Research Article

Fmoc-Conjugated PEG-Vitamin E₂ Micelles for Tumor-Targeted Delivery of Paclitaxel: Enhanced Drug-Carrier Interaction and Loading Capacity

Yifei Zhang,^{1,2,3} Yixian Huang,^{1,2,3} Wenchen Zhao,² Jianqin Lu,^{1,2,3} Peng Zhang,^{1,2,3} Xiaolan Zhang,^{1,2,3} Jiang Li,^{1,2,3} Xiang Gao,^{1,2,3} Raman Venkataraman,^{2,3} and Song Li^{1,2,3,4}

Received 25 June 2014; accepted 22 July 2014; published online 6 September 2014

Abstract. The purpose of this study is to develop an improved drug delivery system for enhanced paclitaxel (PTX) loading capacity and formulation stability based on PEG_{5K}-(vitamin E)₂ (PEG_{5K}-VE₂) system. PEG_{5K}-(fluorenylmethoxycarbonyl)-(vitamin E)₂ (PEG_{5K}-FVE₂) was synthesized using lysine as the scaffold. PTX-loaded PEG_{5K}-FVE₂ micelles were prepared and characterized. Fluorescence intensity of Fmoc in the micelles was measured as an indicator of drug-carrier interaction. Cytotoxicity of the micelle formulations was tested on various tumor cell lines. The therapeutic efficacy and toxicity of PTX-loaded micelles were investigated using a syngeneic mouse model of breast cancer (4T1.2). Our data suggest that the PEG_{5K}-FVE₂ micelles have a low CMC value of 4 µg/mL and small sizes (~60 nm). The PTX loading capacity of PEG_{5K}-FVE₂ micelles was much higher than that of PEG_{5K}-VE₂ micelles. The Fmoc/PTX physical interaction was clearly demonstrated by a fluorescence quenching assay. PTX-loaded PEG_{5K}-FVE₂ micelles exerted more potent cytotoxicity than free PTX or Taxol formulation *in vitro*. Finally, intravenous injection of PTX-loaded PEG_{5K}-FVE₂ micelles showed superior anticancer activity compared with PEG_{5K}-VE₂ formulation with minimal toxicity in a mouse model of breast cancer. In summary, incorporation of a drug-interactive motif (Fmoc) into PEG_{5K}-VE₂ micelles represents an effective strategy to improve the micelle formulation for the delivery of PTX.

KEY WORDS: drug delivery; drug-interactive motif; micelles; paclitaxel; vitamin E derivative.

INTRODUCTION

Paclitaxel (PTX) is a potent anticancer agent acting through the stabilization of microtubules (1). However, the clinical benefit of PTX is limited by its poor water solubility. The widely-used clinical formulation Taxol® contains alcohol and Cremophor EL which can cause irritation and

hyperactivity reactions (2). Therefore, substantial efforts have been made to develop alternative formulations to improve the solubility of PTX. Polymeric micelles have attracted significant attention due to their unique advantages for the intravenous delivery of paclitaxel and other hydrophobic drugs. The small size (10–100 nm) of micelles and hydrophilic corona shells of PEG are critical for the long blood circulation time and effective passive targeting to tumor tissues through enhanced permeability and retention (EPR) effect (3), leading to enhanced antitumor efficacy and decreased toxicity. Several micelle formulations of PTX have been developed and hold great promise for clinical application, such as NK105 (PTX-loaded polyethylene glycol-(L-aspartic acid) micelles) (4) and Genexol-PM (a PTX incorporated PEG-PLA micelle system) (5).

D- α -tocopheryl polyethylene glycol (PEG)-1000 succinate (TPGS) is a PEG derivative of vitamin E that has been used as an excipient in various drug formulations (6–9). TPGS has intrinsic P-gp inhibitory effect which helps to overcome multidrug resistance (10,11). TPGS is self-assembled into micelles in aqueous solution; however, the high critical micelle concentration (CMC) decreases the stability of micelles in physiological environment (12). We and others have synthesized a series of PEG-vitamin E conjugates that differ in PEG molecular weight (2 vs. 5 K) and the number of vitamin E molecules (1 vs. 2) (13,14). By increasing the PEG length and including one additional VE, the PEG_{2K}-VE₂

Electronic supplementary material The online version of this article (doi:10.1208/s12248-014-9651-2) contains supplementary material, which is available to authorized users.

¹ Center for Pharmacogenetics, School of Pharmacy, University of Pittsburgh, 639 Salk Hall, Pittsburgh, PA 15261, USA.

² Department of Pharmaceutical Sciences, School of Pharmacy, University of Pittsburgh, Pittsburgh, PA 15261, USA.

³ University of Pittsburgh Cancer Institute, University of Pittsburgh, Pittsburgh, PA 15261, USA.

⁴ To whom correspondence should be addressed. (e-mail: sol4@pitt.edu)

ABBREVIATIONS: PTX, Paclitaxel; PEG_{5K}-VE₂, PEG_{5K}-(vitamin E)₂; PEG_{5K}-FVE₂, PEG_{5K}-(fluorenylmethoxycarbonyl)-(vitamin E)₂; EPR, Enhanced permeability and retention; TPGS, D- α -tocopheryl polyethylene glycol (PEG)-1000 succinate; CMC, Critical micelle concentration; DLS, Dynamic light scattering; DLC, Drug loading capacity; DLE, Drug loading efficiency; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; AST, Aspartate aminotransferase; ALT, Alanine aminotransferase; PEI, Polyethylenimine.

micelles achieved lower CMC value, higher loading capacity for doxorubicin, and enhanced anticancer effect compared with PEG_{2K}-VE and traditional TPGS (13). In addition, our study showed that PEG_{5K}-(vitamin E)₂ (PEG_{5K}-VE₂) was the most stable and effective micellar formulation of PTX compared with PEG_{2K}-VE, PEG_{2K}-VE₂, and PEG_{5K}-VE (14). The tumor growth inhibitory effect of PTX-loaded PEG_{5K}-VE₂ was significantly improved in comparison to Taxol formulation or other three PTX micellar formulations *in vivo* (14). Nevertheless, the drug loading capacity of PTX-loaded PEG_{5K}-VE₂ micelles was still inadequate, which warranted further improvement.

