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Design and Characterization of PEG-Derivatized Vitamin E as a Nanomicellar Formulation for Delivery of Paclitaxel

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Abstract

Various PEG-Vitamin E conjugates including D-alpha-tocopheryl polyethylene glycol succinate 1000 (TPGS) have been extensively studied as a nonionic surfactant in various drug delivery systems. However, limited information is available about the structure-activity relationship of PEG-Vitamin E conjugates as a micellar formulation for paclitaxel (PTX). In this study, four PEG-Vitamin E conjugates were developed that vary in the molecular weight of PEG (PEG_{2K} vs PEG_{5K}) and the molar ratio of PEG/Vitamin E (1/1 vs 1/2) in the conjugates. These conjugates were systematically characterized with respect to CMC, PTX loading efficiency, stability, and their efficiency in delivery of PTX to tumor cells *in vitro* and *in vivo*. Our data show that PEG_{5K}-conjugates have lower CMC values and are more effective in PTX loading with respect to both loading capacity and stability. The conjugates with two Vitamin E molecules also worked better than the conjugates with one molecule of Vitamin E, particularly for PEG_{2K}-system. Furthermore, all of the PEG-Vitamin E conjugates can inhibit P-gp function with their activity being comparable to that of TPGS. More importantly, PTX-loaded PEG_{5K}-VE_2 resulted in significantly improved tumor growth inhibitory effect in comparison to PTX formulated in PEG_{2K}-VE or PEG_{2K}-VE_2, as well as Cremophor EL (Taxol) in a syngeneic mouse model of breast cancer (4T1.2). Our study suggests that PEG_{5K}-Vitmin E_2 may hold promise as an improved micellar formulation for in vivo delivery of anticancer agents such as PTX.

Keywords

Nanomicelles; Paclitaxel; TPGS; Controlled and sustained drug delivery; Cancer; Nanotechnology

INTRODUCTION

The poor clinical efficacy and the associated severe side effects of conventional chemotherapy in cancer treatment have stimulated the development of novel and effective drug delivery systems. Recently, increasing efforts have been placed on the development of nanotechnology-based drug delivery platforms. Polymeric micelles, liposomes, dendrimers

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Supporting Information Available: HPLC trace, ^1H NMR spectra and MALDI-TOF of PEG_{2K}-VE, PEG_{2K}-VE_2, PEG_{5K}-VE and PEG_{5K}-VE_2. Size distribution and TEM of PEG_{2K}-VE, PEG_{2K}-VE_2 and PEG_{5K}-VE. These materials are available free of charge via the Internet at http://pubs.acs.org.

and nanoparticles of biodegradable polymers have been extensively studied as delivery systems to improve cancer treatment.[1] Among the many studied delivery systems, polymeric micelles have drawn considerable attention as a versatile nanotherapeutic platform, owing to ease of preparation, good biocompatibility, and relatively high efficiency in drug delivery.[2,3] It is well known that polymeric micelles can improve the aqueous solubility of poorly water-soluble chemotherapeutic agents by packing them in the hydrophobic core of the micelles. Besides, the blood circulation times of drug-loaded micelles can be significantly prolonged due to the steric hindrance imposed by the presence of the long hydrophilic PEG shell.[2,3] Furthermore, compared to other delivery systems, micelles are highly effective in passive tumor targeting through the leaky vasculature via enhanced permeability and retention effect (EPR) because of their extremely small sizes ranging from 10 to 100 nm, resulting in favorable biodistribution and improved therapeutic index.[4,5] Nevertheless, most polymeric micellar formulations employ “inert” excipients that not only lack therapeutic activity, but also potentially impose safety concern.[6]

D-alpha-tocopheryl polyethylene glycol succinate 1000 (TPGS) is a hydrophilic derivative of natural Vitamin E, which is generated via coupling of polyethylene glycol (PEG) to Vitamin E succinate via an ester linkage.[7] Over the last decade, TPGS has been intensively studied in various types of delivery systems: TPGS has been used as an effective emulsifier, solubilizer, additive, permeability enhancer as well as absorption enhancer.[8,9] As an inhibitor of P-gp, TPGS has also been utilized as an excipient to overcome multidrug resistance (MDR) and improve the bioavailability of anticancer drugs.[9–12] Examples of TPGS application in nanomedicine platform include TPGS-emulsified PLGA nanoparticles, nanoparticles of TPGS-based copolymers, and TPGS-based micelles, liposomes, and prodrugs.[3,13–19] In addition, several new derivatives of improved performance have been reported including $TPGS_{5K}$, $TPGS_{2K}$, and PEG_{2K}-Vitamin E_2 conjugate.[3,15,19], However, the optimal structure of PEG-Vitamin E conjugates as a micellar delivery system remains incompletely understood.

We have recently developed a PEG-derivatized embelin-based micellar system that is suitable for delivery of poorly water-soluble drugs such as PTX.[20] Structurally, PEG-embelin conjugate is very similar to TPGS. Embelin has various biological activities including anti-inflammatory, anti-diabetic, and hepatoprotective effect.[21–23] Embelin also has antitumor activity and synergizes with other anticancer agents through blocking the activity of X-linked inhibitor of apoptosis protein (XIAP).[20,21,24–27] Thus, similar to TPGS, PEG-embelin also functions as a dual functional system for delivery of anticancer agents but with different mechanism of action.[20] Optimization of PEG-embelin system has shown that a conjugate with two embelin molecules coupled to PEG is significantly more effective than the conjugate with a 1: 1 molar ratio of PEG and embelin.[28] In addition, the embelin conjugates with PEG_{5K} worked better than the $PEG_{3.5K}$ conjugates.[28] This has prompted us to conduct similar study with TPGS micellar system. We have developed four PEG-Vitamin E conjugates that vary in the molecular weight of PEG (PEG_{2K} vs PEG_{5K}) and the molar ratio of PEG/Vitamin E (1/1 vs 1/2) in the conjugates. Our data show that PEG_{5K}-conjugates have lower CMC values and are more effective in PTX loading with respect to both loading capacity and stability. The conjugates with two Vitamin E molecules also worked better than the conjugates with one molecule of Vitamin E, particularly for PEG_{2K}-system. All of the four PEG-Vitamin E conjugates showed the P-gp inhibition activity with their efficiency being comparable to that of TPGS. More importantly, PTX-loaded PEG_{5K}-VE_2 resulted in significantly improved tumor growth inhibitory effect in comparison to PTX formulated in PEG_{2K}-VE or PEG_{2K}-VE_2, as well as Cremophor EL (Taxol) in a syngeneic mouse model of breast cancer (4T1.2).

EXPERIMENTAL SECTION

Materials

Paclitaxel (98%) was purchased from AK Scientific Inc. (CA, USA). Dulbecco's phosphate buffered saline (DPBS) was purchased from Lonza (MD, USA). Methoxy-$PEG_{2,000}$-OH, Methoxy-$PEG_{5,000}$-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) and succinate anhydride were all purchased from Sigma-Aldrich (MO, USA). Fetal bovine serum (FBS) and penicillin-streptomycin solution were from Invitrogen (NY, USA). D-alpha-tocopheryl was purchased from Tokyo Chemical Industry (OR, USA). DCC was purchased from Alfa Aesar (MA, USA). DMAP was purchased from Calbiochem-Novabiochem Corporation (CA, USA). All solvents used in this study were HPLC grade.

Synthesis of PEG_{2K}-VE, PEG_{2K}-VE_2, PEG_{5K}-VE and PEG_{5K}-VE_2

PEG_{5K}-VE_2 was synthesized via solution phase condensation reactions from MeO-PEG-OH with a molecular weight of 5000 Da. (Boc)lysine(Boc)-OH (2 equ.) was coupled onto the terminal-OH of PEG using DCC (2 equ.) and DMAP (0.1 equ.) as coupling reagents in DCM overnight. Di-Boc lysyl-PEG_{5K} ester was precipitated and washed three times with cold ethanol and ether, respectively. Then, Boc groups were removed via treatment with 50% trifluoroacetic acid in DCM, and the lysyl-PEG_{5K} ester was precipitated and washed three times by cold ethanol and ether, respectively. White powder precipitate was dried under vacuum. Vitamin E succinate was coupled to the deprotected amino groups of lysine with the assistance of DCC (2 equ.) and DMAP (0.1 equ.), resulting in PEG_{5K}-VE_2. This compound was subsequently dialyzed against water and lyophilized to yield a white powder. PEG_{2K}-VE_2 was similarly synthesized as PEG_{5K}-VE_2. PEG_{2K}-VE ($TPGS_{2K}$) and PEG_{5K}-VE ($TPGS_{5K}$) were synthesized following the literature.[3]

Preparation and characterization of free or PTX-loaded micelles

PTX-solubilized micelles were prepared by the following method. PTX (10 mM in chloroform) was added to different PEG-Vitamin E conjugates (10 mM in chloroform), respectively, with various carrier/drug molar ratios. The organic solvent was first removed by steady nitrogen flow to form a thin dry film of drug/carrier mixture. The film was further dried under high vacuum for 2 h to remove any traces of remaining solvent. Drug-loaded micelles were formed by suspending the film in DPBS. The drug-free micelles were similarly prepared as described above. The mean diameter of four different micelles with or without loaded drug was assessed by dynamic light scattering (DLS). The morphology and size distribution of PEG_{2K}-VE, PEG_{2K}-VE_2, PEG_{5K}-VE and PEG_{5K}-VE_2 micelles were observed, respectively, using transmission electron microscopy (TEM) after negative staining. The concentration of PTX in PTX-loaded micelles was evaluated by HPLC as described previously.[20] The drug loading capacity (DLC) and drug loading efficiency (DLE) were calculated according to the following formula:

$$\text{DLC (\%)} = [\text{weight of drug used}/(\text{weight of polymer} + \text{drug used})] \times 100\%$$

$$\text{DLE (\%)} = (\text{weight of loaded drug}/\text{weight of input drug}) \times 100\%$$

Stability study of micelles

A series of PTX-loaded micelles with different carrier/PTX molar ratios were prepared as described above and the PTX concentration in all samples was kept at 1 mg/mL. The sizes of samples were measured at different time points following the sample preparation. To examine the effect of serum on the particle stability, the samples were mixed with serum

(FBS) at a final serum concentration of 50%. Size changes were monitored by DLS and measurement was terminated when the change of size reached significant difference.

