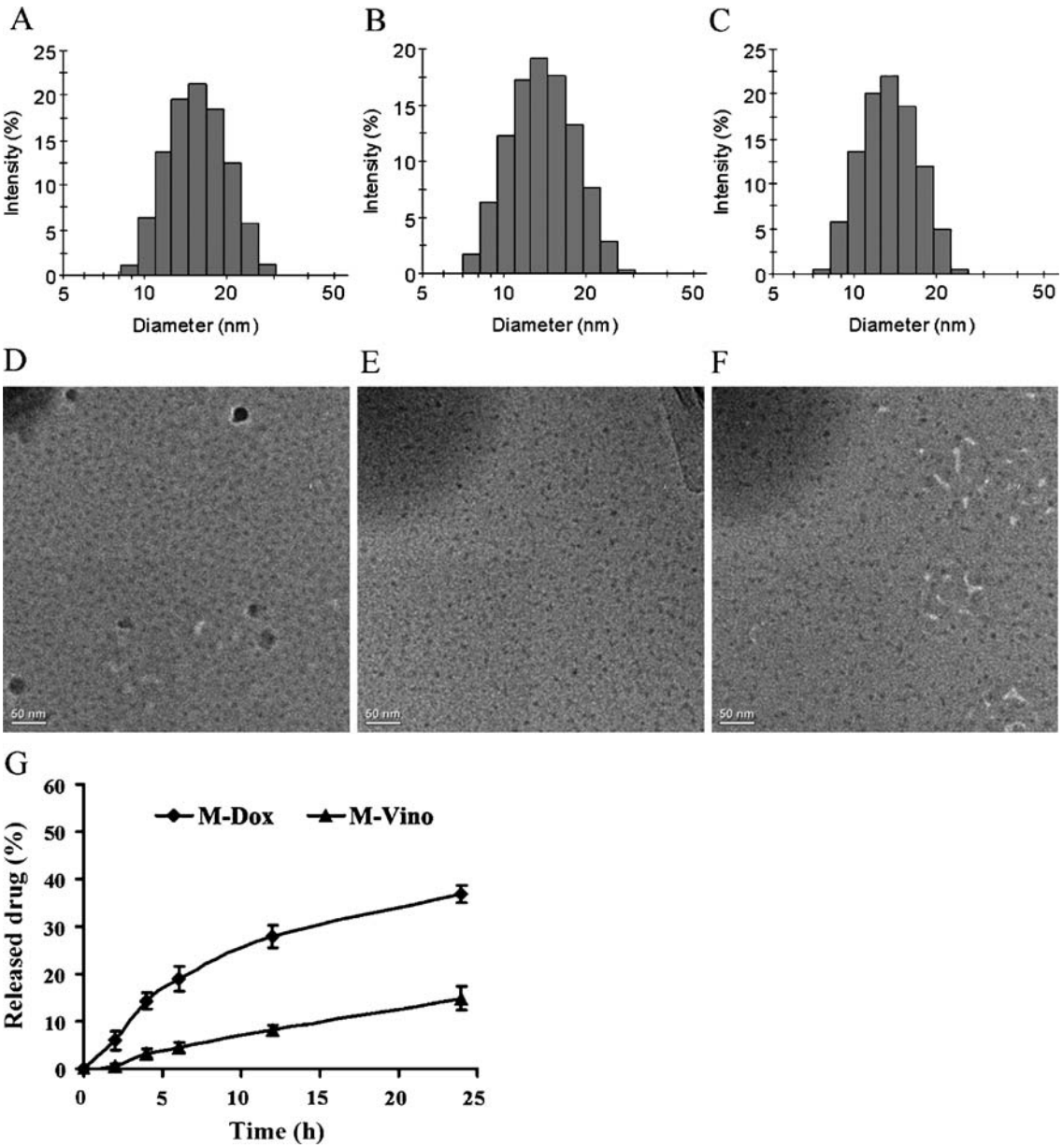

Fig. 3. Characterization of micelles in terms of particle size distribution, morphology and drug release kinetics. (**A**) Particle size of empty micelle examined by dynamic light scattering analysis. (**B**) Particle size of doxorubicin-loaded micelle. (**C**) Particle size of vinorelbine-loaded micelle. (**D**) Cryo-TEM image of empty micelle examined by cryogenic transmission electron microscopy. (**E**) Cryo-TEM image of doxorubicin-loaded micelle. (**F**) Cryo-TEM image of vinorelbine-loaded micelle. Scale bar is 50 nm. (**G**) *In-vitro* release profiles of drug-loaded micelles in pH 7.4 PBS buffer. Data represent mean ± SD (n=3) from individual experiments.

