Published in final edited form as:
*Bioconjug Chem*. 2012 July 18; 23(7): 1443–1451. doi:10.1021/bc3000468.

# PEG-Derivatized Embelin as a Dual Functional Carrier for the Delivery of Paclitaxel

**Yixian Huang**[*,1,2], **Jianqin Lu**[*,1,2], **Xiang Gao**[1,2], **Jiang Li**[1,2], **Wenchen Zhao**[2], **Ming Sun**[3], **Donna Beer Stolz**[3], **Raman Venkataramanan**[2], **Lisa Cencia Rohan**[2,4], and **Song Li**[1,2]

[1]Center for Pharmacogenetics, University of Pittsburgh, Pittsburgh, PA, 15261, USA

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

[3]Department of Cell Biology and Physiology, School of Medicine, University of Pittsburgh, Pittsburgh, PA, 15261, USA

[4]Magee Women's Research Institute, University of Pittsburgh, Pittsburgh, PA, 15261, USA

## Abstract

Embelin, identified primarily from the *Embelia ribes* plant, has been shown to be a natural small molecule inhibitor of X-linked inhibitor of apoptosis protein (XIAP). It is also a potent inhibitor of NF-κB activation, which makes it a potentially effective suppressor of tumor cell survival, proliferation, invasion, angiogenesis, and inflammation. However, embelin itself is insoluble in water, which makes it unsuitable for *in vivo* applications. In this work, we developed a novel micelle system through conjugating embelin to a hydrophilic polymer, polyethylene glycol 3,500 ($PEG_{3.5K}$) through an aspartic acid bridge. The $PEG_{3.5k}$-embelin$_2$ ($PEG_{3.5k}$-$EB_2$) conjugate readily forms micelles in aqueous solutions with a CMC of 0.0205mg/mL. Furthermore, $PEG_{3.5k}$-$EB_2$ micelles effectively solubilize paclitaxel (PTX), a model hydrophobic drug used in this study. Both drug-free and drug-loaded micelles were small in sizes (20 ~ 30 nm) with low polydispersity indexes. *In vitro* cytotoxicity studies with several tumor cell lines showed that $PEG_{3.5k}$-$EB_2$ is comparable to embelin in antitumor activity and synergizes with PTX at much lower doses. Our results suggest that PEG-derivatized embelin may represent a novel and dual-functional carrier to facilitate the *in vivo* applications of poorly water-soluble anticancer drugs such as PTX.

## INTRODUCTION

Low water-solubility, high protein-binding and relatively short half-life are major problems in the clinical applications of many potent anti-cancer drugs such as paclitaxel (PTX).[1,2] Currently a variety of drug delivery systems such as liposomes, dendrimers, microcapsules and polymeric micelles have been developed to address these problems and further to promote sustained, controlled and targeted delivery of poorly water-soluble anti-cancer drugs.[3] Of all these delivery systems, polymeric micelles have gained considerable attention as a versatile nanomedicine platform due to their technical ease, high biocompatibility, and high efficiency in drug delivery.[4,5] Polymer micelles have been demonstrated to improve the

Address correspondence to: Song Li, M.D., Ph.D. Center for Pharmacogenetics University of Pittsburgh School of Pharmacy 639 Salk Hall Pittsburgh, PA 15261 Tel: 412-383-7976 Fax: 412-648-1664 sol4@pitt.edu.
*Authors contributed equally to this work

**Supporting Information Available:** HPLC trace, $^1$H NMR spectra and MALDI-TOF of $PEG_{3.5K}$-$EB_2$, and the cytotoxicity of embelin, PTX, and the combination of embelin and PTX against human prostate cancer cells DU145. This materials is available free of charge via the Internet at http://pubs.acs.org.

aqueous solubility of chemotherapeutic agents and prolong their *in vivo* half-lives, owing to the steric hindrance provided by a hydrophilic shell.[4,5] Moreover, compared with other delivery systems, micelles show advantages in passive tumor targeting through the leaky vasculature via the enhanced permeability and retention (EPR) effect due to their small size ranging from 10-100 nm.[6,7]