Determination of the critical micelle concentration (CMC)

The CMCs of four different micelles were determined by employing pyrene as a fluorescence probe.[29] A drug-free micelle solution in DPBS (2.5 mg/mL) was prepared via solvent evaporation method. A series of 2-fold dilutions was then made for PEG_{2K}-VE, PEG_{2K}-VE_2, PEG_{5K}-VE and PEG_{5K}-VE_2 micelles, with concentrations ranging from 2×10^{-4} to 0.5mg/mL. At the same time, aliquots of 50 μL of 4.8×10^{-6} M pyrene in chloroform were added into separate vials. The chloroform was first removed by nitrogen flow to form a thin film. The film was further dried under high vacuum for 2 h to remove any traces of remaining solvent. Then, the pre-prepared micelle solutions (400 μL in DPBS) of varying concentrations were added to the pyrene film to obtain a final pyrene concentration of 6×10^{-7} M in each vial. The solutions were kept on a shaker at 37 °C for 24 h to reach equilibrium before fluorescence measurement. The fluorescence intensity of samples was measured at the excitation wavelength of 334 nm and emission wavelength of 390 nm by Synergy H1 Hybrid Multi-Mode Microplate Reader (Winooski, VT). The CMC is determined from the threshold concentration, where the sharp increase in pyrene fluorescence intensity is observed.

In vitro drug release study

An *in vitro* drug release study was carried out by dialysis using DPBS (PH = 7.4) containing 0.5% (w/v) Tween 80 as the release medium. Two mL of PTX-loaded micelles (PEG_{2K}-VE, PEG_{2K}-VE_2, PEG_{5K}-VE or PEG_{5K}-VE_2) (1 mg PTX/mL) were sealed in dialysis tubes (MWCO = 12 KDa, Spectrum Laboratories) which were then immersed in 200 mL release medium in a beaker covered with parafilm. The beakers were placed in an incubator shaker at 100 rpm and 37°C. The concentration of PTX remaining in the dialysis tubes at various time points was measured by HPLC with the detector set at 227 nm. Values were reported as the means from triplicate samples.

Cell culture

DU145 and PC-3 are two androgen-independent human prostate cancer cell lines. 4T1.2 is a mouse metastatic breast cancer cell line. MCF-7 and MDA-MB-231 are human breast cancer cell lines. NCI/ADR-RES is Adriamycin (ADR)-resistant cell line. All cell lines were cultured in DMEM containing 10% FBS and 1% penicillin-streptomycin in a humidified environment at 37 °C with 5% CO_2.

Hemolytic effect of micelles

Fresh blood samples were collected through cardiac puncture from rats. Heparin was immediately added into 10 mL of blood to prevent coagulation. Red blood cells (RBCs) were separated from plasma by centrifugation at 1500 rpm for 10 min at 4 °C. RBCs were washed three times with 30 mL ice-cold DPBS. RBCs were then diluted to 2% w/v with ice-cold DPBS and utilized immediately for the hemolysis assay. One mL of diluted RBC suspension was treated with various concentrations (0.0001, 0.001, 0.01, 0.1 and 1.0 mg/mL) of PEG_{2K}-VE, PEG_{2K}-VE_2, PEG_{5K}-VE or PEG_{5K}-VE_2 micelles, and PEI, respectively, and then incubated at 37 °C in an incubator shaker for 4 h. The samples were centrifuged at 1500 rpm for 10 min at 4 °C, and 100 μL of supernatant from each sample was transferred into a 96-well plate. The release of hemoglobin was determined by the absorbance at 540 nm using a microplate reader. RBCs treated with Triton X-100 (2%) and DPBS were considered as the positive and negative controls, respectively. Hemoglobin release was calculated as $(OD_{sample}\text{-}OD_{negative\ control})/(OD_{positive\ control}\text{-}OD_{negative\ control}) \times 100\%$

In vitro cytotoxicity study

The cytotoxicity of PTX formulated in PEG_{2K}-VE, PEG_{2K}-VE_2, PEG_{5K}-VE or PEG_{5K}-VE_2 micelles was assessed with two cancer cell lines (4T1.2 and NCI/ADR-RES) and compared to Taxol formulation. Briefly, 4T1.2 (1000 cells/well) or NCI/ADR-RES (3000 cells/well) cells were seeded in 96-well plates followed by 24 h of incubation in DMEM with 10% FBS and 1% streptomycin-penicillin. Various dilutions of PTX-loaded PEG_{2K}-VE, PEG_{2K}-VE_2, PEG_{5K}-VE or PEG_{5K}-VE_2 micelles, and Taxol (at the equal concentrations of PTX) were added to cells. Cells were incubated for 72 h and cell viability was assessed by MTT assay as described previously.[20] The cytotoxicity of PEG_{2K}-VE, PEG_{2K}-VE_2, PEG_{5K}-VE or PEG_{5K}-VE_2 micelles alone was similarly tested in 4T1.2 (1000 cells/well), NCI/ADR-RES (3000 cells/well), MCF-7 (5000 cells/well), MDA-MB-231 (2000 cells/well) and PC-3 (5000 cells/well) cells as described above.

P-gp ATPase assay

The modulation of P-gp ATPase activity by PEG-derivatized Vitamin E conjugates was conducted by using P-gp-Glo™ assay system (Promega, USA). This assay system provides the necessary reagents for performing luminescent P-gp ATPase assay. Compounds that interact with P-gp can be identified as stimulator or inhibitor of the ATPase activity. The P-gp-Glo™ assay detects the effects of compounds on recombinant human P-gp in a cell membrane fraction. Essentially, the assay relies on an ATP-dependent light-generating reaction of firefly luciferase. The effect of PEG-derivatized Vitamin E conjugates on P-gp ATPase activity was evaluated on a verapamil-stimulated ATPase activity. In this assay, sodium orthovanadate (Na_3VO_4) was employed as a selective inhibitor of P-gp. First, test samples containing verapamil (50μM) and PEG-derivatized Vitamin E conjugates (final concentrations at 10 and 100 μM, respectively) or Na_3VO_4 were added to 96-well plates and incubated with P-gp membrane for 5 min at 37 °C. Then, the reaction was initiated by the addition of MgATP followed by another 40 minutes' incubation at 37°C. Afterwards, the samples were removed from 37°C incubator and then ATP detection reagent was added in order to develop the luminescence. Signals were measured 20 minutes later on a plate reading luminometer (Victor^2 1420 multilabel counter). The changes of relative light unit (ΔRLU) were determined as follows: ΔRLU = (luminescence of Na_3VO_4-treated group) – (luminescence of the samples treated by the mixture of verapamil and PEG-derivatized Vitamin E conjugates).

Animals

Female BALB/c mice, 10–12 weeks were purchased from Charles River (Davis, CA). 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.

In vivo therapeutic study

A syngeneic murine breast cancer model (4T1.2) was used to examine the therapeutic effect of PTX formulated in PEG_{2K}-VE, PEG_{2K}-VE_2, PEG_{5K}-VE or PEG_{5K}-VE_2 micelles, and Taxol. 2×10^5 4T1.2 cells in 200 μL PBS were inoculated s.c. at the right flank of female BALB/c mice. Treatments were initiated when tumors in the mice reached a tumor volume around 50 mm^3 and this day was designated as day 1. On day 1, mice were randomly divided into six groups (n=5) and received i.v. administration of PTX formulated in PEG_{2K}-VE, PEG_{2K}-VE_2, PEG_{5K}-VE or PEG_{5K}-VE_2 micelles, as well as Taxol (10 mg PTX/kg), respectively on days 1, 3, 5, 9, and 12, while control mice received saline. Tumor sizes were measured with digital caliper on days 1, 3, 5, 9, 12, 15 and 18, and calculated according to

the following formula: $(L \times W^2)/2$, where L is the longest and W is the shortest in tumor diameters (mm). 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 first treatment). Mice were sacrificed when tumor reached 2000 mm^3 or developed ulceration. To monitor the potential toxicity, the body weights of all mice from different groups were measured on days 1, 3, 5, 9, 12, 15 and 18.

Statistical analysis

In all statistical analysis, the significance level was set at a probability of $P < 0.05$. All results were reported as the mean ± standard deviation (SD) unless otherwise indicated. Statistical analysis was performed by Student's t-test for two groups, and one-way ANOVA for multiple groups.

RESULTS

Synthesis of PEG_{2K}-VE, PEG_{2K}-VE_2, PEG_{5K}-VE or PEG_{5K}-VE_2 conjugates

Lysine-linked di-tocopherol polyethylene glycol 5000 succinate was synthesized via solution phase reaction with two Vitamin E succinates attached to mPEG-5000 through the linker of lysine. The synthetic scheme is presented in Fig.1.

Initially, (Boc)lysine(Boc)-OH was coupled onto the terminal −OH of PEG using DCC and DMAP as coupling reagents in DCM. Boc groups were removed by the treatment with 50% trifluoroacetic acid in DCM. Vitamin E succinate was coupled to the amino groups of lysine, yielding PEG_{5K}-VE_2. The structure of PEG_{5K}-VE_2 was confirmed by ^1H NMR in CDCl3 (Fig.S1A).The intense peak at 3.66 ppm was assigned to the methane protons of the polyethylene glycol. The proton peaks below 3.0 ppm were ascribed to the section of Vitamin E succinate.