Table I. Effect of the Ratio of Lipid to Drug on the Encapsulation Efficiency

Dox:PEG-PE (molar ratio)	0.5:1	1:1	2:1	3:1	4:1
EE_d [a] (%)	99.8	99.2	50.5	33.8	24.0
EE_p [b] (%)	100	100	50	33	25
Correlation equation & coefficient	$EE_d=0.9917\times EE_p+0.2803$, R=0.9994				

[a] EE_d: the determined encapsulation efficiency.
[b] EE_p: the predicted encapsulation efficiency based on the presumption that the interaction of doxorubicin and PEG-PE would occur at a molar ratio of 1:1.

Table II. Effect of DOTAP Incorporation on Zeta Potentials of Micelles and Drug Encapsulation Efficiency

PEG-PE:DOTAP (molar ratio)	DOX	Zeta potential (mV)	EE (%)
1:0	0	−25.9±2.1	–
1:0.5	0	−15.3±3.2	–
	0.5	−5.9±1.6	98.4±1.3
	1	−2.5±1.1	54.0±3.7
1:1	0	−1.2±0.5	–
	0.5	3.2±1.3	35.7±4.5
	1	7.7±2.6	16.8±2.1

paclitaxel, to incubate with PEG-PE micelles under the same conditions as doxorubicin loading. Only 3.5% paclitaxel was encapsulated into PEG-PE micelles (Fig. 4B). These results suggested that the hydrophobic core of PEG-PE is not for all drugs with hydrophobic or hydrophobic moiety to provide stable reservoir, but is mostly structure-dependent.

We also determined other factors that affect the encapsulation efficiency, such as incubation temperature and time. The results are shown in Fig. 4C. We changed incubation temperatures from 60°C to 4°C and incubation time from 30 min to 30 s, and measured the doxorubicin encapsulation efficiency at the 1:1 molar ratio of doxorubicin to PEG-PE. More than 99% doxorubicin was encapsulated into PEG-PE micelles under all above conditions, indicating that assembling process of doxorubicin and PEG-PE is both time- and temperature-independent.

NMR Spectra Prove Drugs Intercalated into PEG-PE Micelles

To interpret in detail the mechanism of assembly of doxorubicin and PEG-PE, we performed the NMR experiments. We first characterized the physical state and the chemical composition of PEG-PE using 1D ^{1}H NMR, 2D ^{1}H-^{1}H NOESY, and ^{31}P NMR spectra. The critical micelle concentration (CMC) of PEG-PE in water is about 10^{-5}M (10). The concentration of PEG-PE (10^{-2}M) in the NMR samples for the present study was much higher than the CMC value. Therefore, all of PEG-PE molecules in the sample are supposedly in a form of the micelle.

1D ^{1}H NMR spectrum of 5 mM PEG-PE was in good agreement with the published spectrum of a mixture of DPPC/DPPG/PEG-DSPE (21). Thus, the resonances in ^{1}H NMR spectrum of PEG-PE were assigned by comparing the chemical shifts and intensities of signals with those previously assigned. The assigned proton resonances for chemical groups