We have recently shown that incorporation of a drug-interactive domain into a lipid surfactant can improve both drug-loading capacity and formulation stability. This concept was experimentally tested when we were formulating JP4-039, a mitochondria-targeted antioxidant (15). We have synthesized a series of PEG-lipopeptides that have a PEG hydrophilic headgroup, one or several oleoyl chains, and amino acids derivatized with various functional motifs at the interfacial region. Among several functional groups tested, 9-fluorenylmethoxycarbonyl (α -Fmoc) was identified to be the most effective drug-interactive motif that not only enhances the JP4-039 loading capacity but also improves the stability of JP4-039-loaded emulsion and micellar formulations (15). The improved drug/carrier interaction can be attributed to the hydrophobic/hydrophobic interaction, hydrogen bonding, and π - π stacking between the Fmoc-containing carrier and JP4-039 (15).

We hypothesize that inclusion of Fmoc moiety shall similarly improve the drug loading capacity of PEG_{5K}-VE₂ micelles for PTX. In the current work, we synthesized a novel amphiphilic polymer, PEG_{5K}-(fluorenylmethoxycarbonyl)-(vitamin E)₂ (PEG_{5K}-FVE₂) by introducing an Fmoc motif into PEG_{5K}-VE₂ conjugate. To evaluate the ability of PEG_{5K}-FVE₂ micelles for delivery of PTX, the drug-loading capacity of those micelles was investigated. The interaction between Fmoc moiety and PTX molecule was analyzed by measuring the change of Fmoc fluorescence intensity at different drug to carrier ratios. Cytotoxicity of blank micelles and PTX-loaded micelles was studied against free PTX using various tumor cell lines. Finally, the therapeutic efficacy and toxicity of PTX-loaded micelles were investigated using a syngeneic mouse model of breast cancer (4T1.2).

MATERIALS AND METHODS

Materials

PTX (98%) was purchased from AK Scientific Inc. (CA, USA). Dulbecco's phosphate-buffered saline (DPBS) was purchased from Lonza (MD, USA). Methoxy-PEG 5000-OH, dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Triton X-100, Dulbecco's modified Eagle's medium (DMEM), succinate anhydride, Boc-lys-(Boc)-OH, and Fmoc-lys-(Boc)-OH were all purchased from Sigma-Aldrich (MO, USA). Fetal bovine serum (FBS) and penicillin-streptomycin solution were from Invitrogen (NY, USA). D- α -tocopheryl was purchased from Tokyo Chemical Industry (OR, USA). *N,N'*-dicyclohexylcarbodiimide (DCC) was purchased from Alfa

Aesar (MA, USA). 4-Dimethylaminopyridine (DMAP) was purchased from Calbiochem-Novabiochem Corporation (CA, USA). All solvents used in this study were HPLC grade.

Synthesis of PEG_{5K}-Fmoc-VE₂ (PEG_{5K}-FVE₂)

PEG_{5K}-FVE₂ was synthesized via solution-phase condensation reactions from MeO-PEG-OH with a molecular weight of 5,000 Da. Fmoc-lys-(Boc)-OH (4 eq.) was coupled onto the terminal -OH of PEG using DCC (4 eq.) and DMAP (0.2 eq.) as coupling reagents in DCM for overnight. Fmoc-lys-(Boc) PEG ester was precipitated and washed three times with cold ethanol and ether, respectively. Then, Boc groups were removed by the treatment with 50% trifluoroacetic acid in DCM, and the Fmoc-lysyl PEG ester was precipitated and washed three times with cold ethanol and ether, respectively. Boc-lys-(Boc)-OH (2 eq.) was coupled onto the terminal -NH₂ of Fmoc-lysyl PEG ester using DCC (2 eq.) and DMAP (0.1 eq.) as coupling reagents in DCM for overnight. The di-Boc PEG ester was precipitated and washed three times with cold ethanol and ether, respectively. Then, Boc groups were removed by the treatment with 50% trifluoroacetic acid in DCM, and the di-NH₂ PEG ester was precipitated and washed three times with cold ethanol and ether, respectively. The resulting white powder precipitate was dried under vacuum. Vitamin E succinate (4 eq.) was coupled to the deprotected amino groups of lysine with the assistance of DCC (4 eq.) and DMAP (0.2 eq.). The resulting PEG_{5K}-FVE₂ was precipitated and washed three times with cold ethanol and ether, respectively. The final product was subsequently dialyzed against water and lyophilized to yield white powder.

Preparation and Characterization of PEG_{5K}-FVE₂ Micelles

Drug-free or PTX-loaded PEG_{5K}-FVE₂ micelles were prepared according to published procedures (14). Briefly, PTX solution (10 mM in chloroform) was mixed with PEG_{5K}-FVE₂ (10 mM in chloroform) at various molar ratios. The chloroform was removed by nitrogen flow and a thin dry film of drug/carrier mixture was formed. High vacuum was then applied to remove any traces of remaining solvent. DPBS was added into each vial to hydrate the film. The samples were then probe-sonicated for 2 min at 3 W. The blank micelles were similarly prepared except that PTX was not added.