Favorable drug biodistribution and improved therapeutic index can be achieved by using the micelle delivery system.[3,4] However, most of the polymeric systems use “inert” excipients that lack therapeutic activity. The presence of large amounts of carrier materials not only adds to the cost but also imposes additional safety issue.[8] One of the most sophisticated designs of drug delivery systems is that the components forming the carriers can also be of therapeutic effects. The carrier materials may be capable of counteracting the side effects caused by the loaded anticancer drugs.[9] Also, it is possible that the carrier may collaborate with the loaded drug to achieve synergistic effects to better treat the tumor.[5] However, the strategy of using highly water-insoluble drugs themselves as the hydrophobic region of polymeric micelle is rarely reported. One example is the pegylated vitamin E, D-α-tocopheryl polyethylene glycol succinate (Vitamin E TPGS or TPGS).[10-12] Vitamin E shows antitumor activity against a number of types of cancers through various mechanisms such as induction of apoptosis, inhibition of tumor cell proliferation and differentiation, suppression of nuclear factor-kappa B (NF-κB) activation etc.[13,14] The pegylated vitamin E is a highly water soluble amphiphilic molecule comprising lipophilic alkyl tail and hydrophilic polar head portion. In addition to its antitumor activity, it is effective in solubilizing various hydrophobic drugs such as PTX. Synergistic actions between the TPGS-based carrier and delivered anticancer agents have been reported.[5]

In this study, we report the development of PEG-derivatized embelin as another novel and dual-functional carrier for delivery of poorly water-soluble anticancer drugs. Embelin is a naturally occurring alkyl substituted hydroxyl benzoquinone compound and a major constituent of *Embelia ribes* BURM. It has been shown to possess antidiabetic, anti-inflammatory, and hepatoprotective activities.[15-17] Embelin also shows antitumor activity in various types of cancers.[15,18-21] One major mechanism involves the inhibition of the activity of X-linked inhibitor of apoptosis protein (XIAP).[22] XIAP is overexpressed in various types of cancers cells, particularly drug-resistant cancer cells and inhibition of XIAP has been explored as a new approach for the treatment of cancers.[23,24] XIAP plays a minimal role in normal cells and therefore embelin shows significantly less toxicity on normal cells. Embelin also downregulates the expression of survivin, XIAP, IAP1/2, TRAF1, cFLIP, Bcl-2, and Bcl-$x_L$ through the inhibition of NF-κB activation.[25] Embelin is poorly water soluble and PEG modification was originally explored by us as an approach to increase its solubility. Interestingly, PEG-derivatized embelin forms micelles that are highly efficient in solubilizing other compounds such as PTX. Preparation of PEG-derivatized embelin can be readily achieved with commercially available embelin. In addition, we have developed an efficient synthesis strategy to prepare PEG-embelin conjugate. Our *in vitro* studies showed that PEG-embelin has similar activity as free embelin with $IC_{50}$ in the low μM range. More importantly, PEG-embelin synergizes with PTX at much lower doses (~nM) in a number of cancer cell lines tested.

## EXPERIMENTAL PROCEDURES

### Materials

Paclitaxel (98%) was purchased from AK Scientific. Inc. (CA, USA). 2,5-dihydroxy-3-undecyl-1,4-benzoquinone (embelin 98%) was purchased from 3B Scientific Corporation (IL, USA). Dulbecco’s phosphate buffered saline (DPBS) was purchased from Lonza (MD, USA). Methoxy-PEG3,500-OH, dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-

yl)-2,5-diphenyl tetrazolium bromide (MTT), trypsin-EDTA solution, Triton X-100, and Dulbecco's Modified Eagle's Medium (DMEM) were all purchased from Sigma-Aldrich (MO, USA). Fetal bovine serum (FBS) was purchased from Gibco Life Technologies (AG, Switzerland). Penicillin-streptomycin solution was from Invitrogen (NY, USA). All solvents used in this study were HPLC grade.

### Cell culture

DU145 and PC3 are two androgen-independent human prostate cancer cell lines. MDA-MB-231 is a human breast adenocarcinoma cell line. 4T1 is a mouse metastatic breast cancer 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% $CO_2$.

### Synthesis of $PEG_{3.5K}$-Embelin$_2$ ($PEG_{3.5K}$-$EB_2$)

Scheme 1 shows the synthesis sequence of $PEG_{3.5K}$-$EB_2$ conjugate. Synthesis of the intermediates and structural characterizations are detailed below.

Compound **2:** Sesamol (1.52 g, 24 mmol) in 30 mL of methanol was added to a rapidly stirred solution of Fremy's salt (7.96 g, 30 mmol) and 5.49 g (40 mmol) of $KH_2PO_4$ in 400 mL water at 5 °C. The color of the solution changed from light brown to bright yellow within 5 min. The mixture was stirred for another 30 min and then extracted with 4 × 40 mL of ethyl acetate. The ethyl acetate phase was treated with a solution of $Na_2S_2O_4$ (9.0 g, 52 mmol) in water (30 mL), and the yellow color changed to a colorless solution. The organic layer was acidified with HCl (1 N), extracted with ethyl acetate (3 × 30 mL), washed with water (20 mL), dried with anhydrous $MgSO_4$, and concentrated to give 1.1 g (65%) of **2** as a light pink solid. $^1$H NMR($(CD_3)_2CO$): δ7.49 (s, 2H), 6.45 (s, 2H), 5.79 (s, 2H).