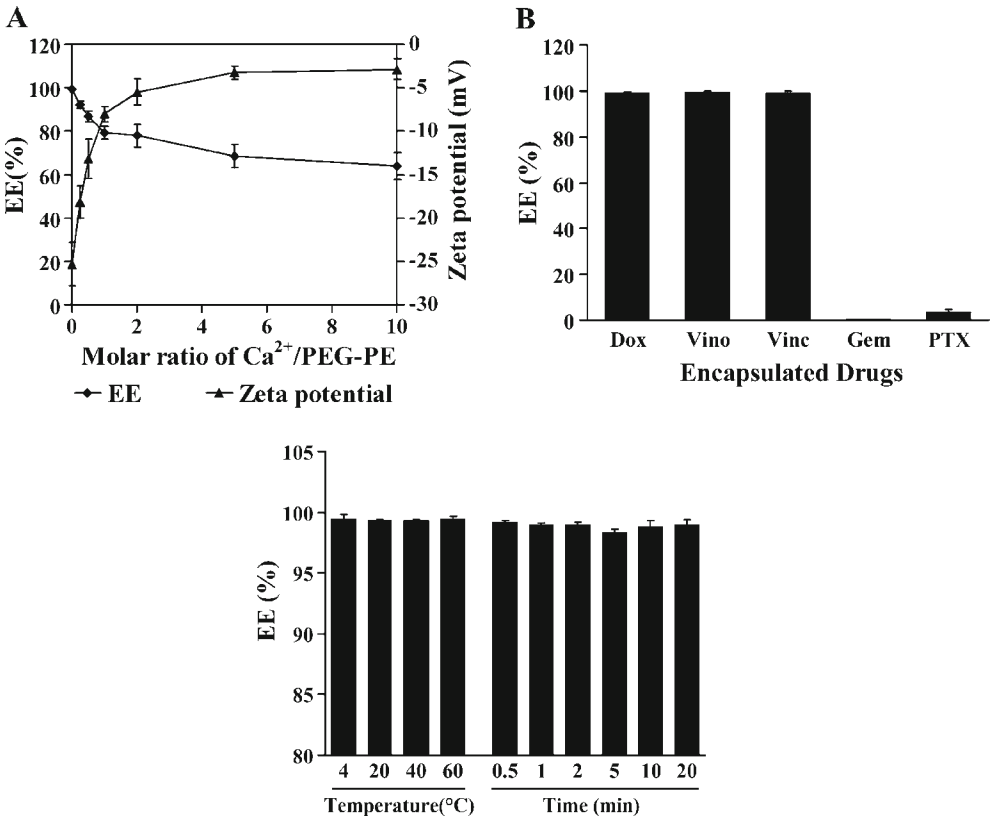

Fig. 4. Ca^{2+}, structure of drug, incubation time and temperature effect on encapsulation of drugs in PEG-PE micelles. (**A**) Corresponding correlation between zeta potentials and doxorubicin encapsulation efficiency in the presence of Ca^{2+}. (**B**) Self-assembly of PEG-PE and drugs with different structures in aqueous solution. Doxorubicin hydrochloride, vinorelbine tartrate, vincristine sulfate, gemcitabine hydrochloride and paclitaxel were abbreviated as Dox, Vino, Vinc, Gem and PTX, respectively. (**C**) Effect of incubation time and temperature on the self-assembly of PEG-PE and doxorubicin hydrochloride.

of PEG-PE were indicated in (Fig. 5C). The signals of protons in 1-CH_3, 3-CH_2, and 4-CH_2 of PE chains were much broader than those in 14-CH_3O and 11-CH_2 of PEG chain at the concentration of PEG-PE higher than its CMC value. The ^{1}H resonances of bulk 2*-CH_2 of PE chains also showed broader peak than those of bulk 13*-CH_2O of PEG chain. The difference in ^{1}H spectral linewidth of PEG from PE chains implies that PEG chain has higher mobility than PE chains in PEG-PE micelles. Thus, the hydrophobic PE chains construct a hydrophobic inner core, while the extended PEG chains form a hydrophilic outer shell in aqueous media.

As shown in Fig. 5A, the ^{1}H-NMR spectrum of M-Dox in D_2O also suggests the formation of the core-shell structure. The micelle shell composed of PEG block was hydrated to a high degree in D_2O and showed the specific peak of PEG, while the resonances of the PE core were significantly reduced because of the insufficient mobility in D_2O. Free Dox showed intrinsic specific peaks at 1.0–8.0 ppm (Fig. 5B). In contrast, Dox was intercalated into the PE core of micelles, and the specific peaks of Dox molecules disappeared as shown in Fig. 5A.