The size of micelles was measured by dynamic light scattering (DLS) method using a Malvern Zetasizer (Nano ZS; Malvern instruments Ltd., Worcestershire, UK). The PTX concentration was kept at 1 mg/mL for DLS measurements. To measure the PTX loading efficiency, unincorporated free PTX was removed by filtering through a syringe filter with pore size of 0.22 μ m (16). The amount of PTX loaded into micelles was analyzed by HPLC according to a published method (16). The drug loading capacity (DLC) and drug loading efficiency (DLE) were calculated according to the following formula (16):

$$\begin{aligned} \text{DLC (\%)} &= [\text{weight of drug used}/(\text{weight of polymer} + \text{drug used})] \times 100\% \\ \text{DLE (\%)} &= (\text{weight of loaded drug}/\text{weight of input drug}) \times 100\% \end{aligned}$$

CMC Measurement

The CMC of PEG_{5K}-FVE₂ was determined by employing pyrene as a fluorescence probe as described previously (16). Briefly, aliquots of 50 μ L pyrene solution (4.8×10^{-6} M) were mixed with various amount of PEG_{5K}-FVE₂ in chloroform. The chloroform was then removed by nitrogen flow and high vacuum to form a thin film in the vials. Each sample was hydrated with 400 μ L DPBS to give a final pyrene concentration of 6×10^{-7} M with PEG_{5K}-FVE₂ concentrations ranging from 6×10^{-5} to 0.25 mg/mL. The samples were kept shaking at 37°C for 24 h to reach equilibrium, followed by fluorescence measurement at the wavelength of em/ex=334 nm/390 nm using Synergy H1 Hybrid Multi-Mode Microplate Reader (Winooski, VT). The fluorescence intensity was plotted against PEG_{5K}-FVE₂ concentration. The CMC was determined from the threshold concentration, where the fluorescence intensity begins to increase dramatically.

Fluorescence Measurement of Blank and PTX-Loaded Micelles

Micelle formulations with or without PTX were prepared in 500 μ L of water as described (17). Freshly prepared micelles were added to 96-well plate (100 μ L/well), and the fluorescence intensity was measured immediately with a Synergy H1 Hybrid reader (BioTek). The emission spectra were recorded ranging from 300 to 400 nm with a fixed excitation at 270 nm.

Hemolytic Effect of Micelles

Rat blood was collected via cardiac puncture with heparin as the anticoagulant. The blood sample was centrifuged at 1,500 rpm for 10 min at 4°C to collect the red blood cells (RBCs). The RBCs were washed with 30-mL ice-cold DPBS for three times and diluted to 2% w/v with ice-cold DPBS. One milliliter of diluted RBC suspension was mixed with blank PEG_{5K}-VE₂ or PEG_{5K}-FVE₂ micelles to obtain the final polymer concentrations of 0.2 or 1.0 mg/mL. The mixture was incubated at 37°C with constant shaking for 4 h and then centrifuged at 1,500 rpm for 10 min at 4°C. One hundred microliter of supernatant from each sample was transferred into a 96-well plate. The absorbance at 540 nm was measured using a PerkinElmer LS 50B luminescence spectrometer (Waltham, MA) to determine the release of hemoglobin from RBCs. Triton X-100 (2%) and DPBS were also incubated with RBCs as positive and negative controls, respectively. The percentage of hemoglobin release was calculated as $(OD_{\text{sample}} - OD_{\text{negative control}}) / (OD_{\text{positive control}} - OD_{\text{negative control}}) \times 100\%$ (16).

Cell Culture

Huh-7 is a human hepatocellular carcinoma cell line and Hepa 1-6 is a mouse hepatoma cell line. 4T1.2 is a mouse metastatic breast cancer cell line and MCF-7 is a human breast cancer cell line. All cell lines were cultured in DMEM containing 10% FBS and 1% penicillin-streptomycin at 37°C in a humidified incubator with 5% CO₂.

In Vitro Cytotoxicity Study

The PTX-loaded PEG_{5K}-FVE₂ micelles were examined for their cytotoxicity against four cancer cell lines using PTX-loaded PEG_{5K}-VE₂ micelles, Taxol® formulation, and PTX dissolved in DMSO as control groups. The 4T1.2 cells were seeded in 96-well plates at a density of 1,000 cells per well. After incubation for 24 h, the cells were treated with various formulations at the indicated PTX concentrations for 72 h, followed by MTT assay as described previously (16). Briefly, 20 μ L of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in DPBS (5 mg/mL) was added to each well and the cells were further incubated for 4 h. The medium was then removed and 150 μ L DMSO was added into each well to solubilize the MTT formazan. The absorbance at 570 nm was detected by PerkinElmer LS 50B luminescence spectrometer (Waltham, MA). Data were shown as the percentages of cell viability calculated from triplicate wells using the formula: $[(OD_{\text{treat}} - OD_{\text{blank}}) / (OD_{\text{untreat}} - OD_{\text{blank}})] \times 100\%$. The cytotoxicity with Huh-7, Hepa 1-6, and MCF-7 cells was similarly performed except that the cells were seeded at a density of 5,000 cells per well.

Animals

Female BALB/c mice, 10–12 weeks and male Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA). All animals were housed under pathogen-free conditions according to AAALAC guidelines. All animal-related experiments were performed in full compliance with institutional guidelines and approved by the animal use and care administrative advisory committee at the University of Pittsburgh.

Tissue Distribution of PTX-Loaded PEG_{5K}-FVE₂ Micelles

4T1.2 cells (2×10^5 in 200 μ L PBS) were inoculated s.c. at the right flank of female BALB/c mice. Once the tumor volume reached approximately 400–600 mm³, 15 mice were divided into 3 groups randomly and treated via tail vein injection with PTX-loaded formulations including PEG_{5K}-VE₂, PEG_{5K}-FVE₂ micelles, and Taxol at a PTX dose of 10 mg/kg. Mice were sacrificed 24 h after injection. The liver, lung, and tumor tissues were collected, weighed, and stored at –80°C.

Analysis of Tissue Samples

Mouse tissue was placed in glass tubes and homogenized with 2 mL solvent (acetonitrile to H₂O=1:1, v/v). The samples were centrifuged at 4°C, 3,500 rpm for 15 min, and the supernatant was transferred to clean tubes. The tissue homogenates were dried by evaporation under nitrogen flow, then the residue was dissolved in 200 μ L solvent (MeOH to H₂O=1:1, v/v) and centrifuged at 4°C, 14,500 rpm for 6 min. The supernatant was mixed with equal volume of MeOH and centrifuged again at 4°C, 14,500 rpm for 6 min. Then, 20 μ L of the clear supernatant was injected into HPLC system (16).