Compound **3:** A solution of **2** (1.54 g, 10 mmol) in water (30 mL) was treated with NaOH (0.4 g, 10 mmol) while the flask was kept in an ice bath. The reaction mixture was stirred for 15 min after which MeI (1.41 g, 10 mmol) was added dropwise. The reaction mixture was then heated under reflex for 1 h, allowed to cool down to room temperature and the solvent was removed via a rotary evaporator. The crude product was purified by flash chromatography with silica gel (ethyl acetate: petroleum ether, 1: 5) and pure **3** was obtained as an amber oil with a yield of 99% (1.68 g). $^1$H NMR($CDCl_3$): δ 6.11 (m, 2H), 5.88 (s, 2H), 5.35 (s, 1H), 3.72 (s, 3H).

Compound **4:** To a solution of *N*-(*tert*-Butoxycarbonyl)-L-aspartic acid (Boc-Asp-OH) (2.33 g, 10 mmol) in $CH_2Cl_2$ (40 mL) was added dicyclohexylcarbodiimide (DCC) (6.2 g, 30 mmol), 4-dimethyamineopyridine (DMAP) (0.61 g, 5 mmol), and compound **3** (3.36 g, 20 mmol) at room temperature. The reaction mixture was stirred overnight at room temperature. After the reaction was completed, 100 mL $Et_2O$ was added to the mixture. The mixture was filtered to remove the insoluble DCU byproduct and the organic phase of the filtrate was concentrated under vacuum. The resulting residue was purified by silica gel flash chromatography (MeOH: $CH_2Cl_2$, 1:10) to give pure **4** as an oil in 62% yield (3.31 g). $^1$H NMR ($CDCl_3$): δ 6.11 (m, 4H), 5.88 (m, 4H), 5.40 (m, 1H), 4.65 (m, 1H), 3.74 (s, 3H), 3.72 (s, 3H), 2.88 (m, 1H), 2.64 (m, 1H), 1.42 (s, 9H). ESI-MS *m/z* 534.2 ($[M+H]^+$).

Compound **5**: To a solution of **4** (5.33 g, 10 mmol) in acetonitrile (MeCN, 10 mL) at 0-5°C, dry dimethylformamide (DMF) (0.73 g, 10 mmol) and $POCl_3$ (1.78 g, 11 mmol) were added with constant stirring over 0.5 h. The salt formed was filtered, washed with cold MeCN, dissolved in 20 mL of water, heated at 50 °C for 0.5 h, and then cooled. The mixture was extracted with 3 × 40 mL of $CH_2Cl_2$, the combined organic phase was washed with brine, dried over anhydrous $Na_2SO_4$, and concentrated in vacuum. The crude residue was purified

by silica gel flash chromatography (MeOH: $CH_2Cl_2$, 1: 10) to give pure **5** as an oil in 80% yield (4.82 g).$^1$H NMR ($CDCl_3$): δ 10.21 (s, 1H), 10.18 (s, 1H), 6.65 (m, 2H), 5.92 (m, 4H), 5.37 (m, 1H), 4.51 (m, 1H), 3.75 (s, 3H), 3.72 (s, 3H), 2.85 (m, 1H), 2.60 (m, 1H), 1.40 (s, 9H). ESI-MS *m/z* 590.5 ($[M+H]^+$).

Compound **6:** A solution of sodium bis(trimethylsilyl)amide (12 mL, 2 M solution in THF) was added dropwise to a stirred solution of decanyltriphenylphosphonium bromide (9.67g, 20 mmol) in 40 mL THF at room temperature. The resulting mixture was stirred for 30 min at room temperature and then cooled to −78°C. To this mixture was added compound **5** (6.03 g, 10 mmol). The reaction mixture was stirred for 2 h at −78°C and then warmed up to room temperature. The reaction mixture was quenched with saturated solution of $NH_4Cl$, extracted with ethyl acetate. The combined organic layer was washed with brine, dried over anhydrous $Na_2SO_4$, and concentrated in vacuum. The crude residue was purified by silica gel flash chromatography (MeOH: $CH_2Cl_2$, 1: 10) to give pure **6** as an oil in 90% yield.$^1$H NMR ($CDCl_3$): δ 6.48 (m, 2H), 6.39 (m, 2H), 6.02 (m, 2H), 5.86 (m, 4H), 5.35 (m, 1H), 4.53 (m, 1H), 3.75 (s, 3H), 3.72 (s, 3H), 2.83 (m, 1H), 2.61 (m, 1H), 2.15 (m, 4H), 1.40 (s, 9H), 1.29 (m, 28H), 0.91 (m, 6H). ESI-MS *m/z* 838.4 ($[M+H]^+$)