For detecting the interactions of doxorubicin with PEG-PE, 10 mM PEG-PE was titrated into the samples containing 5 mM doxorubicin and then measured by ^{1}H NMR (Fig. 6). The linewidths of ^{1}H resonances for doxorubicin increased with increasing molar ratios of PEG-PE in the mixtures of doxorubicin and PEG-PE. The changes in linewidth of the ^{1}H resonances of anthracycline ring (2-CH, 3-CH, and 4-CH) and of amino sugar (1′-CH, 5′-CH, and 5′-CH_3) in doxorubicin molecule were obviously observed. Usually, intermolecular ^{1}H-^{1}H dipolar interactions give rise to a large amount of homogeneous line broadening. Thus, the increase of the linewidths of ^{1}H resonances for doxorubicin reflects the increase of ^{1}H-^{1}H dipolar interactions between PEG-PE and doxorubicin with increasing molar ratios of PEG-PE.

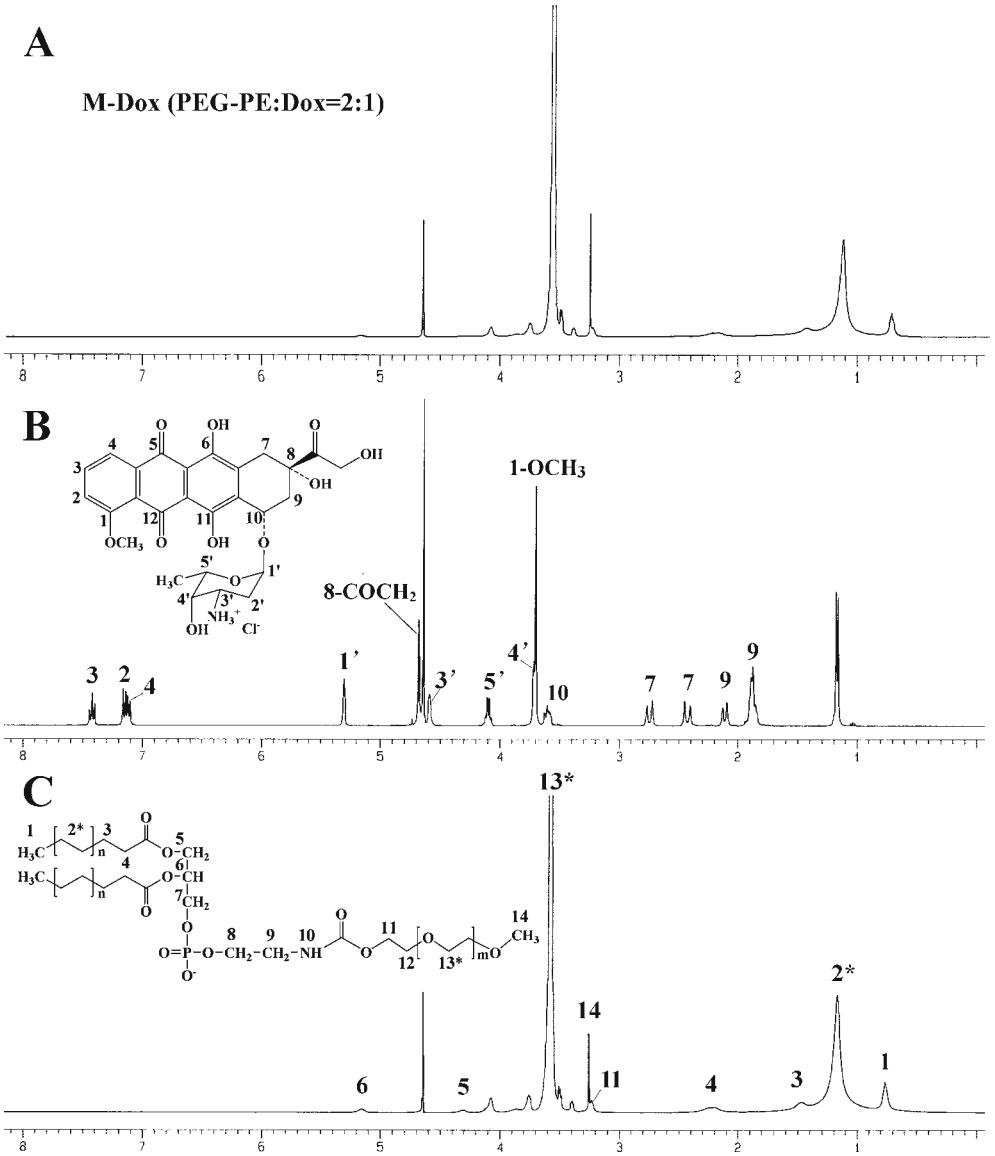

Fig. 5. ^{1}H-NMR spectra of (**A**) Doxorubicin-loaded PEG-PE micelles at molar ratio of PEG-PE to doxorubicin at 2:1. (**B**) Doxorubicin hydrochloride. (**C**) PEG-PE copolymer in D_2O.