MALDI-TOF suggested that two Vitamin E succinates were successfully attached to mPEG5000 with the linker of lysine. (Fig. S2A). PEG_{2K}-VE_2 was synthesized following the same synthesis route of PEG_{5K}-VE_2. PEG2K-VE and PEG_{5K}-VE were synthesized according to the literature.[3] Their structures were confirmed by ^1H NMR and MALDI-TOF as reported (Figs. S1 & S2). The purity for each conjugates was assessed via HPLC and shown in Fig. S3.

Size & size distribution of micelles

In aqueous solution, the four PEG-derivatized Vitamin E conjugates readily self-assemble to form micellar nanoparticles with the particle sizes of around 20 nm as determined by DLS analysis (Table.1).

Fig. 2A shows a single peak for PEG_{5K}-VE_2 micelles in size distribution. Negative EM staining revealed spherical particles of uniform size (Fig.2B).

The sizes of the micelles observed under TEM were quite consistent with those measured by DLS. Similar results were shown for the other three micelles (Figs. S4 & S5).

Critical micelle concentration (CMC)

Fig. 3 shows the CMC measurements of PEG_{2K}-VE, PEG_{2K}-VE_2, PEG_{5K}-VE, and PEG_{5K}-VE_2 micelles using pyrene as a fluorescence probe.

Upon incorporation into the micelles, the fluorescence intensity of pyrene increases substantially at the concentration of micelles above the CMC.[30] Based on the partition of the

pyrene, the CMC of micelles was obtained by plotting the fluorescence intensity versus logarithm concentration of the polymer. The CMCs of PEG-derivatized micelles were determined from the crossover point at the low concentration range. The CMCs of the PEG_{5K}-VE and PEG_{5K}-VE_2 conjugates are 0.58 μM and 0.30 μM, respectively, which are lower than those of PEG_{2K}-VE (3.56 μM) and PEG_{2K}-VE_2 (1.15 μM).

Drug loading efficiency (DLE)

DLE is one of the important parameters in drug delivery systems. The PTX loading efficiency of PEG_{2K}-VE, PEG_{2K}-VE_2, PEG_{5K}-VE, and PEG_{5K}-VE_2 micelles with different carrier to drug molar ratios was determined by HPLC (Table. 2). The sizes of micelles were also examined under corresponding conditions.

PEG_{5K}-VE and PEG_{5K}-VE_2 were comparable with respect to DLE at all carrier/drug ratios examined. Both effectively solubilized PTX in aqueous solution in a molar ratio as low as 0.5:1 with particle size remaining around 20 nm. However, these drug-loaded particles were only stable for less than 1 h. At a carrier/drug ratio of 7.5/1, they formed stable mixed micelles with PTX that were stable for about one day in DPBS. Increasing the carrier/drug ratio to 10:1 led to formation of particles that are stable over 65 h in DPBS. Essentially, all of the added PTX was incorporated into the micelles. In addition, the sizes of the particles remained the same following lyophilization and reconstitution with water (data not shown).

For PEG_{2K}-VE and PEG_{2K}-VE_2 micelles, a minimal carrier/drug ratio of 2.5/1 (m/m) was required to solubilize the drug. PEG_{2K}-VE_2 was more effective than PEG_{2K}-VE in solubilizing PTX with higher DLE at all carrier/drug ratios examined. At a carrier/drug ratio of 10/1, PTX-loaded PEG_{2K}-VE_2 micelles were significantly more stable than PTX formulated in PEG_{2K}-VE micelles (55.5 vs 4.2 h). In addition to evaluating the stability of PTX-loaded micelles in DPBS, their stability in 50% FBS over time was also examined. All of the formulations tested were less stable in serum than in DPBS. Addition of serum to PEG_{5K}-VE_2/PTX (10/1, m/m) mixed micelles resulted in an increase of the particle size from 19.6 nm to 31.7 nm, which stayed stable for 45 h. Again, PEG_{5K}-VE_2/PTX shows the best stability in serum among the 4 mixed micelles tested. Overall, the four conjugates were ranked in the order of PEG_{5K}-VE_2 > PEG_{5K}-VE > PEG_{2K}-VE_2 > PEG_{2K}-VE with respect to their efficiency in forming stable mixed micelles with PTX in both DPBS and 50% FBS.

In vitro PTX release kinetics

A dialysis method was used to evaluate the release kinetics of PTX from PEG_{2K}-VE, PEG_{2K}-VE_2, PEG_{5K}-VE or PEG_{5K}-VE_2 micelles with DPBS (PH = 7.4) containing 0.5% w/v Tween 80 as the release medium. As shown in Fig. 4, PTX formulated in PEG_{5K}-VE and PEG_{5K}-VE_2 micelles exhibited significantly better stability than PTX-loaded PEG_{2K}-VE and PEG_{2K}-VE_2 micelles.

For the first 7 h, there was no significant difference among the 4 micellar systems, during which a burst release due to the relatively high drug concentrations at the very beginning may account for this result. However, significant differences were observed among the 4 formulations during the remaining experimental period. The size of PEG significantly affects the release kinetics: the two conjugates with PEG_{5K} showed significantly slower release kinetics compared to the two conjugates with PEG_{2K}. In addition, the conjugates with two molecules of Vitamin E gave better stability than the PEG-VE conjugates of 1: 1 molar ratio, particularly for PEG_{2K} conjugates. Overall, the four conjugates were ranked in the order of PEG_{5K}-VE_2 > PEG_{5K}-VE > PEG_{2K}-VE_2 > PEG_{2K}-VE with respect to their stability in the release study.

Hemolytic effect of micelles

One concern for micellar systems is whether or not the surface activity of the surfactants affects cell membrane integrity. Therefore, free PEG_{2K}-VE, PEG_{2K}-VE_2, PEG_{5K}-VE and PEG_{5K}-VE_2 micelles were examined for the hemolytic activity and compared to polyethylenimine (PEI), a cationic polymer with potent cell surface activity. As shown in Fig. 5, treatment with PEI resulted in significant hemolysis in a dose-dependent manner. In contrast, only a very low level of hemolysis (~5%) was observed for all four blank micelles at the high doses (0.1 and 1 mg/mL) examined. The negligible hemolytic activity suggests that all of the 4 conjugates are mild surfactants that can be suitable for *in vivo* delivery of potent hydrophobic anticancer drugs.

In vitro cytotoxicity of free and PTX-loaded micelles

The cytotoxicity of carriers alone was examined in 4T1.2, NCI/ADR-RES, MCF-7, MDA-MB-231, and PC-3 cells, respectively. It was apparent that the single Vitamin E conjugates (PEG_{2K}-VE and PEG_{5K}-VE) showed significantly higher levels of cytotoxicity than those of double Vitamin E conjugates (PEG_{2K}-VE_2 and PEG_{5K}-VE_2) in all five cancer cell lines tested (Fig. 6).

In MDA-MB-231 cells, the IC50 for PEG_{2K}-VE_2 and PEG_{5K}-VE_2 is 6 and 4.8 times higher than their single Vitamin E counterparts (Table. 3). Similar results were shown for the other four cancer cell lines (Table. 3). It is also apparent that NCI/ADR-RES cells were more sensitive than the other four cancer cell lines to all of the conjugates (Fig. 6 & Table. 3). Again, the single Vitamin E conjugates showed more potent cytotoxicity than the double Vitamin E conjugates in this drug-resistant cell line (Fig. 6 & Table. 3).

Fig. 7A shows the *in vitro* cytotoxicity of PTX formulated in PEG_{2K}-VE, PEG_{2K}-VE_2, PEG_{5K}-VE and PEG_{5K}-VE_2 micelles in comparison with Taxol in 4T1.2 cancer cells. All of the four PTX mixed micelles were less active than Taxol in antitumor activity. Interestingly, different from the study of carriers alone in which single Vitamin E conjugates were more active, PTX-loaded PEG_{5K}-VE_2 micelles were more potent than PTX formulated in the other three micelle formulations. Similar to the study of carriers alone, NCI/ADR-RES tumor cells are also more sensitive than 4T1.2 cancer cells to PTX formulated in either PEG-Vitamin E micelles or Cremophor/ethanol (Taxol) (Fig. 7B).

Again, PTX-loaded PEG_{5K}-VE_2 micelles showed the highest level of in vitro cytotoxicity followed by PTX-loaded PEG_{5K}-VE micelles. PTX-PEG_{2K}-VE and PTX-PEG_{2K}-VE_2 are comparable in antitumor activity. However, all of the four PTX micellar formulations were more active than Taxol in NCI/ADR-RES tumor cells, which is quite different from the data in 4T1.2 cells. The IC_{50} of Taxol and several PTX-loaded micelles in the two cancer cell lines were summarized in Table. 4.

Inhibition of P-gp ATPase

Fig. 8 shows that P-gp ATPase activity was significantly inhibited by TPGS in a concentration-dependent manner. The P-gp ATPase activity was also significantly inhibited by the four PEG-Vitamin E conjugates although they were less active compared to TPGS, which was consistent with the published work in the literature[31].

In vivo therapeutic study

The *in vivo* therapeutic activity of PTX formulated in PEG_{2K}-VE, PEG_{2K}-VE_2, and PEG_{5K}-VE_2 micelles was evaluated in a syngeneic murine breast cancer model (4T1.2), and compared to Taxol.

4T1.2 is a highly metastatic breast cancer cell line and was selected to rigorously assess the *in vivo* therapeutic efficacy of different PTX formulations. As shown in Fig. 9A, Taxol formulation showed moderate effect in inhibiting the tumor growth at a dose of 10 mg PTX/kg. Compared to Taxol treatment group, PTX formulated in PEG_{2K}-VE or PEG_{2K}-VE_2 exhibited similar tumor growth inhibitory effect. In contrast, PTX formulated in PEG_{5K}-VE_2 micelles showed a significantly more pronounced antitumor activity at the same dosage. No significant changes in body weight were noticed in all treatment groups compared to PBS control group (Fig. 9B), suggesting that significant therapeutic effect can be achieved with minimal toxicity.