Compound **7**: The double bond in compound **6** (8.37 g, 10 mmol) was saturated by catalytic hydrogenolysis with Pd/C (10%, 500 mg) under $H_2$ (1 atm) in a methanol solution (50 mL) at room temperature for 2 h. The solution was filtered to remove Pd/C and concentrated under vacuum. The resulting product was then dissolved in the solution of 10 mL of water, 10 mL MeCN, and 20 mmol CAN (ammonium ceric nitrate) (10.96 g). The mixture was cooled to 0°C and stirred for another 2 h. MeCN was then removed via evaporation under reduced pressure, 100 mL $CH_2Cl_2$ was added to the remaining aqueous solution. The organic phase was washed with brine and then concentrated under vacuum. 10 mL dioxane and 10 mL HCl were then added to the residue. The mixture was stirred at room temperature for 24 h. The reaction mixture was quenched with saturated solution of $NaHCO_3$, extracted with ethyl acetate. The organic layer was washed with brine, dried over anhydrous $Na_2SO_4$, and concentrated under vacuum. The crude product was purified by silica gel flash chromatography (MeOH: $CH_2Cl_2$, 1:10) to give pure **7** as an oil in 42% yield (2.89 g).$^1$H NMR($CDCl_3$): δ 8.16 (m, 2H), 6.75 (m, 2H), 5.35 (m, 2H), 4.53 (m, 1H), 2.85 (m, 1H), 2.60 (m, 1H), 2.43 (m, 4H), 1.25 (m, 36H), 0.89 (m, 6H). ESI-HRMS calcd for $C_{38}H_{55}NO_{10}Na$ ($[M+Na]^+$) 708.4766, found 708.4747.

Compound **8:** A solution of MeO-$PEG_{3.5k}$-$CO_2H$ (3.5 g, 1 mmol) in $CH_2Cl_2$ (5 mL) was treated with DCC (0.41 g, 2 mmol), DMAP (0.12 g, 1 mmol), and compound **7** (689 mg, 1 mmol) at room temperature. The reaction mixture was stirred overnight. After the reaction was completed, 100 mL of $Et_2O$ was added and the mixture was filtered and concentrated under vacuum. The resulting residue was purified by silica gel flash chromatography (MeOH: $CH_2Cl_2$, 1:10) to give pure **8 (**$PEG_{3.5K}$-$EB_2$**)** as a wax solid in ~50% yield (2.1 g). $^1$H NMR ($CDCl_3$): δ 8.14 (m, 2H), 6.72 (m, 2H), 5.57 (m, 1H), 4.98 (m, 1H), 3.35 (s, 3H), 2.60 (m, 10H), 1.25 (m, 36H), 0.89 (m, 6H).

## Formation of micelles

PTX-solubilized micelles were prepared by the following method. PTX (10 mM in chloroform) was added to $PEG_{3.5K}$-$EB_2$ (10 mM in chloroform) with various carrier/drug ratios. The organic solvent was first removed by nitrogen flow to form a thin 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.

### Measurement of size and zeta potential

Zetasizer (Zetasizer Nano ZS instrument, Malvern, Worcedtershire, UK) was used to measure the particle size and zeta potential of drug-free and drug-loaded micelles. Micelles were stored at 4 °C, and the samples were tested for changes in particle size and size distribution.

### Determination of PTX loading efficiency

PTX-solubilized micelles were prepared at an input PTX concentration of 1.07, 2.14, and 3.21 mg/mL respectively. Aliquots of samples were filtered through 0.45 μm PVDF syringe filter. PTX in the filtered and non-filtered micelles was extracted using methanol and measured by high performance liquid chromatography (HPLC, Waters). A reverse phase column (C18) was employed. The detection was performed by using UV detector at 227 nm, 70% methanol as a mobile phase, flow rate at 1.0 mL/min. Drug loading capacity (DLC) and drug loading efficiency (DLE) were calculated according to the following formula:

DLC (%) = [weight of drug used/(weight of polymer + drug used)] ×100%

DLE (%) = (weight of loaded drug/weight of input drug) ×100%

### Determination of the critical micelle concentration (CMC)

The CMC of $PEG_{3.5K}$-$EB_2$ was determined by employing pyrene as a fluorescence probe.[26] 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 with $PEG_{3.5K}$-$EB_2$ concentrations ranging from $7.63\times10^{-5}$ to 2.5mg/mL. At the same time, aliquots of 50 μL of $4.8\times10^{-6}$ M pyrene in chloroform were added into 15 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 $PEG_{3.5K}$-$EB_2$ concentrations were added to the pyrene film to obtain a final pyrene concentration of $6\times10^{-7}$ M for 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.