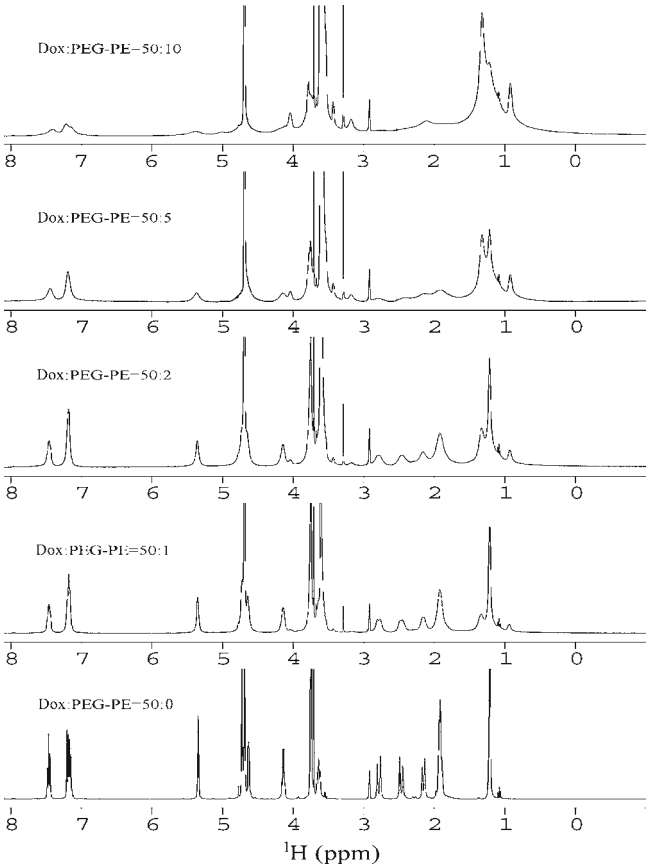

Fig. 6. ^{1}H-NMR spectra of 5 mM doxorubicin in the presence of increasing ratio of PEG-PE in the mixture. The molar ratio of doxorubicin to PEG-PE is indicated in each spectrum.

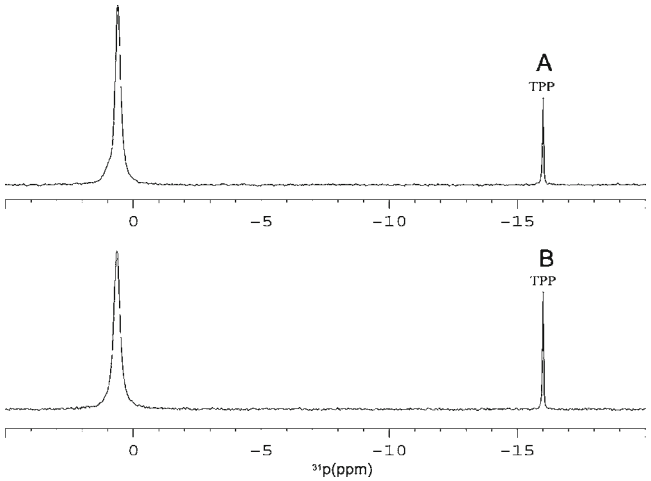

Fig. 8. ^{31}P NMR spectra of PEG-PE collected in the absence (**A**) and presence (**B**) of doxorubicin. The peak at about −16 ppm is a signal of external standard TPP.

The 2D ^{1}H NOESY spectra recorded for the mixture of Dox and PEG-PE (molar ratio of doxorubicin: PEG-PE= 50:5) at 298 K were shown in Fig. 7. The peaks indicated with arrows in the NOESY spectrum of the mixture described the NOEs between the protons of anthracycline ring of doxorubicin (2-CH, 3-CH, and 4-CH) and the protons of PE chain (1-CH_3 and 2*-CH_2). The NOEs built between them indicate that the aromatic ring of doxorubicin is close to PE chains in the mixture. PE chains having fatty acid methyl (1-CH_3) and bulk methylene (2*-CH_2) groups and aromatic ring (2-CH, 3-CH, and 4-CH) remote from amino sugar of doxorubicin construct the hydrophobic core of the micelle. Therefore, observed NOEs between the protons of doxorubicin and PEG-PE suggest strongly that anthracycline ring of doxorubicin inserts into hydrophobic core of PEG-PE micelle.