DISCUSSION

We have systematically compared the biophysical property and *in vitro* and *in vivo* efficiency of PTX delivery of four PEG-Vitamin E conjugates that differ in the size of PEG motif (PEG_{2K} vs PEG_{5K}) and the molar ratio of PEG/Vitamin E (1/1 vs 1/2) in the conjugates. Our data showed that PEG_{5K}-conjugates were significantly more effective than PEG_{2K}-conjugates in forming stable mixed micelles with PTX and in mediating delivery of PTX to tumor cells, particularly *in vivo*. In addition, conjugates with two Vitamin E molecules work better than the conjugates with one molecule of Vitamin E.

It is likely that various mechanisms are involved in the carrier/drug interaction for the Vitamin E-based micellar system. Vitamin E has a benzene ring and a long alkyl chain. In addition to hydrophobic interaction with PTX, the hydrogen bonding and the π-π stacking may also contribute to the overall carrier/PTX interaction. The close proximity of two Vitamin E molecules in PEG-VE_2 conjugates is likely to facilitate the formation of a binding pocket that enhances the interaction between the carriers and PTX. This is supported by data from our recent work that inclusion of a drug-interactive motif at the interfacial region of surfactants significantly improves the carrier-drug interaction, leading to improvement in both drug-loading capacity and formulation stability[32]. Recently, Wang and colleagues reported a similar work in which they showed that PEG_{2K}-Vitamin E_2 conjugate was more effective than PEG_{2K}-Vitamin E in mediating delivery of doxorubicin to tumors.[15] In an independent study with two similar delivery systems based on PEG-embelin and PEG-farnesylthiosalicylic acid (FTS) conjugates, we also showed that conjugates with two embelin or FTS molecules were more effective than conjugates with one embelin or FTS molecule regardless whether $PEG_{3.5K}$ or PEG_{5K} was used.[20,27,34]

As a hydrophilic motif of amphiphilic molecules, the size of PEG also critically affects the performance of the micelles. PEG provides steric hindrance, which is critical for ensuring long circulation property of the micelles. PEG decoration has also been shown to facilitate the penetration of nanoparticles through the mucus layer[19,35] In this regard, PEGs of higher MW are expected to be more effective than those of lower MW. However, the size of PEG also affects the CMC which in turn significantly affects the performance of the micelles, particularly *in vivo*. Different micellar systems appear to be differentially affected by the size of PEG.[3,20,28,33] In a systematic study on the SAR of PEG-cholic acid cluster-based micellar system, PEG_{2K} was shown to be the optimal hydrophilic motif.[33] Our data clearly showed that PEG_{5K}-conjugates (with either one or two Vitamin E molecules) were more active than PEG_{2K}-conjugates in forming stable mixed micelles with PTX. PEG_{5K}-conjugates formed stable complexes with PTX at lower carrier/PTX molar ratios compared to PEG_{2K}-conjugates. In addition, PTX formulated in PEG_{5K}-micelles displayed much slower release kinetics. We have similarly demonstrated the advantages of PEG_{5K} over $PEG_{3.5K}$ in PEG-embelin and PEG-FTS micellar systems.[20,28,34] In the study by Hanes and colleagues, PLGA particles coated with $TPGS_{5K}$ were more effective than the particles decorated with $TPGS_{1K}$ in penetrating human cervicovaginal mucus[19].

The four different PEG-Vitamin E conjugates showed varied levels of activity by themselves in four cancer cell lines. Overall, the single Vitamin E conjugates were more potent than the conjugates with two Vitamin E molecules in all cell lines tested. The more potent activity of single Vitamin E conjugates is unlikely due to the more active surface activity of the single chain conjugates as all of the four conjugates showed minimal hemolytic activity at much higher concentrations tested. It is possible that active Vitamin E is more readily released from the single Vitamin E conjugates than the ones with two Vitamin E molecules due to less steric hindrance to intracellular esterases. More studies are needed in the future to examine if the single Vitamin E conjugates indeed yield greater amounts of active free Vitamin E intracellularly.

Different from the cytotoxicity profiles of the conjugates alone, PTX formulated in PEG_{5K}-VE_2 micelles showed higher levels of cytotoxicity than PTX formulated in other three micellar systems in both 4T1.2 and NCI/ADR-RES tumor cell lines. This might be attributed to a more efficient intracellular delivery of PTX via PEG_{5K}-VE_2 micelles as PEG_{5K}-VE_2 formed the most stable mixed micelles with PTX among the four micellar systems tested. Despite the difference in the levels of cytotoxicity among the four types of micellar PTX, all of them were less active than Taxol formulation in 4T1.2 tumor cells. Interestingly we saw a reversal of the pattern in NCI/ADR-RES tumor cells: all of the four micellar PTX were more active than Taxol in this drug resistant cell line. The improved *in vitro* cytotoxicity of the PTX micellar formulations in this drug resistant cell line can be ascribed to the well-known inhibitory effect of P-gp efflux pump by Vitamin E derivatives and thus an improved bioavailability of PTX inside the tumor cells[9–12]. This hypothesis was supported by the P-gp ATPase activity assay in this work (Fig. 8). Our results were consistent with previous studies with various types of delivery system that involve the use of TPGS.[9–12]

In vivo therapy study clearly showed a significantly higher level of antitumor activity for PTX formulated in PEG_{5K}-VE_2 micelles compared to either Taxol or other two micellar formulations. This is likely due to the significantly improved loading capacity and stability for PEG_{5K}-VE_2 micelles, which shall lead to more effective delivery of PTX to tumor tissue *in vivo*. No significant difference was noticed between PEG_{2K}-Vitamin E_2 and PEG_{2K}-Vitamin E in antitumor activity despite the demonstrated advantages of PEG_{2K}-Vitamin E_2 over PEG_{2K}-Vitamin E in biophysical property. This might be due to the aggressive nature of 4T1.2 tumor model, which requires significant improvement of the formulation to achieve a significant gain in the therapeutic benefit.

CONCLUSION

In summary, we have shown that PEG_{5K}-conjugates have lower CMC values and are more effective in PTX loading with respect to both loading capacity and stability. The conjugates with two Vitamin E molecules also worked better than the conjugates with one molecule of Vitamin E. All of the four PEG-Vitamin E were comparable to TPGS in inhibiting the function of P-gp. More importantly, PTX-loaded PEG_{5K}-VE_2 resulted in significantly improved tumor growth inhibitory effect in comparison to PTX formulated in PEG_{2K}-VE or PEG_{2K}-VE_2, as well as Taxol in a syngeneic mouse model of breast cancer (4T1.2). More studies on the SAR of the Vitamin E-based micellar system and the mechanism of antitumor effect may lead to further improvement in this system for *in vivo* delivery of anticancer agents such as PTX.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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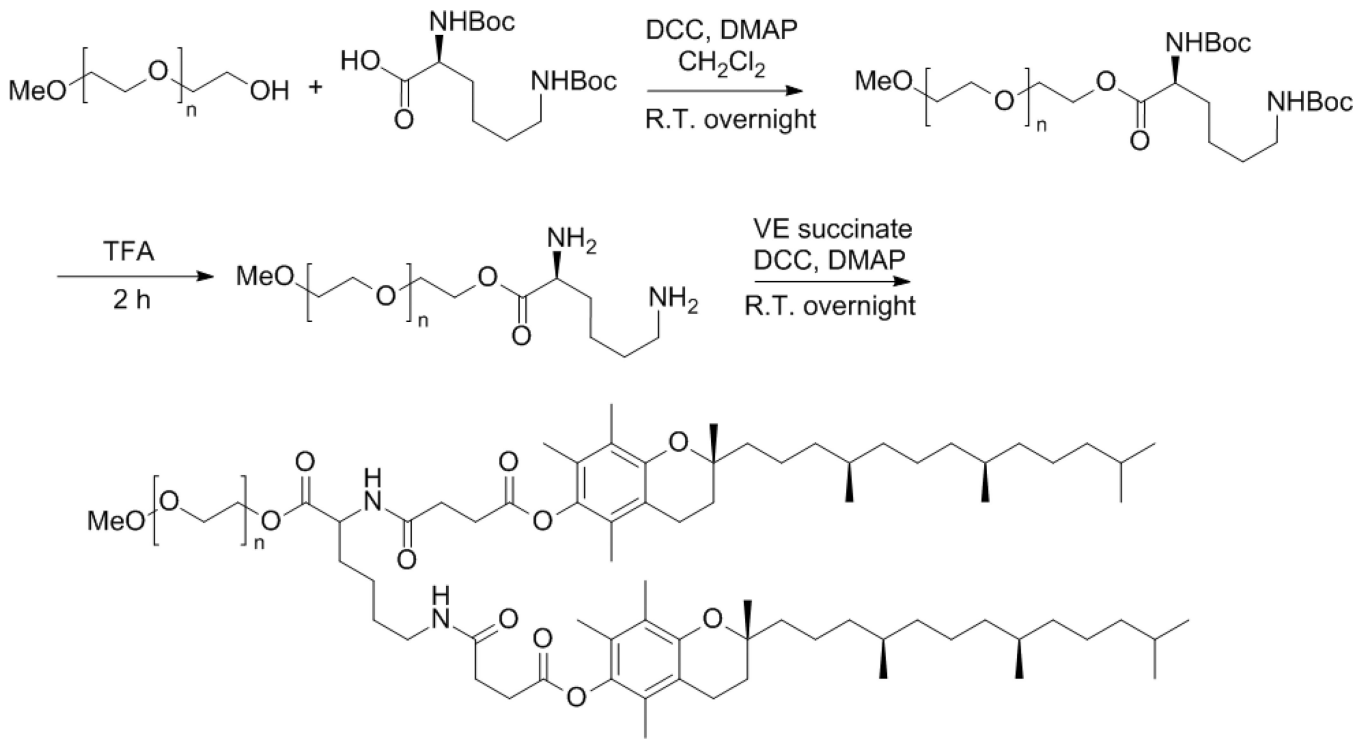

Fig. 1.