In Vivo Therapeutic Study

A syngeneic murine breast cancer model was used to evaluate the therapeutic efficacy of PTX-loaded PEG_{5K}-FVE₂ micelles. 4T1.2 cells (2×10^5 in 200 μ L PBS) were inoculated s.c. at the right flank of female BALB/c mice. Treatments were initiated when tumors in the mice reached a volume around 50 mm³ and this day was designated as day 0. On day 0, mice were randomly divided into five groups ($n=5$) and treated with tail vein injection of saline (control), blank PEG_{5K}-FVE₂ micelles, PTX-loaded PEG_{5K}-VE₂ micelles (PEG_{5K}-VE₂ to PTX=5:1, mol/mol), PTX-loaded PEG_{5K}-FVE₂ micelles (PEG_{5K}-FVE₂ to PTX=5:1, mol/mol), and Taxol® (all at 10 mg PTX/kg), respectively. Blank PEG_{5K}-FVE₂ micelles were given at the equivalent dosage of the carrier in the group of PTX-loaded micelles. The treatment was performed three times a week for 2 weeks. Tumor size was measured with digital caliper three times a week. Tumor volume was calculated by the formula: $(L \times W^2)/2$, where L is the longest and W is the shortest in tumor diameters (mm). Mice were sacrificed at the end of experiment. To compare between groups, relative tumor volume (RTV) was calculated at each measurement time point (where RTV equals the tumor volume at a given time point divided by the tumor volume prior to the first treatment). Mice were euthanized when tumor volume reached 2,000 mm³. To monitor potential toxicity, the body weight of each mouse was measured three times a week. In addition, serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were examined at the completion of the study.

Statistical Analysis

Statistical analysis was performed by Student's t test for two groups, and one-way ANOVA for multiple groups,

followed by Newman-Keuls test if $P < 0.05$. All results were reported as the mean $\pm$ standard deviation (SD) unless otherwise indicated. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Synthesis and Biophysical Characterization of PEG_{5K}-FVE₂

PEG_{5K}-FVE₂ polymer was synthesized by conjugating PEG_{5K} with vitamin E succinate using dendritic oligo-lysine as the scaffold. Fmoc-lys-(Boc) and Boc-lys-(Boc) were sequentially linked to PEG_{5K} by condensation reaction, followed by conjugation with succinate VE. The synthetic scheme is shown in Fig. 1. The structure of PEG_{5K}-FVE₂ was confirmed by ¹H NMR (Fig. S1A) and MALDI-TOF (Fig. S1B). In Fig. S1A, the peaks between 7.2 and 8.5 ppm were assigned to the Fmoc group. The peak at 3.3 ppm corresponded to the -OCH₃ on the end of mPEG, the intense peaks at 3.5 ppm could be assigned to the methane protons of the polyethylene glycol. The proton peaks below 3.0 ppm were assigned to the vitamin E succinate. MALDI-TOF indicated that the molecular weight of PEG_{5K}-FVE₂ was around 6,500 (Fig. S1B), which is in agreement with the calculated molecular weight of 6,502.

The sizes of PTX-loaded PEG_{5K}-FVE₂ micelles at different carrier to drug molar ratios were determined by DLS analysis. The sizes of blank and drug-loaded micelles were both around 60 nm (Table I). PTX could be loaded into PEG_{5K}-FVE₂ micelles at a carrier to drug molar ratio as low as 0.5:1. This is in contrast to PEG_{5K}-VE₂ micelles for which a minimal carrier to drug molar ratio of 2.5:1 was required to solubilize PTX (14). In addition, the drug loading efficiency as determined by HPLC was also improved by increasing the carrier to drug ratios. More than 95% of the input PTX was

Fig. 1. Synthesis scheme of PEG_{5K}-FVE₂ conjugate

Table I. Comparison of PTX-loaded PEG_{5K}-VE₂ and PEG_{5K}-FVE₂ Micelles (*n*=3)

Micelle	Molar ratio ^a	Size (nm) ^b	PDI ^c	DLC (%) ^d	DLE (%) ^e
^f PEG _{5K} -VE ₂	–	18.8±0.12	0.09	–	–
PEG _{5K} -FVE ₂	–	60.12±2.99	0.208	–	–
^f PEG _{5K} -VE ₂ :PTX	2.5:1	20.1±0.72	0.05	5.4	86.4
	5:1	19.7±0.38	0.04	2.8	88.4
PEG _{5K} -FVE ₂ :PTX	0.5:1	55.32±1.84	0.256	20.8	87.0
	1:1	61.60±3.55	0.213	11.6	96.0
	2.5:1	68.64±2.90	0.224	5.0	95.4
	5:1	70.06±6.84	0.226	2.6	98.9

^a The PTX concentration was 1 mg/mL in all the drug-loaded samples above

^b Data represents the mean±standard deviation (*n*=3)

^c PDI: polydispersity index

^d DLC: drug loading capacity

^e DLE: drug loading efficiency

^f PEG_{5K}-VE₂: data from our previous publication (14)

incorporated into the micelles at a carrier to drug molar ratio of 1:1 and above (Table I).

The CMC of PEG_{5K}-FVE₂ polymer was measured with pyrene as the fluorescent probe. A series of pyrene-loaded PEG_{5K}-FVE₂ micelles were prepared with a fixed concentration of pyrene and different concentrations of polymer. When the concentration of a polymer reaches its CMC, the micellization is marked by an abrupt increase in fluorescence intensity of pyrene at the wavelength of 390 nm (18). Based on this principle, the fluorescence intensity was recorded and plotted against the logarithmic concentration of the PEG_{5K}-FVE₂ polymer, and the CMC value was determined. Figure 2 shows that the CMC of PEG_{5K}-FVE₂ micelles was 4 µg/mL, which was slightly higher than that of PEG_{5K}-VE₂ (1.7 µg/mL) (14), but still significantly lower than that of TPGS (0.2 mg/mL) (12).