The ^{31}P NMR spectra of the PEG-PE micelles with inserted doxorubicin (PEG-PE: doxorubicin = 2: 1) and without doxorubicin showed a single symmetric peak (Fig. 8), indicating that the insertion of the anthracycline ring of doxorubicin between the PE lipids does not disturb the arrangement of PE headgroup. This result was in good agreement with that provided by TEM. Taken together, the schematic nano-architecture of M-Dox was illustrated in Fig. 2B. Doxorubicin is loaded in PEG-PE micelle in such a way that the anthracycline ring of doxorubicin is inserted

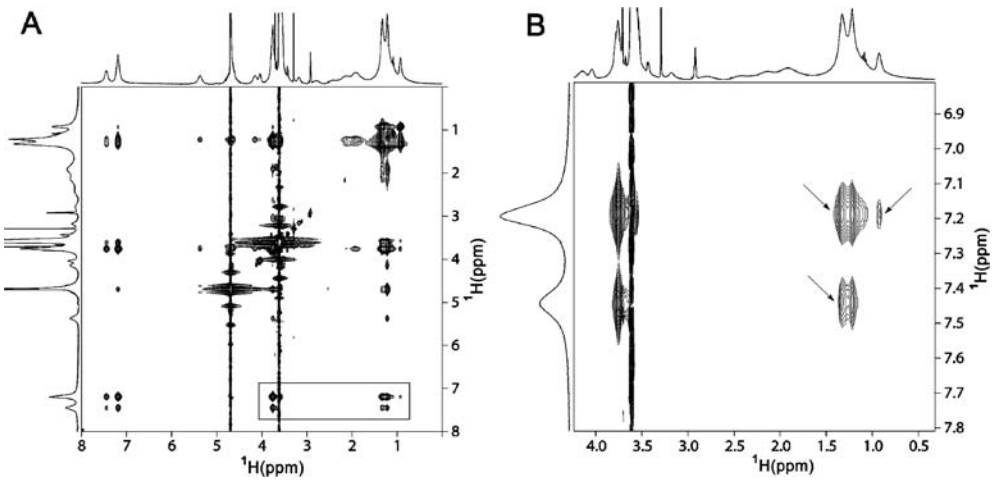

Fig. 7. (**A**) 2D ^{1}H-^{1}H NOESY spectrum of the mixture of doxorubicin and PEG-PE at a molar ratio of 50:5 for doxorubicin: PEG-PE. (**B**) Expansion of the small box in panel A.

between PE phospholipids, locating closely to glycerol backbone of PE hydrophobic core, and amino sugar of doxorubicin is in the outer shell of micelle between PEG chains.

DISCUSSION

The development of new drug delivery system of anticancer drugs is a major challenge in tumor therapy. It implies a full understanding of the drug-carrier interaction. In the present work, water-soluble drugs were entrapped into PEG-PE micelles using one-step self-assembly procedure. In the past decades, the solubilization of poorly soluble drugs by micelles has been extensively investigated (22). Organic solvents, such as ethanol and chloroform, and equipments were used in the preparation of hydrophobic drug-loaded micelles. In addition, a novel one-step freeze-drying procedure was developed to prepare paclitaxel and docetaxel-loaded micelles; however, *tert*-butanol was used in this process (23). In the present work, we prepared water-soluble drug-loaded micelles with high drug encapsulation efficiency using a very simple procedure. Organic solvents and rotary evaporator were not required in this process.

Earlier, we reported some research data on the doxorubicin-loaded PEG-PE micelles (M-Dox) *in vitro* and *in vivo* (19). In that paper, M-Dox was successfully prepared by thin film hydration method using chloroform and methanol as solvents in the presence of triethylamine. Based on the extensive investigation on the structure of PEG-PE molecule and PEG-PE micelles as well as our previous data, we inferred that the hydrophobic interaction and polar and electrostatic interaction may play important roles in imparting this high encapsulation efficiency. Interestingly, Sarthak *et. al*. had investigated the roles of nonpolar and polar intermolecular interactions in the improvement of the hydrophobic drug loading capacity of PEG-PCL with increasing PCL content (24).