The synthesis scheme of PEG_{5K}-VE_2. First, PEG_{5K} reacted with di-Boc-protected lysine to obtain PEG_{5K}-conjugated di-Boc lysine. Then TFA was employed to remove the Boc groups in order to get free amine. Finally, free amine reacted with Vitamin E succinate to attain PEG_{5K}-VE_2.

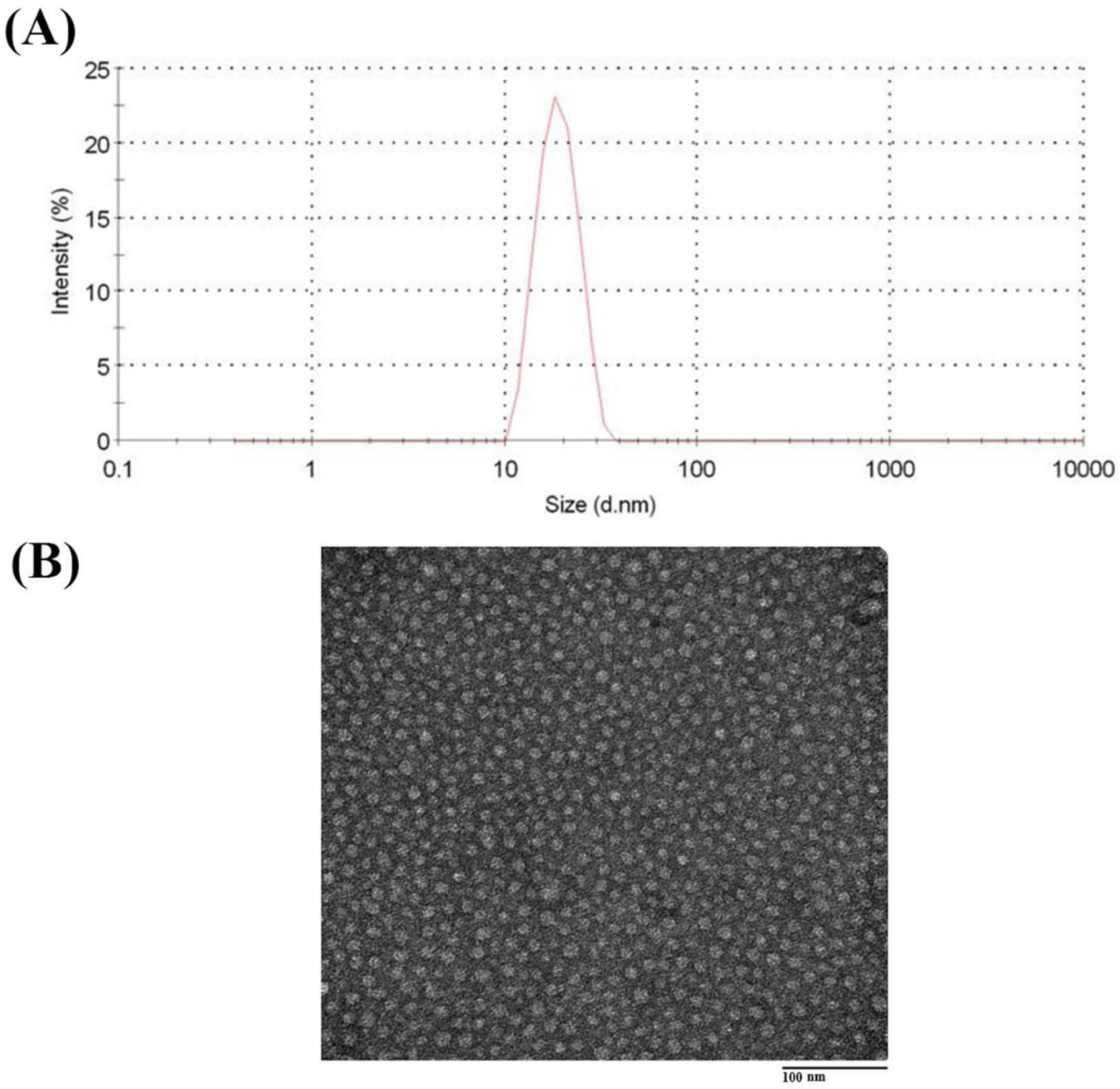

Fig. 2.

(A) The size distribution of free PEG_{5K}-VE_2 nanoparticles in DPBS measured by dynamic light scattering (DLS) and (**B**) Transmission electron microscopic (TEM) images of PEG_{5K}-VE_2 micelles. The spherical micelles with diameter around 20 nm were observed. The concentration of PEG_{5K}-VE_2 was kept at 20 mg/mL during the measurements.

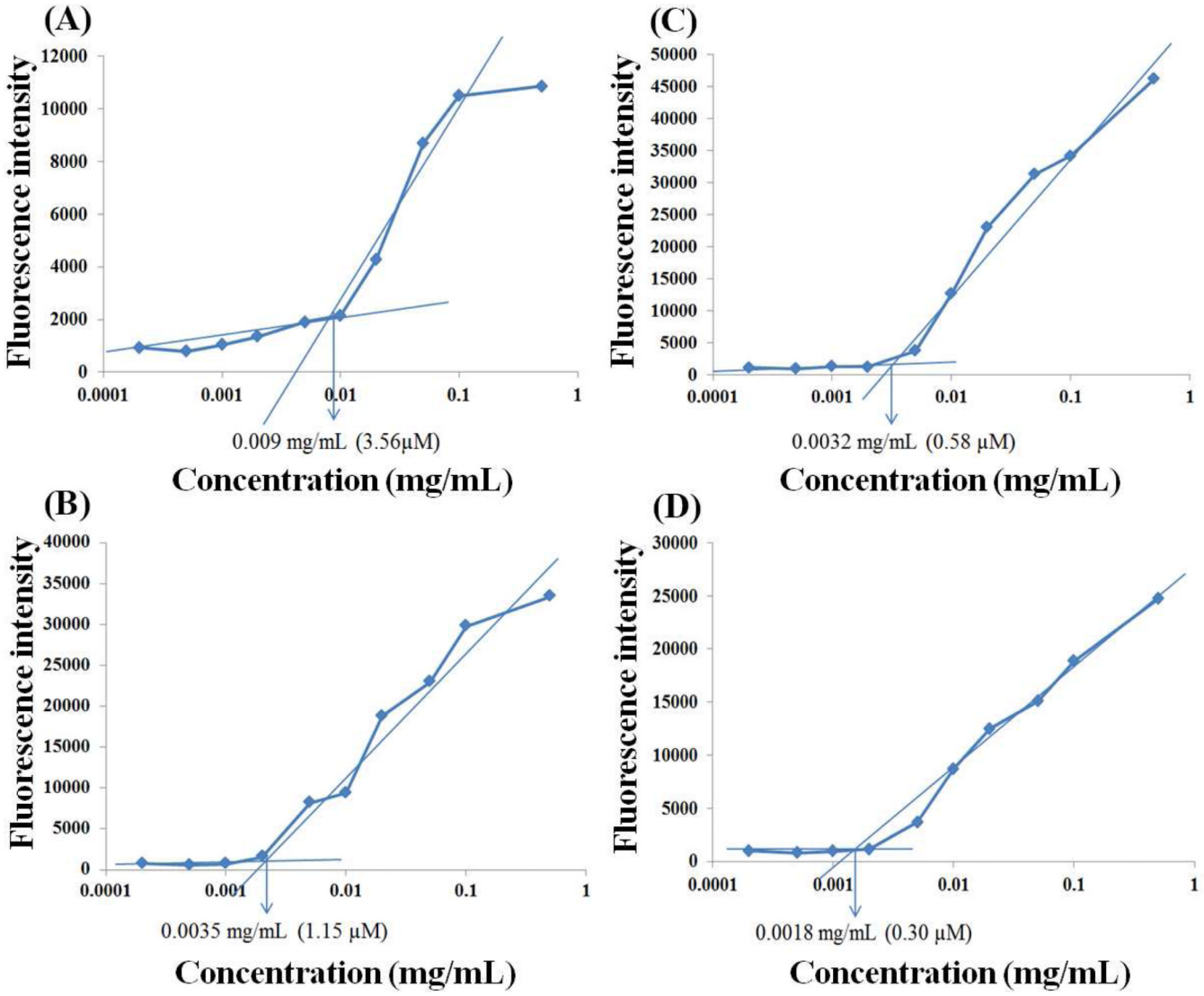

Fig. 3.

Critical micelle concentration (CMC) measurements of PEG_{2K}-VE (A), PEG_{2K}-VE_2 (B), PEG_{5K}-VE (C) and PEG_{5K}-VE_2 (D) by using pyrene as a hydrophobic fluorescence probe. The fluorescence intensity of pyrene was collected at the excitation wavelength of 334 nm and the emission wavelength of 390 nm. The fluorescence intensity was plotted as a function of logarithmic concentration of micelles. [pyrene] =6 × 10−7 mol/L.