Fluorescence Quenching Assay

The improved PTX loading capacity of PEG_{5K}-FVE₂ over PEG_{5K}-VE₂ suggests a role of Fmoc/PTX interaction in the formation of PTX-loaded PEG_{5K}-FVE₂ micelles. To test this hypothesis, we studied the Fmoc/PTX interaction via fluorescence quenching assay. We chose an excitation

Fig. 2. CMC determination of the PEG_{5K}-FVE₂ micelles using pyrene as a hydrophobic fluorescence probe. The fluorescence intensity of pyrene was measured (Ex/Em=334 nm/390 nm) and plotted against the logarithmic concentration of PEG_{5K}-FVE₂ micelles. The concentration of pyrene was kept at 6×10^{-7} mol/L. Values reported are the means±SD, *n*=3

wavelength of 270 nm and examined the emission spectra of PEG_{5K}-FVE₂, PEG_{5K}-VE₂, and free PTX ranging from 300 to 400 nm. As shown in Fig. 3a, there is a distinct fluorescence peak at the emission wavelength of 305 nm for PEG_{5K}-FVE₂. This fluorescence appeared to be highly specific for Fmoc because minimal fluorescence intensity was detected for either PEG_{5K}-VE₂ or free PTX (Fig. 3a). We then prepared several PTX-loaded PEG_{5K}-FVE₂ micelles with constant PEG_{5K}-FVE₂ concentration but varying amounts of PTX, and similarly measured the fluorescence intensity. As shown in Fig. 3b, the fluorescence intensity of Fmoc group at 305 nm was dramatically reduced with increased amount of PTX. We also conducted a similar experiment with several different mixed micelles that had a fixed concentration of PTX (0 or 0.1 mg/mL) but varying amounts of PEG_{5K}-FVE₂. As shown in Fig. 3c, at each concentration of PEG_{5K}-FVE₂, the Fmoc fluorescence intensity at 305 nm was dramatically reduced in the presence of PTX. Figure 3d summarizes the changes of fluorescence intensity at 305 nm from the above fluorescence studies. It is apparent that the carrier to drug molar ratio affected the degree of reduction in fluorescence, which became more dramatic with increased amount of PTX.

Hemolytic Effect of Micelles

The PEG_{5K}-FVE₂ polymer is amphiphilic and could solubilize lipids or insert into phospholipid membranes to induce membrane destabilization (19). Detrimental interaction between the polymeric materials and blood constituents such as red blood cells must be avoided for intravenous drug delivery. Therefore, the hemolytic effect of PEG_{5K}-FVE₂ micelles was evaluated as an indicator of the hematocompatibility. Polyethylenimine (PEI), a cationic polymer with potent cell surface activity, was included as the positive control. As shown in Fig. 4, incubation of RBC with PEI caused significant hemolysis in a dose-dependent manner. In contrast, PEG_{5K}-FVE₂ caused less than 5% hemolysis at both 0.2 and 1 mg/mL, which is considered as nonhemolytic according to the Guiding Principles of Hemolysis Test [H]GPT4-1 (20). These data suggest that the PEG_{5K}-FVE₂ polymer is hemocompatible and suitable for intravenous administration.

In Vitro Cytotoxicity of PTX-loaded Micelles

The cytotoxicity of PTX-loaded PEG_{5K}-FVE₂ micelles was investigated in breast cancer cell lines such as 4T1.2, MCF-7, and liver cancer cell lines including Hepa 1-6 and Huh-7 cells (Fig. 5a-d). PTX-loaded PEG_{5K}-VE₂ micelles, free PTX dissolved with DMSO, and Taxol were included as control formulations. PTX-loaded PEG_{5K}-FVE₂ and PEG_{5K}-VE₂ micelles showed comparable tumor cell killing effect in all of the cell lines tested (Fig. 5a-d). In 4T1.2 cancer cells, both PTX-loaded PEG_{5K}-VE₂ and PEG_{5K}-FVE₂ micellar formulations were more cytotoxic than free PTX, but less active than Taxol formulation (Fig. 5a). However, the two PTX micellar formulations showed higher level of cytotoxicity than both Taxol and free PTX towards MCF-7 cells (Fig. 5b), Hepa 1-6 cells (Fig. 5c), and Huh-7 cells (Fig. 5d). The IC₅₀ of various formulations in the four tumor cell lines tested were summarized in Table S1. Carrier alone (PEG_{5K}-FVE₂ or PEG_{5K}-VE₂) showed minimal cytotoxicity at the concentrations used for PTX-loaded micelles (Fig. S2).

Tissue Distribution of PTX-loaded Micelles

Female BALB/c mice bearing 4T1.2 breast tumor were used for tissue distribution study. PTX-loaded PEG_{5K}-VE₂ micelles, PEG_{5K}-FVE₂ micelles, and Taxol formulation were injected at the same PTX dose of 10 mg/kg. At 24-h

Fig. 4. Hemolytic activity of PEG_{5K}-FVE₂ compared to PEG_{5K}-VE₂ and PEI. The polymers (0.2 or 1 mg/mL) were incubated with rat red blood cells (RBCs) for 4 h at 37°C in an incubator shaker. The degree of RBCs lysis was determined by measuring the absorbance of solution at 540 nm after centrifuge. 2% Triton X-100 and DPBS were used as positive and negative control, respectively. Values are presented as means $\pm$ SD, $n=3$

Fig. 3. Change of Fmoc fluorescence intensity in PEG_{5K}-FVE₂ loaded with various amount of PTX. The fluorescence emission spectra at 300–400 nm were recorded with a fixed excitation wavelength of 270 nm (a–c). **b** Fluorescence spectra at different drug to carrier molar ratios with fixed concentration of PEG_{5K}-FVE₂ at 3.81 mg/mL. **c** Fluorescence spectra at different drug to carrier molar ratios with fixed concentration of PTX at 0.1 mg/mL. **d** Summary of changes in the fluorescence intensity of various formulations in **c** at 305 nm

postinjection, liver, lung, and tumors were excised for the analysis of drug concentration. As shown in Fig. 6, PTX concentration in the tumor of mice treated with PEG_{5K}-FVE₂/PTX micelles was significantly higher than that of PEG_{5K}-VE₂/PTX and Taxol groups (Fig. 6).