To design and prepare optimal pharmaceutical formulations of drug-loaded micelles, a full understanding of the interactions of PEG-PE with water-soluble drugs as well as molecular architecture of micelles is very important. To answer the above question, we used various NMR techniques to analyze the molecular assembly mechanism of M-Dox. NMR data demonstrated that anthracycline ring of doxorubicin was inserted between PE phospholipids, and its amino sugar located in the outer shell of micelle between PEG chains. Furthermore, we also found that the electrostatic interactions played an important role in the process of self-assembly (Fig. 4A and Table II). This result was consistent with the fact that the interaction of doxorubicin and PEG-PE occurred at a molar ratio of 1:1, because the charges of doxorubicin hydrochloride and PEG-PE are +1 and −1, respectively (Table I). To further prove our hypothesis that electrostatic interaction and hydrophobic forces play important roles in the intercalation of water-soluble drugs into PEG-PE micelles, we selected different types of anticancer drugs to perform drug loading experiments. As shown in Fig. 4B, vinorelbine tartrate and vincristine sulfate incorporated into PEG-PE micelles showed high encapsulation efficiency of 99.4% (molar ratio of Vino to PEG-PE is 1:2) and 99.2% (molar ratio of Vinc to PEG-PE is 1:2), respectively. However, gemcitabine hydrochloride and paclitaxel had negligible incorporation into PEG-PE micelles. These results confirmed the importance of electrostatic and hydrophobic interactions in the process of self-assembly of PEG-PE and water-soluble drugs.

Based on the understanding of the interactions of PEG-PE with drugs, we successfully prepared vinorelbine-loaded PEG-PE micelles (M-Vino). Vinorelbine incorporated into PEG-PE micelle has high encapsulation efficiency (99.8% at 1:4 molar ratio of Vino to PEG-PE). After intravenous injection of M-Vino (5 mg/kg vinorelbine tartrate and 50 mg/kg PEG-PE micelles), M-Vino significantly reduced lung metastases in mice bearing 4T1 tumors and increased their survival with low systemic toxicity (to be published elsewhere).

CONCLUSION

In conclusion, we have shown that a number of water-soluble drugs can be effectively incorporated into micelles made from PEG-PE conjugates with high efficiency of >99.0% using a one-step self-assembly procedure. The molecular architecture of drug-loaded micelles, such as M-Dox, was determined by NMR, showing that anthracycline ring of doxorubicin was inserted between PE phospholipids, and its amino sugar located in the outer shell of micelle between PEG chains. In addition, we found that electrostatic interaction and hydrophobic forces are responsible for the intercalation of water-soluble drugs into PEG-PE micelles. These findings can be applied in the optimal design of drug nano-carrier for cancer therapy or other disease therapeutic applications.

ACKNOWLEDGEMENTS

This work was supported by grants from the National Nature Sciences Foundation of China (No. 90606019, 30901869), China Postdoctoral Science Foundation (No. 20090450598), State Key Development Plan Project (2006CB933305, 2007CB935801) and China-Finland Inter-Governmental S&T Cooperation Project (2008DFA01510).

REFERENCES

1. Maeda H, Wu J, Sawa T, Matsumura Y, Hori K. Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. J Control Release. 2000;65:271–84.
2. Kwon GS. Polymeric micelles for delivery of poorly water-soluble compounds. Crit Rev Ther Drug Carrier Syst. 2003;20:357–403.
3. Torchilin VP. Targeted polymeric micelles for delivery of poorly soluble drugs. Cell Mol Life Sci. 2004;61:2549–59.
4. Matsumura Y, Hamaguchi T, Ura T, Muro K, Yamada Y, Shimada Y, *et al*. Phase I clinical trial and pharmacokinetic evaluation of NK911, a micelle-encapsulated doxorubicin. Br J Cancer. 2004;91:1775–81.
5. Kuroda J, Kuratsu J, Yasunaga M, Koga Y, Saito Y, Matsumura Y. Potent antitumor effect of SN-38-incorporating polymeric micelle, NK012, against malignant glioma. Int J Cancer. 2009;124:2505–11.
6. Matsumura Y. Poly (amino acid) micelle nanocarriers in preclinical and clinical studies. Adv Drug Deliv Rev. 2008;60:899–914.
7. Danson S, Ferry D, Alakhov V, Margison J, Kerr D, Jowle D, et al. Phase I dose escalation and pharmacokinetic study of pluronic polymer-bound doxorubicin (SP1049C) in patients with advanced cancer. Br J Cancer. 2004;90:2085–91.
8. Lee KS, Chung HC, Im SA, Park YH, Kim CS, Kim SB, *et al*. Multicenter phase II trial of Genexol-PM, a Cremophor-free,