### Transmission electron microscope (TEM)

The morphology of micelles was observed on a Jeol 1011 transmission electron microscope (TEM). The aqueous micelle solution (1.0 mg/mL) was added onto copper grids coated with Formvar, and then stained with 1% uranyl acetate. The sample processing and imaging was performed at room temperature.

### Hemolysis assay

Fresh blood samples were collected through cardiac puncture from rats. Ten mL blood was added with EDTA-$Na_2$ immediately to prevent coagulation. Red blood cells (RBCs) were separated from plasma by centrifugation at 1500 rpm for 10 min at 4 °C. The 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 (0.2 and 1.0 mg/mL) of $PEG_{3.5k}$-$EB_2$ 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* cell cytotoxicity

DU145 (2000 cells/well), PC-3 (5000 cells/well), MDA-MB-231 (2000 cells/well), or 4T1 (1000 cells/well) were seeded in 96-well plates followed by 24 h of incubation in DMEM with 10% FBS and 1% streptomycin-penicillin. Then various concentrations of PTX (dissolved in DMSO or formulated in $PEG_{3.5K}$-$EB_2$ micelles) were added in quadruplicate and cells were incubated for 72 h. Twenty μL of 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) in PBS (5mg/mL) was added and cells were further incubated for 4 h. The medium in the plates was removed and MTT formazan was solubilized by DMSO. The absorbance was measured by microplate reader with wavelength at 550 nm and reference wavelength at 630 nm. Untreated groups were used as controls. Cell viability was calculated as $[(OD_{treat}\text{-}OD_{blank})/(OD_{control}\text{-}OD_{blank}) \times 100\%]$.

## RESULTS

### Synthesis of $PEG_{3.5K}$-$EB_2$ conjugates

We have developed a strategy to synthesize $PEG_{3.5K}$-$EB_2$ conjugate in which two molecules of embelin were coupled to one molecule of PEG via a linker of aspartic acid. This is modified from the scheme reported by Wang's group[27] for the total synthesis of embelin. This involves the synthesis of benzoquinone followed by coupling to carboxyl groups of aspartic acid. Undecyl side chains were then installed onto each of the two benzoquinone rings. Finally PEG was coupled to aspartic acid-$EB_2$ through the deprotected amino group. HPLC shows that the purity of the final product ($PEG_{3.5K}$-$EB_2$) is 94% (Fig. S1). $^1$H NMR spectrum of $PEG_{3.5K}$-$EB_2$ shows signals at 3.63 ppm attributable to the methylene protons of PEG, the embelin proton signals at 8.14 and 6.72 ppm and the carbon chain singles at 1.05—1.25 ppm. The aspartate signals were identified at 5.57, 4.98 and 2.60 ppm (Fig. S2). The molecular weight of the $PEG_{3.5K}$-$EB_2$ conjugate from MALDI-TOF MS (4197) is very similar to the theoretical value (4203) (Fig. S3). These results suggest successful synthesis of $PEG_{3.5K}$-$EB_2$ conjugate.

### Biophysical characterization of micelles

Micelles were readily prepared from $PEG_{3.5K}$-$EB_2$ conjugate via solvent evaporation method. $PEG_{3.5K}$-$EB_2$ conjugate can be dissolved in water at concentration up to 750 mg/mL (data not shown). Dynamic light scattering (DLS) measurements showed that these micelles had hydrodynamic sizes around 22 nm at the concentration of 20 mg/mL (Fig. 1A), which shall ensure efficient passive targeting to the solid tumors.

PTX, a potent hydrophobic anticancer agent, was readily loaded into $PEG_{3.5K}$-$EB_2$ micelles. Fig. 1C shows the DLS size measurement of PTX-loaded $PEG_{3.5K}$-$EB_2$ micelles at a drug concentration of 1 mg/mL. There were little changes in sizes when PTX was loaded into micelles at a carrier/drug ratio of 7.5/1 (m/m).

Fig. 1B and 1D show the TEM images of drug-free and PTX-loaded micelles after staining with 1% uranyl acetate. Spherical particles of uniform size were observed for both drug-free and PTX-loaded micelles. The sizes of the micelles observed under TEM are consistent with those measured by DLS.