Fig. 5. In vitro cytotoxicity of free PTX, PTX-loaded PEG_{5K}-FVE₂ micelles, PTX-loaded PEG_{5K}-VE₂ micelles, and Taxol formulation against several cancer cell lines including 4T1.2 (a), MCF-7 (b), Hepa 1-6 (c), and Huh-7 (d). Cells were treated with various formulations for 72 h followed by MTT assay. Data are reported as mean $\pm$ SD, $n=3$

In Vivo Therapeutic Study

The *in vivo* tumor growth inhibitory effect of PTX-loaded PEG_{5K}-FVE₂ micelles were investigated in a syngeneic murine breast cancer model (4T1.2). As shown in Fig. 7a, blank PEG_{5K}-FVE₂ micelles showed minimal inhibitory

Fig. 6. Tissue distribution of PTX 24-h postinjection. PTX-loaded PEG_{5K}-VE₂ and PEG_{5K}-FVE₂ micelles were injected into female BALB/c mice bearing 4T1.2 breast tumor at the dose of 10 mg PTX/kg, respectively. Taxol was also injected as a control. Values are presented as means $\pm$ SD, $n=5$

effect on tumor growth compared with the control group treated with saline. Taxol exhibited moderate effect in inhibiting the tumor growth at the PTX dose of 10 mg/kg. In contrast, at the same PTX dose, a significant improvement in tumor growth inhibition was achieved in the mice treated with PTX-loaded PEG_{5K}-VE₂ micelles. Furthermore, the antitumor effect of PTX-loaded PEG_{5K}-FVE₂ micelles was even higher than that of PTX-loaded PEG_{5K}-VE₂ micelles.

The toxicity of all of the formulations was evaluated by following their effects on body weight and serum levels of AST and ALT. No significant changes in body weight occurred in all of the groups (Fig. 7b). In addition, serum levels of AST and ALT in the mice treated with PTX-loaded PEG_{5K}-FVE₂ micelles were comparable to those in saline-treated group (Fig. 7c, d), suggesting the negligible toxicity of PTX-loaded PEG_{5K}-FVE₂ *in vivo*.

DISCUSSION

We have previously shown that inclusion of drug-interactive Fmoc moiety into a lipopeptide surfactant can dramatically enhance its ability for loading JP4-039, a poorly water-soluble antioxidant (15). In this study, we applied this concept in the optimization of PEG_{5K}-VE₂ polymeric material and synthesized a novel compound PEG_{5K}-FVE₂. Our data showed that PEG_{5K}-FVE₂ is more effective for the loading of PTX compared with PEG_{5K}-VE₂.

Drug solubility in amphiphilic di-block copolymer micelles is affected by the compatibility between the drug and the micelle core (21). Accumulating data have demonstrated

Fig. 7. **a** Enhanced antitumor activity of PTX formulated in PEG_{5K}-FVE₂ micelles *in vivo*. Balb/c mice were inoculated s.c. with 4T1.2 cells (2×10^5 cells/mouse). After the tumor volume reach 50 mm³, the mice received various treatments via tail vein injection three times a week for 2 weeks as indicated by arrows in the graph. Tumor volume was measured and plotted as relative tumor volume. **b** Change of body weight in mice receiving different treatments. The ALT (**c**) and AST (**d**) enzyme activity in serum was measured at the conclusion of experiment. Data are presented as mean $\pm$ SD. * $p < 0.05$, PTX-loaded PEG_{5K}-FVE₂ micelles versus PTX-loaded PEG_{5K}-VE₂ micelles. $n = 5$

that the drug loading capacity and stability of micelles can be greatly improved by tailoring the inner core segment of micelles for stronger drug-carrier interaction. Besides hydrophobic interaction, the compatibility between drug and carrier was shown to be improved by various additional noncovalent bonding. For example, hydrogen-bonding of micelles with doxorubicin significantly enhanced their loading capacity and stability (22, 23). In addition, acid-base interaction between hydrophobic segments of micelles and guest molecules containing carboxylic acid groups also dramatically improved the loading of indomethacin and ibuprofen (24). Furthermore, metal-ligand coordination facilitated the encapsulation of cisplatin into polymeric micelles (25, 26). In particular, Park's group showed that inclusion of "hydrotropic motifs" into the hydrophobic domain of polymeric micelles represented a versatile strategy to enhance the compatibility of carrier with poorly water-soluble drugs (27, 28). Similarly, inclusion of aromatic end groups into the hydrophobic chain of diblock copolymers improved the stable incorporation of

camptothecin (29) and PTX (15, 30). Data from this study and another recent study (17) suggest that incorporation of a drug-interactive motif (Fmoc) at the interfacial region represents an effective strategy in improving the performance of lipid-core micellar systems in loading hydrophobic drugs such as PTX.

Various mechanisms are likely to be involved in the carrier/drug interactions of PTX-loaded PEG_{5K}-FVE₂ micelles, such as hydrophobic interaction with PTX, hydrogen bonding, and the π - π stacking effect (14). The enhanced PTX loading capacity was most likely due to excellent Fmoc-PTX compatibility, which results from the rigid aromatic structure of Fmoc moieties that may interact with those in PTX. This hypothesis was further supported by the change in Fmoc fluorescence intensity in the presence of PTX, which indicated the close proximity between Fmoc and PTX. The decrease of Fmoc fluorescence intensity likely results from the fluorescence quenching caused by intermolecular π - π interactions (31). This information not only supports a role of

Fmoc in the improved PTX loading capacity of PEG_{5K}-FVE₂ micellar system, but also sheds new light on the design of novel polymeric nanocarriers for the delivery of other aromatic structure-containing drugs such as doxorubicin.