polymeric micelle formulation of paclitaxel, in patients with metastatic breast cancer. Breast Cancer Res Treat. 2008;108: 241–50.
9. Torchilin VP. Recent advances with liposomes as pharmaceutical carriers. Nat Rev Drug Discov. 2005;4:145–60.
10. Lukyanov AN, Gao Z, Mazzola L, Torchilin VP. Polyethylene glycol-diacyllipid micelles demonstrate increased acculumation in subcutaneous tumors in mice. Pharm Res. 2002;19:1424–9.
11. Gao Z, Lukyanov AN, Singhal A, Torchilin VP. Diacyllipid-polymer micelles as nanocarriers for poorly soluble anticancer drugs. Nano Lett. 2002;2:979–82.
12. Musacchio T, Laquintana V, Latrofa A, Trapani G, Torchilin VP. PEG-PE micelles loaded with paclitaxel and surface-modified by a PBR-ligand: synergistic anticancer effect. Mol Pharm. 2009;6:468–79.
13. Sawantand RR, Torchilin VP. Enhanced cytotoxicity of TATp-bearing paclitaxel-loaded micelles *in vitro* and *in vivo*. Int J Pharm. 2009;374:114–8.
14. Noble RL. The discovery of the vinca alkaloids–chemotherapeutic agents against cancer. Biochem Cell Biol. 1990;68:1344–51.
15. Tritonand TR, Yee G. The anticancer agent adriamycin can be actively cytotoxic without entering cells. Science. 1982;217:248–50.
16. Van Vleetand JF, Ferrans VJ. Myocardial diseases of animals. Am J Pathol. 1986;124:98–178.
17. Lobert S. Neurotoxicity in cancer chemotherapy: vinca alkaloids. Crit Care Nurse. 1997;17:71–9.
18. Shenasa H, Calderone A, Vermeulen M, Paradis P, Stephens H, Cardinal R, *et al*. Chronic doxorubicin induced cardiomyopathy in rabbits: mechanical, intracellular action potential, and beta adrenergic characteristics of the failing myocardium. Cardiovasc Res. 1990;24:591–604.
19. Tang N, Du G, Wang N, Liu C, Hang H, Liang W. Improving penetration in tumors with nanoassemblies of phospholipids and doxorubicin. J Natl Cancer Inst. 2007;99:1004–15.
20. Vakiland R, Kwon GS. Effect of cholesterol on the release of amphotericin B from PEG-phospholipid micelles. Mol Pharm. 2008;5:98–104.
21. Vernooij EA, Gentry CA, Herron JN, Crommelin DJ, Kettenes-van den Bosch JJ. 1H NMR quantification of poly(ethylene glycol)-phosphatidylethanolamine in phospholipid mixtures. Pharm Res. 1999;16:1658–61.
22. Lukyanovand AN, Torchilin VP. Micelles from lipid derivatives of water-soluble polymers as delivery systems for poorly soluble drugs. Adv Drug Deliv Rev. 2004;56:1273–89.
23. Fournier E, Dufresne MH, Smith DC, Ranger M, Leroux JC. A novel one-step drug-loading procedure for water-soluble amphiphilic nanocarriers. Pharm Res. 2004;21:962–8.
24. Patel SK, Lavasanifar A, Choi P. Roles of nonpolar and polar intermolecular interactions in the improvement of the drug loading capacity of PEO-b-PCL with increasing PCL content for two hydrophobic Cucurbitacin drugs. Biomacromolecules. 2009;10:2584–91.