Tab. 1 shows the sizes of PTX-loaded micelles at different carrier/drug molar ratios. PTX-loaded $PEG_{3.5K}$-$EB_2$ micelles had relatively large size (~143 nm) at a carrier/drug ratio of 2.5: 1 (m/m) and the particles were stable for less than 1 day. Increasing the input molar ratio of $PEG_{3.5K}$-$EB_2$/PTX led to gradual decrease in the size of PTX-loaded micelles. At the molar ratio of 7.5/1, the size of the PTX-loaded micelles was similar to that of drug-free micelles.

### Drug loading efficiency (DLE)

DLE of PTX-loaded micelles were determined by HPLC and the results are shown in Tab. 2. DLE was as high as 79.89% when PTX was formulated in $PEG_{3.5K}$-$EB_2$ micelles at a carrier/PTX input ratio of 2.5/1 (m/m) and PTX concentration of 1.07 mg/mL. Increasing the carrier/PTX input ratios led to further increase in DLE. $PEG_{3.5K}$-$EB_2$/PTX formed the most stable particles at a carrier/drug ratio of 7.5/1. At this ratio, PTX was quantitatively formulated in the $PEG_{3.5K}$-$EB_2$ micelles when the PTX concentration was less than 2.14 mg/mL. Increasing the PTX concentration to 3.21 mg/mL led to a slight decrease in DLE (81.3%). The surface charges of PTX-loaded $PEG_{3.5K}$-$EB_2$ micelles were close to neutral (+1.89 ~ −2.64) for all particles examined.

### CMC measurements

Fig. 2 shows the results of CMC measurements 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.[28] Based on the partition of the pyrene, the CMC of $PEG_{3.5K}$-$EB_2$ could be obtained by plotting the fluorescence intensity versus logarithm concentration of the polymer. The CMC of $PEG_{3.5K}$-$EB_2$ was determined from the crossover point at the low concentration range. The CMC of the $PEG_{3.5K}$-$EB_2$ conjugate is 4.9 μM, which is similar to most reported micellar delivery systems.

### Hemolysis study

One of the safety concerns for polymeric micelle systems is the hemolytic activity. To address this issue, the hemolytic activity of drug-free $PEG_{3.5K}$-$EB_2$ micelles was examined and compared to a strong detergent Triton X-100 and polyethylenimine (PEI), a cationic polymer known to have significant hemolytic effect.[29] As shown in Fig. 3, PEI induced hemolysis in a dose-dependent manner. In contrast, no observable hemolytic activities (< 5%) were found for $PEG_{3.5K}$-$EB_2$ micelles, suggesting the excellent safety of our new delivery system.

### *In vitro* cytotoxicity

Fig. 4 shows the cytotoxicity of $PEG_{3.5K}$-$EB_2$ in comparison with free embelin (dissolved in DMSO) in 4 cancer cell lines tested including human breast cancer cells MDA-MB-231, murine breast cancer cells 4T1, and two human prostate cancer cell lines PC3 and DU145. $PEG_{3.5K}$-$EB_2$ was comparable to free embelin in antitumor activity in all 4 cancer cell lines with $IC_{50}$ in the low μM range.

Fig. 5A compares the cytotoxicity of free PTX (in DMSO) to that of $PEG_{3.5K}$-$EB_2$-formulated PTX (5/1, m/m) in MDA-MB-231 cells. Drug-free $PEG_{3.5K}$-$EB_2$ did not cause any cytotoxicity to MDA-MB-231 cells due to its relatively low concentrations used in this study. Free PTX exhibited cytotoxicity on MDA-MB-231 cells in a dose-dependent manner. However, formulation of PTX in $PEG_{3.5K}$-$EB_2$ micelles resulted in a significant increase in the cytotoxicity. Similar results were found with three other cancer cell lines (Fig. 5B-D). Table 3 summarizes the $IC_{50}$ of free PTX and $PEG_{3.5K}$-$EB_2$-formulated PTX in the four

different cancer cell lines. Dependent on the cell lines, the $IC_{50}$ was decreased by 1.5- to 8.7-fold when PTX was formulated in $PEG_{3.5K}$-$EB_2$ micelles.

## DISCUSSION

We have developed a new delivery system that consists of an embelin-based hydrophobic domain and a PEG hydrophilic segment. The $PEG_{3.5K}$-$EB_2$ conjugate readily forms micelles in aqueous solutions. More importantly, hydrophobic drugs such as PTX can be loaded into $PEG_{3.5K}$-$EB_2$ micelles.