MTT assay showed that the cytotoxicity of PTX-loaded PEG_{5K}-FVE₂ and PEG_{5K}-VE₂ micelles was more potent than that of free PTX solubilized in DMSO in all the cell lines tested. In Hepa 1-6 and Huh-7 cells, the cell killing effect of PTX-loaded PEG_{5K}-FVE₂ and PEG_{5K}-VE₂ micelles was even higher than that of Taxol formulation at the same PTX concentrations. This might be attributed to the increased cellular uptake of PTX into the cells due to the well-known p-gp inhibitory effect of vitamin E derivatives (10, 14). The fact that the cytotoxicity of PTX-loaded PEG_{5K}-FVE₂ micelles was similar or slightly higher than that of PTX-loaded PEG_{5K}-VE₂ micelles suggests that the PEG_{5K}-FVE₂ retained the function of PEG_{5K}-VE₂ in enhancing the cellular uptake of drug-loaded micelles.

A highly metastatic murine breast cancer cell line 4T1.2 was selected to investigate the *in vivo* therapeutic efficacy of PTX-loaded PEG_{5K}-FVE₂ micelles. PTX-loaded PEG_{5K}-FVE₂ micelles demonstrated significantly higher tumor inhibitory effect compared with both Taxol and PEG_{5K}-VE₂ formulations at the same PTX concentration. The superior antitumor efficacy of PTX-loaded PEG_{5K}-FVE₂ micelles compared with PEG_{5K}-VE₂ micelles can be attributed to the enhanced tumor accumulation of PTX (Fig. 6). To better understand the mechanism for the enhanced tumor uptake of PEG_{5K}-FVE₂ micelles, we observed the morphology of blank and PTX-loaded micelles with transmission electron microscopy (Fig. S3). In contrast to the PEG_{5K}-VE₂ micelles that form typical spherical structures, PEG_{5K}-FVE₂ micelles showed predominantly elongated, worm-like morphology (Fig. S3). This might be attributed to the strong intermolecular interaction among the PEG_{5K}-FVE₂ molecules mediated by Fmoc moieties. Fmoc is known to promote parallel interactions of individual short peptides carrying the same group, which often leads to the formation of elongated nanoassemblies (15). It has been reported that drug carriers with worm-like shape, also known as filomicelles, show longer circulation time compared with spherical nanoparticles composed of the similar materials, leading to improved drug accumulation in tumors (32). More studies are ongoing in our lab to better understand the underlying mechanism for the improved performance of PEG_{5K}-FVE₂ micelles.

Besides measuring the mouse body weight as well as the serum levels of AST and ALT, we have observed that mice treated with Taxol frequently showed transient apathy (33) and decreased overall activity over 10-min postinjection. This is likely caused by Cremophor EL and ethanol in the Taxol formulation. In contrast, there was no noticeable change in activity of mice after injection of PTX-loaded PEG_{5K}-FVE₂ micelles, suggesting the minimal toxicity of PTX-loaded PEG_{5K}-FVE₂ *in vivo*.

CONCLUSIONS

In summary, we have validated and extended our previous study on the significance of introducing a drug-interactive motif at the interfacial region of conventional micellar system. With an improved TPGS-based micellar

carrier, PEG_{5K}-FVE₂, our data clearly showed that incorporation of an Fmoc drug-interactive motif not only enhanced the drug loading capacity of PTX, but also significantly improved the tumor accumulation and therapeutic efficacy. These effects are likely attributed to the high compatibility and interaction between PTX and Fmoc moiety. Our strategy may represent an effective and general approach to improve the performance of various delivery systems including micelles.

ACKNOWLEDGMENTS

This work was supported in part by NIH grants (R01GM102989 and R21CA155983) and a DOD grant (BC09603).

REFERENCES

- Horwitz SB. Taxol (paclitaxel): mechanisms of action. *Ann Oncol.* 1994;5 Suppl 6:S3-6.
- Soga O, van Nostrum CF, Fens M, Rijcken CJ, Schifflers RM, Storm G, *et al.* Thermosensitive and biodegradable polymeric micelles for paclitaxel delivery. *J Control Release.* 2005;103:341-53.
- Gao Z, Lukyanov AN, Singhal A, Torchilin VP. Diacyllipid-polymer micelles as nanocarriers for poorly soluble anticancer drugs. *Nano Lett.* 2002;2:979-82.
- Kato K, Chin K, Yoshikawa T, Yamaguchi K, Tsuji Y, Esaki T, *et al.* Phase II study of NK105, a paclitaxel-incorporating micellar nanoparticle, for previously treated advanced or recurrent gastric cancer. *Invest New Drugs.* 2012;30:1621-7.
- Kim DW, Kim SY, Kim HK, Kim SW, Shin SW, Kim JS, *et al.* Multicenter phase II trial of Genexol-PM, a novel Cremophor-free, polymeric micelle formulation of paclitaxel, with cisplatin in patients with advanced non-small-cell lung cancer. *Ann Oncol.* 2007;18:2009-14.
- Yu L, Bridgers A, Polli J, Vickers A, Long S, Roy A, *et al.* Vitamin E-TPGS increases absorption flux of an HIV protease inhibitor by enhancing its solubility and permeability. *Pharm Res.* 1999;16:1812-7.
- Win KY, Feng SS. In vitro and in vivo studies on vitamin E TPGS-emulsified poly(D, L-lactic-co-glycolic acid) nanoparticles for paclitaxel formulation. *Biomaterials.* 2006;27:2285-91.
- Muthu MS, Kulkarni SA, Xiong JQ, Feng SS. Vitamin E TPGS coated liposomes enhanced cellular uptake and cytotoxicity of docetaxel in brain cancer cells. *Int J Pharm.* 2011;421:332-40.
- Cao N, Feng SS. Doxorubicin conjugated to D-alpha-tocopheryl polyethylene glycol 1000 succinate (TPGS): conjugation chemistry, characterization, in vitro and in vivo evaluation. *Biomaterials.* 2008;29:3856-65.
- Dintaman JM, Silverman JA. Inhibition of P-glycoprotein by D-alpha-tocopheryl polyethylene glycol 1000 succinate (TPGS). *Pharm Res.* 1999;16:1550-6.
- Collnot EM, Baldes C, Schaefer UF, Edgar KJ, Wempe MF, Lehr CM. Vitamin E TPGS P-glycoprotein inhibition mechanism: influence on conformational flexibility, intracellular ATP levels, and role of time and site of access. *Mol Pharm.* 2010;7:642-51.
- Mi Y, Liu YT, Feng SS. Formulation of Docetaxel by folic acid-conjugated D-alpha-tocopheryl polyethylene glycol succinate 2000 (Vitamin E TPGS(2 k)) micelles for targeted and synergistic chemotherapy. *Biomaterials.* 2011;32:4058-66.
- Wang J, Sun J, Chen Q, Gao Y, Li L, Li H, *et al.* Star-shape copolymer of lysine-linked di-tocopherol polyethylene glycol 2000 succinate for doxorubicin delivery with reversal of multi-drug resistance. *Biomaterials.* 2012;33:6877-88.
- Lu J, Huang Y, Zhao W, Chen Y, Li J, Gao X, *et al.* Design and characterization of PEG-Derivatized Vitamin E as a