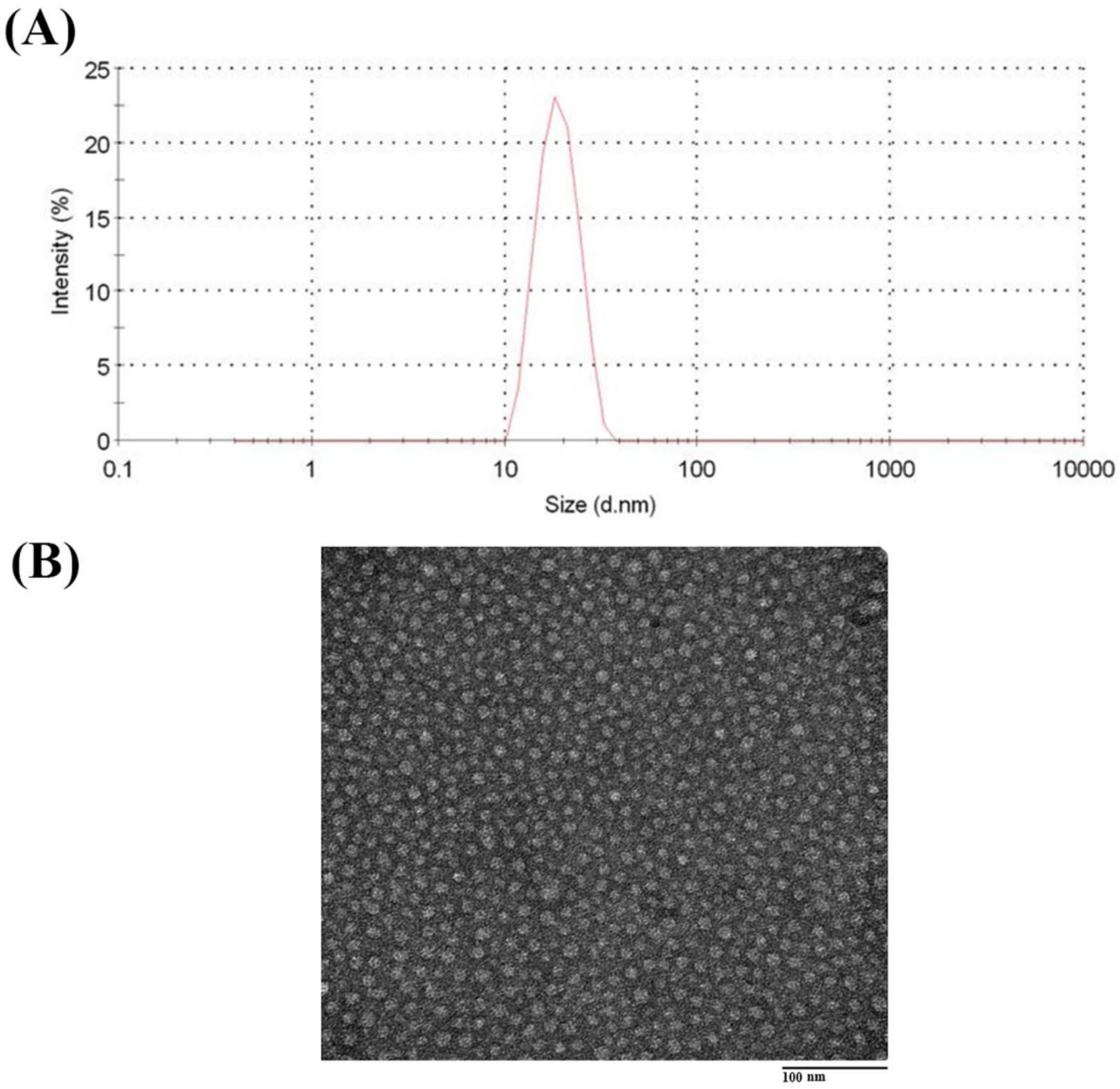

Fig. 4.

Cumulative PTX release profile from PTX-loaded micelles. DPBS (PH = 7.4) containing 0.5% (w/v) Tween 80 was used as the release medium to solubilize released PTX. At various time points, samples from different formulations were collected and measured by HPLC. PTX loading level was 1 mg/mL in micelles. Values are reported as the means ± SD for triplicate samples.

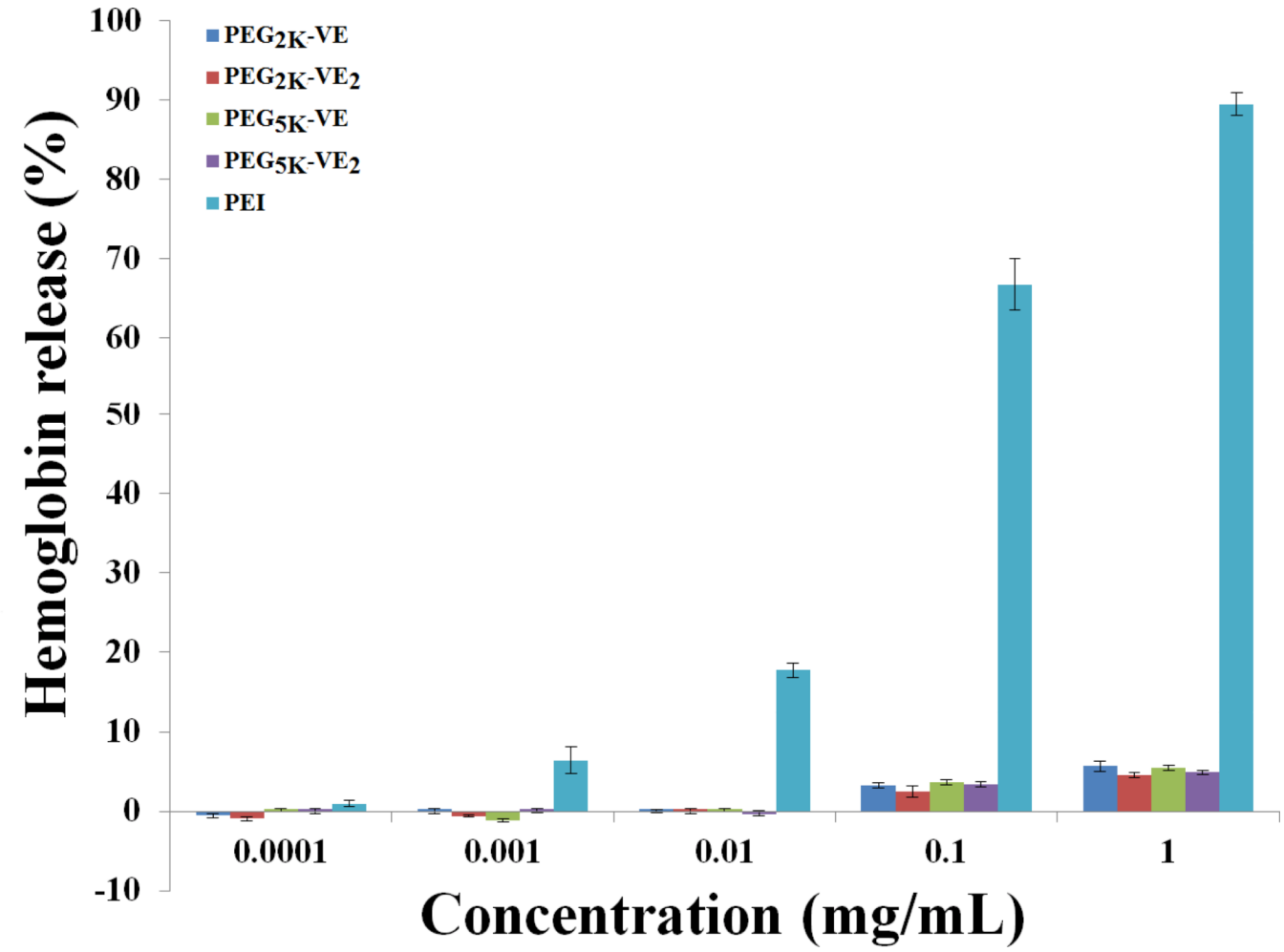

Fig. 5.

In vitro hemolysis assay of PEG-derivatized Vitamin E micelles compared with PEL PEG-VE micelles and PEI of various concentrations were incubated with rat red blood cells (RBCs) for 4 h at 37 °C in an incubator shaker. The degree of RBCs lysis was measured spectrophotometrically (λ=540 nm) according to the release of hemoglobin. 2% Triton X-100 and DPBS were used as a positive and negative control, respectively. Values reported are the means ± SD for triplicate samples.