Various polymeric micelle systems have been reported. Most micellar systems consist of a hydrophobic core that does not have any potential therapeutic effect.[30] In addition, the metabolites of the hydrophobic segments might contribute to some undesired effects, such as inflammation and systemic toxicity.[30,31] The $PEG_{3.5K}$-$EB_2$ conjugate developed in this study represents a dual-functional delivery system that may overcome these limitations. Embelin is a natural product that demonstrates various biological effects including antitumor activity.[15] Embelin also shows excellent safety profiles in animals.[32] Thus, PEG-derivatized embelin may be an attractive delivery system to achieve synergistic activity with anticancer agents while minimizing the carrier-associated toxicity. PEG-embelin conjugates can be synthesized via direct coupling of embelin to PEG via an ester linkage. However, such synthesis is likely to yield a mixture of products with PEG randomly linked to the different hydroxyl groups in the benzene ring. We have developed a strategy to generate $PEG_{3.5K}$-$EB_2$ conjugate via total synthesis (scheme 1). This method was modified from a scheme reported by Wang's group for total synthesis of embelin.[27] Our synthesis ensures generation of structurally well-defined conjugate in which PEG is attached to 1-OH group in the quinone ring. Most of the steps give good yields and the synthesis of PEG-embelin conjugate involves similar number of steps and cost as that of embelin alone.[27]

$PEG_{3.5K}$-$EB_2$ conjugate forms small-sized micelles (20 ~ 30 nm) and loading of PTX did not significantly affect the size of the micelles. It was generally believed that particles in the size of 100 ~ 200 nm can effectively penetrate solid tumors via an EPR effect.[6] A recent study from Lam's group compared the passive targeting of nanoparticles of different sizes in a subcutaneous model of human ovarian cancer xenograft. It was shown that particles with a size of 154 nm were significantly taken up by liver and lungs with limited accumulation at tumor sites. In contrast, particles with respective size of 17 and 64 nm were much more effective in passive targeting to the solid tumor.[33] Cabral and colleagues compared the targeting efficiency of polymeric micelles of different sizes (30, 50, 70 and 100 nm) in both highly and poorly permeable tumours. While all of the tested polymer micelles penetrated highly permeable tumours in mice, only the 30 nm micelles could penetrate poorly permeable pancreatic tumors to achieve an antitumor effect.[34] The small size of our new micelle system suggests its potential for effective tumor targeting *in vivo*, which is currently being evaluated in our laboratory.

*In vitro* cytotoxicity with several cancer cell lines showed that $PEG_{3.5K}$-$EB_2$ is comparable to free embelin in antitumor activity with $IC_{50}$ in the low μM range. More importantly, $PEG_{3.5K}$-$EB_2$ synergizes with PTX in antitumor activity at much lower concentrations (~nM) in all 4 cancer cell lines tested. The $PEG_{3.5K}$-$EB_2$-mediated cytotoxicity is unlikely attributed to its surface activity as $PEG_{3.5K}$-$EB_2$ showed minimal hemolytic activity even at mM concentrations (Fig. 3). Embelin is coupled to PEG via a cleavable ester linkage. It is likely that embelin is released from the conjugate following intracellular delivery and executes the antitumor effect by itself or synergizes with PTX in antitumor activity. These data are consistent with the observation that free embelin synergizes with PTX at

subeffective doses (Fig. S4). More studies are needed to better understand the mechanism by which the $PEG_{3.5K}$-$EB_2$-based delivery system synergizes with PTX *in vitro*.

It should be noted that $PEG_{3.5K}$-$EB_2$ conjugate only represents a model micelle to demonstrate the utility of PEG-derivatized embelin as a dual functional delivery system for hydrophobic anticancer drugs. Considering the flexibility of our synthesis scheme more studies on structure-activity relationship (SAR) can be designed to further improve this new delivery system. These include optimization of the molar ratio of PEG/embelin in the conjugates, the length and structure of the acyl chain in the embelin, and the molecular weight of PEG. Recently embelin derivatives with improved affinity towards XIAP have been developed.[27] The utility of these new derivatives as drug carriers can also be examined and compared to native embelin. Finally, promising candidates identified from these studies need to be further evaluated *in vivo*. These studies are currently ongoing in our laboratory.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

This work was supported in part by NIH grants R21CA128415 and R21CA155983, and a DOD grant BC09603. We would like to thank Dr. Gong Chen at Penn State University for critical reading of this manuscript.