- nanomicellar formulation for delivery of paclitaxel. *Mol Pharm*. 2013;10:2880–90.
15. Gao X, Huang Y, Makhov AM, Epperly M, Lu J, Grab S, *et al*. Nanoassembly of surfactants with interfacial drug-interactive motifs as tailor-designed drug carriers. *Mol Pharm*. 2013;10:187–98.
 16. Lu J, Huang Y, Zhao W, Marquez RT, Meng X, Li J, *et al*. PEG-derivatized embelin as a nanomicellar carrier for delivery of paclitaxel to breast and prostate cancers. *Biomaterials*. 2013;34:1591–600.
 17. Zhang P, Lu J, Huang Y, Zhao W, Zhang Y, Zhang X, *et al*. Design and evaluation of a PEGylated lipopeptide equipped with drug-interactive motifs as an improved drug carrier. *AAPS J*. 2014;16:114–24.
 18. Sezgin Z, Yuksel N, Baykara T. Preparation and characterization of polymeric micelles for solubilization of poorly soluble anticancer drugs. *Eur J Pharm Biopharm*. 2006;64:261–8.
 19. Savic R, Luo L, Eisenberg A, Maysinger D. Micellar nanocontainers distribute to defined cytoplasmic organelles. *Science*. 2003;300:615–8.
 20. Li X, Yang Z, Yang K, Zhou Y, Chen X, Zhang Y, *et al*. Self-assembled polymeric micellar nanoparticles as nanocarriers for poorly soluble anticancer drug etaselen. *Nanoscale Res Lett*. 2009;4:1502–11.
 21. Latere Dwan'Isa JP, Rouxhet L, Préat V, Brewster ME, Ariën A. Prediction of drug solubility in amphiphilic di-block copolymer micelles: the role of polymer-drug compatibility. *Pharmazie*. 2007;62:499–504.
 22. Kim SH, Tan JP, Nederberg F, Fukushima K, Colson J, Yang C, *et al*. Hydrogen bonding-enhanced micelle assemblies for drug delivery. *Biomaterials*. 2010;31:8063–71.
 23. Yang C, Attia AB, Tan JP, Ke X, Gao S, Hedrick JL, *et al*. The role of non-covalent interactions in anticancer drug loading and kinetic stability of polymeric micelles. *Biomaterials*. 2012;33:2971–9.
 24. Giacomelli C, Schmidt V, Borsali R. Specific interactions improve the loading capacity of block copolymer micelles in aqueous media. *Langmuir*. 2007;23:6947–55.
 25. Yokoyama M, Okano T, Sakurai Y, Suwa S, Kataoka K. Introduction of cisplatin into polymeric micelle. *J Control Release*. 1996;39:351–6.
 26. Nishiyama N, Kato Y, Sugiyama Y, Kataoka K. Cisplatin-loaded polymer-metal complex micelle with time-modulated decaying property as a novel drug delivery system. *Pharm Res*. 2001;18:1035–41.
 27. Huh KM, Lee SC, Cho YW, Lee J, Jeong JH, Park K. Hydrotropic polymer micelle system for delivery of paclitaxel. *J Control Release*. 2005;101:59–68.
 28. Kim JY, Kim S, Pinal R, Park K. Hydrotropic polymer micelles as versatile vehicles for delivery of poorly water-soluble drugs. *J Control Release*. 2011;152:13–20.
 29. Opanasopit P, Yokoyama M, Watanabe M, Kawano K, Maitani Y, Okano T. Block copolymer design for camptothecin incorporation into polymeric micelles for passive tumor targeting. *Pharm Res*. 2004;21:2001–8.
 30. Carstens MG, de Jong PH, van Nostrum CF, Kemmink J, Verrijck R, de Leede LG, *et al*. The effect of core composition in biodegradable oligomeric micelles as taxane formulations. *Eur J Pharm Biopharm*. 2008;68:596–606.
 31. Shirai K, Matsuoka M, Fukunishi K. Fluorescence quenching by intermolecular π - π interactions of 2,5-bis(N, N-dialkylamino)-3,6-dicyanopyrazines. *Dyes Pigments*. 1999;42:95–101.
 32. Geng Y, Dalhaimer P, Cai S, Tsai R, Tewari M, Minko T, *et al*. Shape effects of filaments versus spherical particles in flow and drug delivery. *Nat Nanotechnol*. 2007;2:249–55.
 33. Danhier F, Magotteaux N, Ucakar B, Lecouturier N, Brewster M, Preat V. Novel self-assembling PEG-p-(CL-co-TMC) polymeric micelles as safe and effective delivery system for paclitaxel. *Eur J Pharm Biopharm*. 2009;73:230–8.