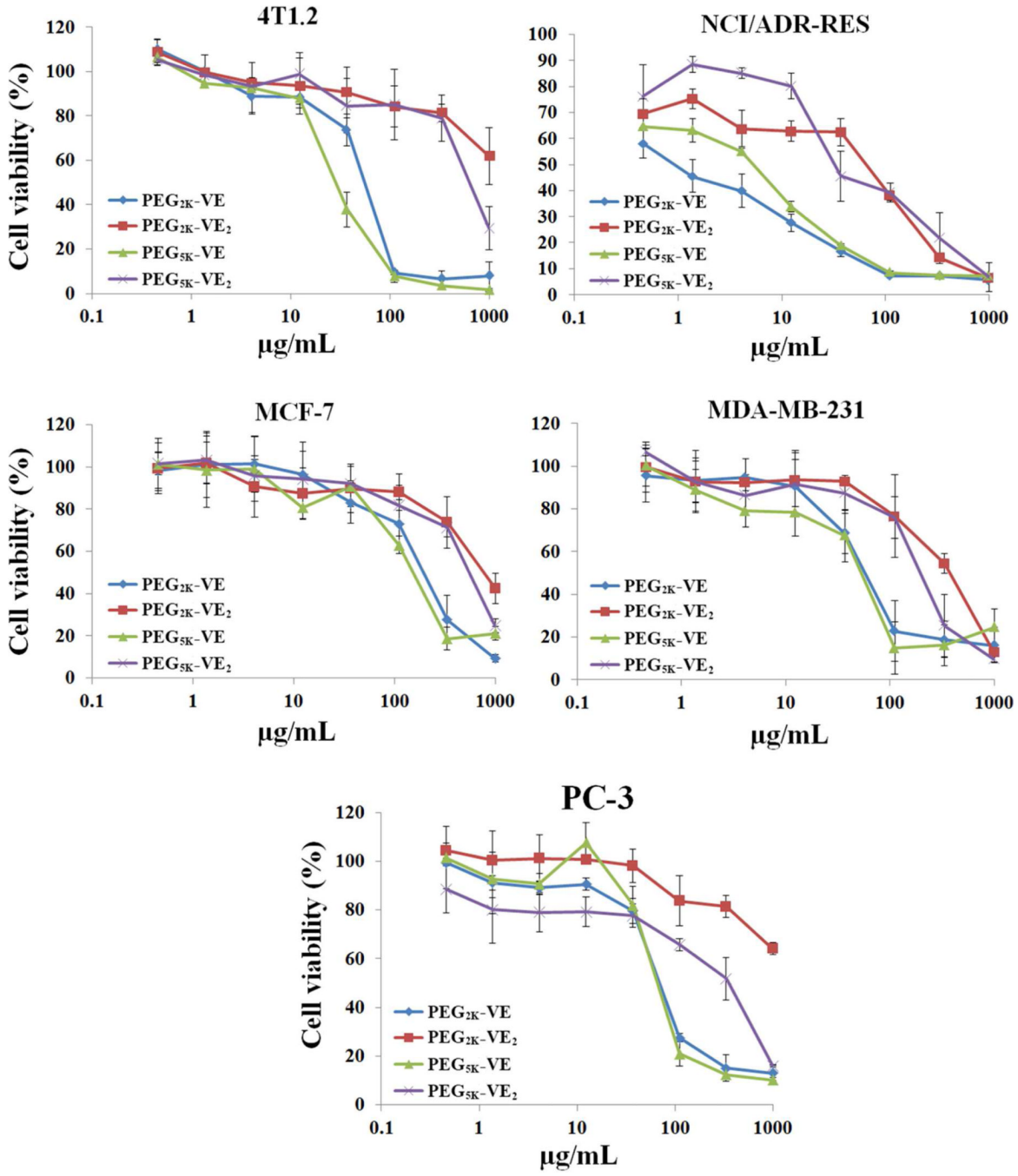

Fig. 6.

Cell viability following treatment with free PEG_{2K}-VE, PEG_{2K}-VE_2, PEG_{5K}-VE, or PEG_{5K}-VE_2 micelles in a mouse breast cancer cell line, 4T1.2, a drug-resistant cell line, NCI/ADR-RES, two human breast cancer cell lines, MCF-7, and MDA-MB-231, and an androgen-independent human prostate cancer cell line. PC-3. Cells were incubated with indicated concentrations of micelles for 72 h and cytotoxicity was determined by MTT assay. Values reported are the means ± SD for triplicate samples

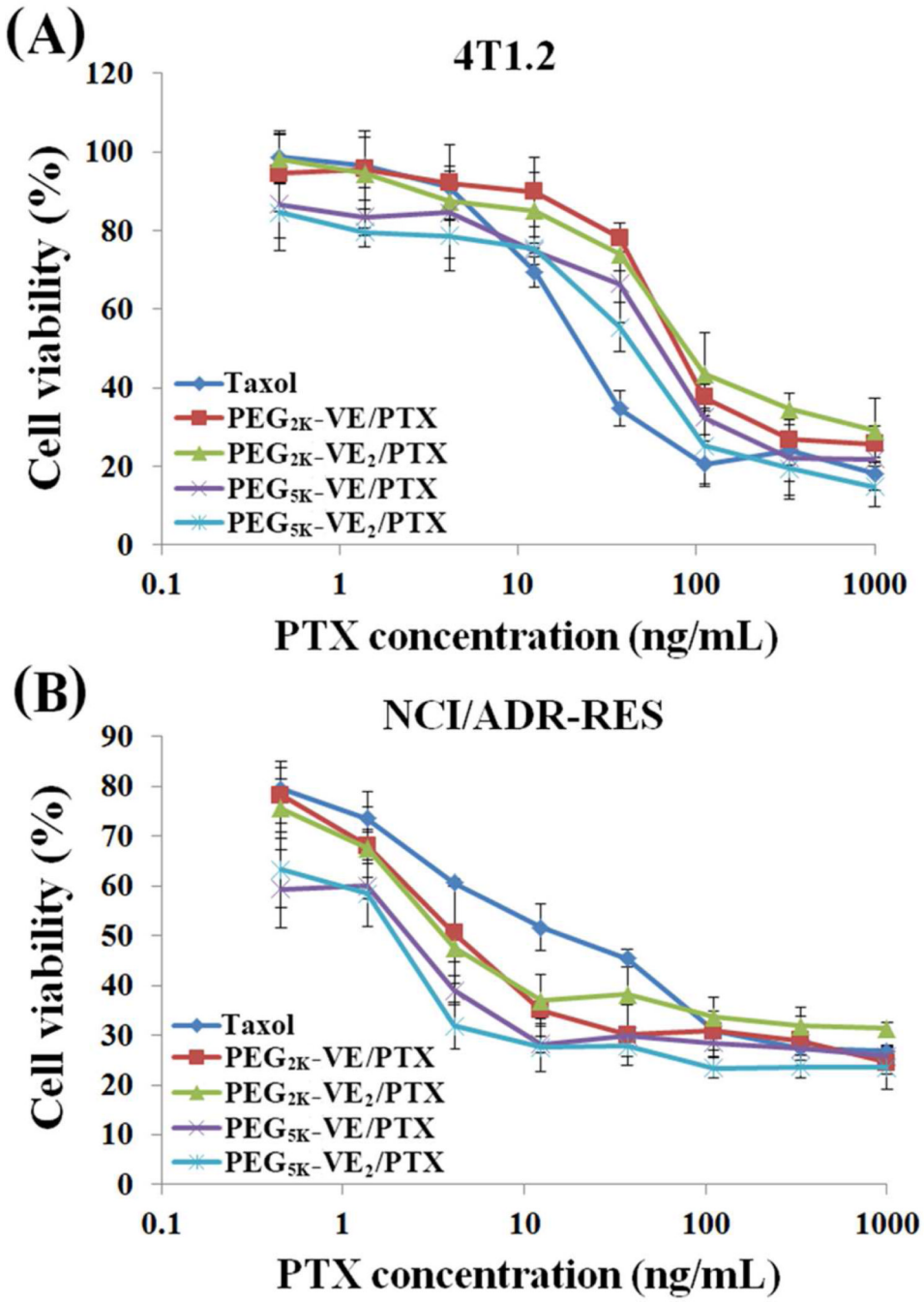

Fig. 7.

The cytotoxicity of PTX-loaded PEG_{2K}-VE, PEG_{2K}-VE_2, PEG_{5K}-VE, or PEG_{5K}-VE_2 micelles against a mouse breast cancer cell line, 4T1.2 **(A)** and a drug-resistant cell line, NCI/ADR-RES **(B)** in comparison to Taxol. Cells were treated with indicated concentrations of different PTX formulations for 72 h and cytotoxicity was then determined by MTT assay. Values reported are the means ± SD for triplicate samples.

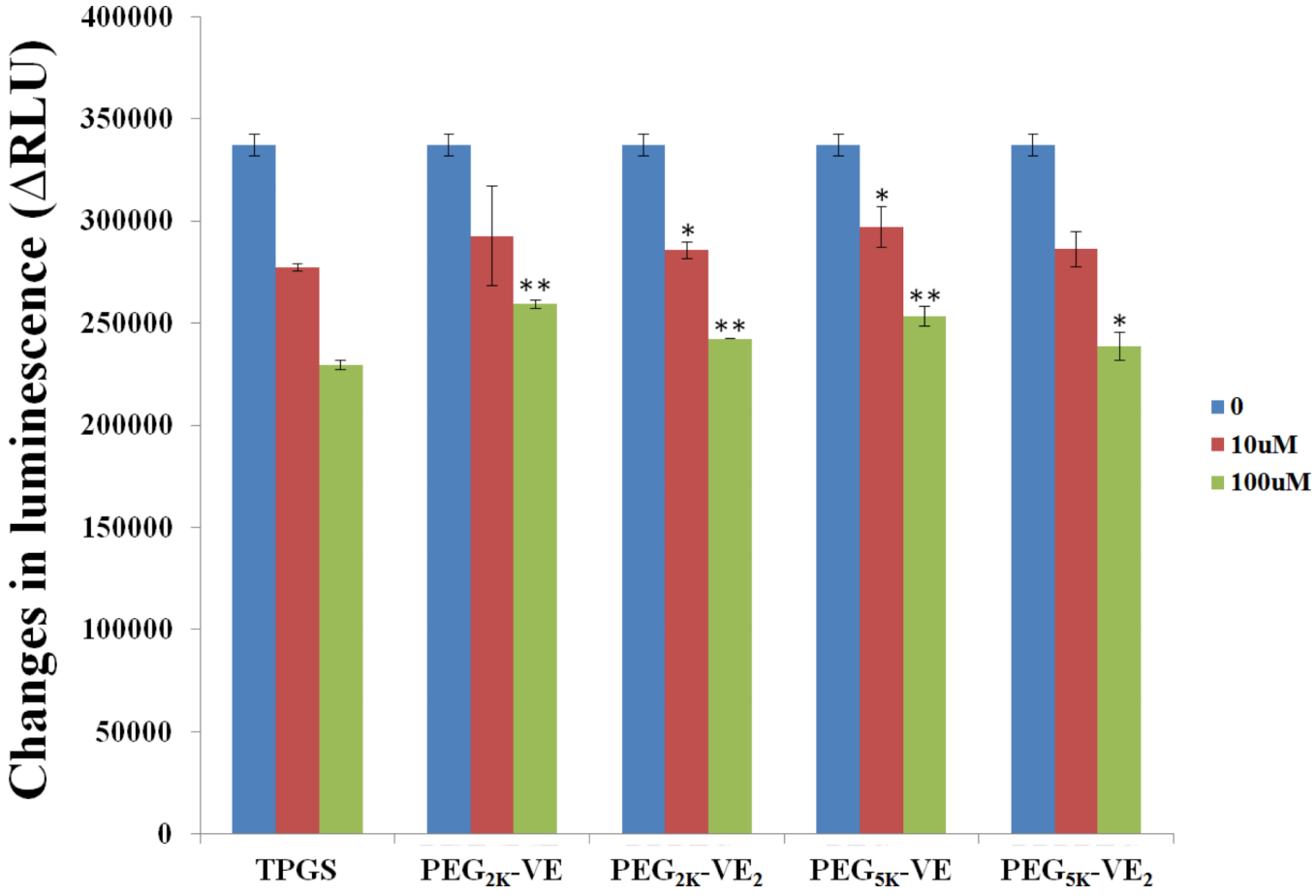

Fig. 8.

Inhibitory effect of TPGS, PEG_{2K}-VE, PEG_{2K}-VE_2, PEG_{5K}-VE or PEG_{5K}-VE_2 on verapamil-stimulated P-gp ATPase activity. Test samples containing verapamil (50μM) and PEG-derivatized Vitamin E conjugates (final concentrations at 10 and 100 μM, respectively) or Na_3VO_4 (a selective inhibitor of P-gp) were incubated with P-gp membrane for 5 min at 37 °C. The reaction was initiated by the addition of MgATP followed by another 40 minutes' incubation at 37°C. ATP detection reagent was then added luminescence was examined. *$p < 0.05$ and **$p < 0.001$ (vs TPGS of equivalent concentration). Values are reported as the means ± SD for triplicate samples.

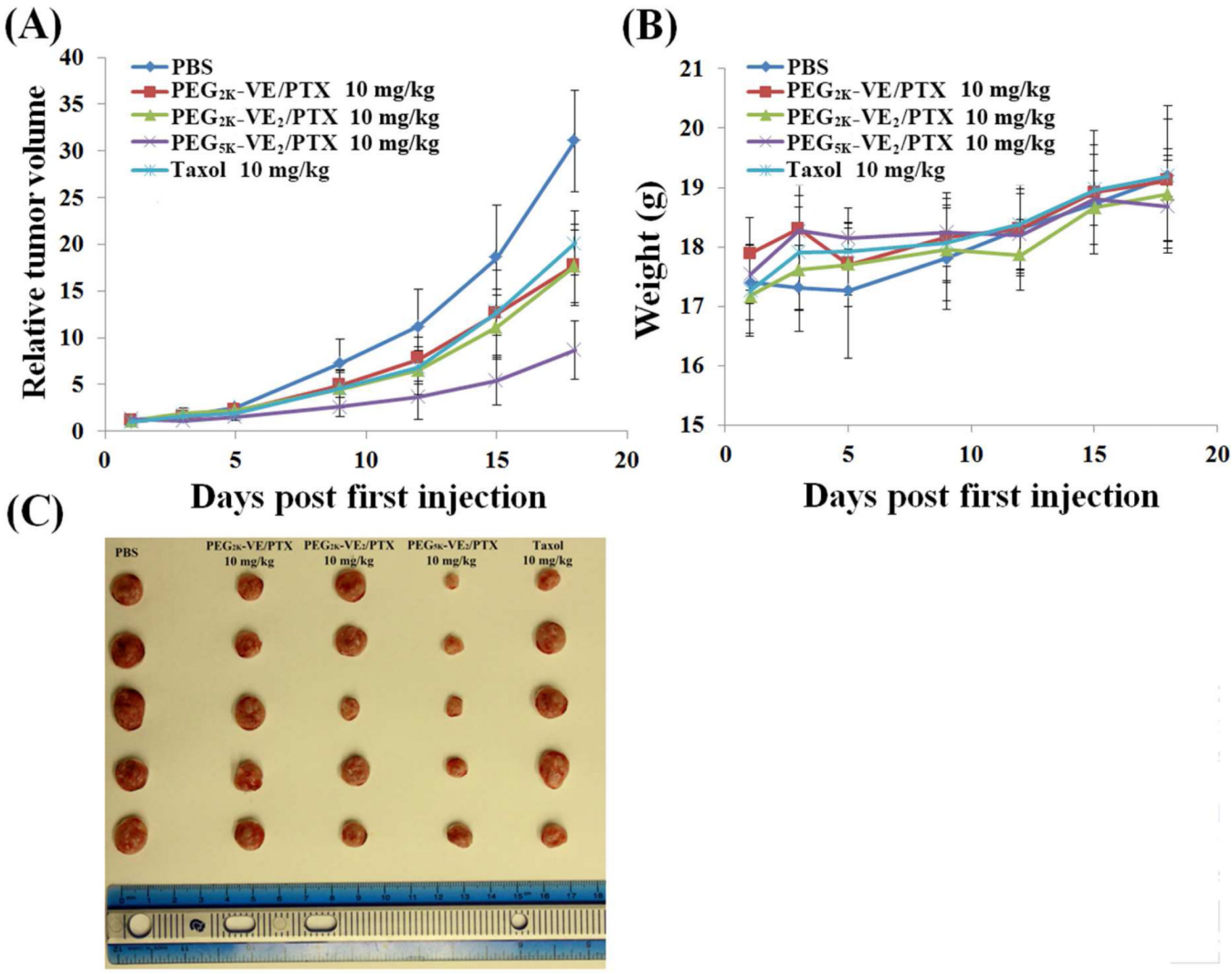

Fig. 9.

(A) Enhanced antitumor activity of PTX formulated in PEG_{5K}-VE_2 micelles. BABL/c mice were inoculated s.c. with 4T1.2 cells (2×10^5 cells/mouse). Five days later, mice received various treatments on days 1, 3, 5, 9, and 12, and tumor growth was monitored and plotted as relative tumor volume. $P < 0.02$ (PEG_{5K}-VE_2/PTX vs. Taxol, PEG_{2K}-VE/PTX or PEG_{2K}-VE_2/PTX), N = 5. **(B)** Changes of body weight in mice receiving different treatments. **(C)** Images of tumors removed from the tumor-bearing mice at the completion of the study.

Table 1

Size of PEG-derivatized Vitamin E micelles

Conjugates	size	PDI
PEG_{2K}-VE	21.5±0.68	0.08
PEG_{2K}-VE_2	18.9±0.08	0.07
PEG_{5K}-VE	19.7±0.09	0.08
PEG_{5K}-VE_2	18.8±0.12	0.09

Table 2

Physicochemical characterization of PTX-loaded miscelles

carrier/PTX ratio	PTX-loaded micelles[a]	size (nm)[b]	PDI[c]	DLE (%)[d]	stability[e] in DPBS (hour)	stability[e] in 50% FBS (hour)
2.5:1	PEG_{2K}-VE/PTX	34.4±1.22	0.34	42.9	0.1	Milky
	PEG_{2K}-VE_2/PTX	26.1±2.42	0.23	64.8	0.2	milky
	PEG_{5K}-VE/PTX	21.3±0.60	0.04	85.4	0.8	0.3
	PEG_{5K}-VE_2/PTX	20.1±0.72	0.05	86.4	1	0.4
5:1	PEG_{2K}-VE/PTX	224±0.36	0.14	66.6	0.8	0.5
	PEG_{2K}-VE_2/PTX	23.5±0.35	0.15	69.8	1	0.7
	PEG_{5K}-VE/PTX	20.3±0.59	0.05	86.4	2.3	1.5
	PEG_{5K}-VE_2/PTX	19.7±0.38	0.04	88.4	3	2.1
7.5:1	PEG_{2K}-VE/PTX	23.6±1.31	0.12	71.6	3	0.9
	PEG_{2K}-VE_2/PTX	22.5±0.78	0.11	72.2	3.5	2.1
	PEG_{5K}-VE/PTX	21.1±0.98	0.19	96.4	22	16.5
	PEG_{5K}-VE_2/PTX	20.9±0.56	0.16	98.2	24	19.3
10:1	PEG_{2K}-VE/PTX	21.9±l.34	0.10	86.4	4.2	2.8
	PEG_{2K}-VE_2/PTX	23.6±0.84	0.16	89.7	55.5	30.8
	PEG_{5K}-VE/PTX	19.8±1.23	0.09	96.9	60	39.5
	PEG_{5K}-VE_2/PTX	19.6±1.15	0.12	98.7	68	45.3

[a] The PTX concentration were kept at 1 mg/mL.

[b] Data represents the mean ± standard deviation (n±3) in DPBS.

[c] Polydispersity index.

[d] Drug Loading efficiency (%) = (weight of Loaded drug/weight of input drug) × 100° 0.

[e] Data means there was no noticeable size change during the follow-up period.

Table 3

IC_{50} of free PEG_{2K}-VE, PEG_{2K}-VE_2, PEG_{5K}-VE and PEG_{5K}-VE_2 micelles in different cancer cell lines

	IC_{50}(μg/mL)			
	PEG_{2K}-VE	**PEG_{2K}-VE_2**	**PEG_{5K}-VE**	**PEG_{5K}-VE_2**
4T1.2	64.30	N/A	30.98	726.44
NCI/ADR-RES	1.04	74.83	6.05	33.83
MCF-7	223.78	839.89	176.29	637.71
MDA-MB-231	66.82	403.93	61.52	224.98
PC-3	79.17	N/A	75.91	366.79

Table 4

IC_{50} of PTX-loaded micelles in 4T1.2 and NCI/ADR-RES cancer cell lines

	IC_{50} (ng/mL)				
	Taxol	**PEG_{2K}-VE/PTX**	**PEG_{2K}-VE_2/PTX**	**PEG_{5k}-VE/PTX**	**PEG_{5K}-VE_2/PTX**
4T1.2	26.16	88.17	95.52	72.75	50.38
NCI/ADR-RES	18.92	4.40	3.78	2.67	2.24