## REFERENCES

(1). Paál K, Müller J, Hegedûs L. High affinity binding of paclitaxel to human serum albumin. Eur. J. Biochem. 2001; 268:2187–2191. [PubMed: 11277943]

(2). Xie Z, Guan H, Chen X, Lu C, Chen L, Hu X, Shi Q, Jing X. A novel polymer-paclitaxel conjugate based on amphiphilic triblock copolymer. J. Control. Release. 2007; 117:210–216. [PubMed: 17188776]

(3). Torchilin VP. Micellar nanocarriers: pharmaceutical perspectives. Pharm. Res. 2007; 24:1–16. [PubMed: 17109211]

(4). Sutton D, Nasongkla N, Blanco E, Gao J. Functionalized micellar systems for cancer targeted drug delivery. Pharm. Res. 2007; 24:1029–1046. [PubMed: 17385025]

(5). Mi Y, Liu Y, Feng SS. Formulation of docetaxel by folic acid-conjugated D-a-tocopheryl polyethylene glycol succinate 2000 (Vitamin E $TPGS_{2k}$) micelles for targeted and synergistic chemotherapy. Biomaterials. 2011; 32:4058–4066. [PubMed: 21396707]

(6). Matsumura Y, Maeda H. A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumouritropic accumulation of proteins and the antitumour agent smancs. Cancer Res. 1986; 46:6387–6392. [PubMed: 2946403]

(7). Gao Z, Lukyanov AN, Singhal A, Torchilin VP. Diacyllipid-polymer micelles as nanocarriers for poorly soluble anticancer drugs. Nano Lett. 2002; 2:979–982.

(8). Croy SR, Kwon GS. Polymeric micelles for drug delivery. Curr. Pharm. Des. 2006; 12:4669–4684. [PubMed: 17168771]

(9). Dong Y, Feng SS. Poly(d,l-lactide-co-glycolide)/montmorillonite nanoparticles for oral delivery of anticancer drugs. Biomaterials. 2005; 30:6068–6076. [PubMed: 15894372]

(10). Zhang Z, Feng SS. Nanoparticles of poly(lactide)/vitamin E TPGS copolymer for cancer chemotherapy: synthesis, formulation, characterization and in vitro drug release. Biomaterials. 2006; 27:262–270. [PubMed: 16024075]

(11). Zhang Z, Lee SH, Gan CW, Feng SS. In vitro and in vivo investigation on PLA-TPGS nanoparticles for controlled and sustained small molecule chemotherapy. Pharm. Res. 2008; 8:1925–1935. [PubMed: 18509603]

(12). Prashant C, Dipak M, Yang CT, Chuang KH, Jun D, Feng SS. Superparamagnetic iron oxide--loaded poly(lactic acid)-D-alpha-tocopherol polyethylene glycol 1000 succinate copolymer nanoparticles as MRI contrast agent. Biomaterials. 2010; 31:5588–5597. [PubMed: 20434210]

(13). Ji X, Wang Z, Geamanu A, Sarkar FH, Gupta SV. Inhibition of cell growth and induction of apoptosis in non-small cell lung cancer cells by delta-tocotrienol is associated with notch-1 down-regulation. J. Cell. Biochem. 2011; 112:2773–2783. [PubMed: 21598300]

(14). Husain K, Francois RA, Yamauchi T, Perez M, Sebti SM, Malafa MP. Vitamin E δ-Tocotrienol augments the antitumor activity of gemcitabine and suppresses constitutive NF-κB activation in pancreatic cancer. Mol. Cancer Ther. 2011; 10:2363–2372. [PubMed: 21971120]

(15). Chitra M, Sukumar E, Suja V, Devi CS. Antitumor, anti-inflammatory and analgesic property of embelin, a plant product. Chemotherapy. 1994; 40:109–113. [PubMed: 7510605]

(16). Bhandari U, Jain N, Pillai KK. Further studies on antioxidant potential and protection of pancreatic beta-cells by Embelia ribes in experimental diabetes. Exp. Diabetes Res. 2007; 2007:15803–15808. [PubMed: 17641739]

(17). Singh D, Singh R, Singh P, Gupta RS. Effect of embelin on lipid peroxidation and free radical scavenging activity against liver damage in rats. Basic Clin. Pharmacol. Toxicol. 2009; 105:243–248. [PubMed: 19496777]

(18). Danquah M, Li F, Duke CB, Miller DD, Mahato RI. Micellar delivery of bicalutamide and embelin for treating prostate cancer. Pharm. Res. 2009; 26:2081–2092. [PubMed: 19415464]

(19). Sreepriya M, Bali G. Chemopreventive effects of embelin and curcumin against N-nitrosodiethylamine/phenobarbital-induced hepatocarcinogenesis in Wistar rats. Fitoterapia. 2005; 76:549–555. [PubMed: 16009505]

(20). Dai Y, Qiao L, Chan KW, Yang M, Ye J, Ma J, Zou B, Gu Q, Wang J, Pang R, Lan HY, Wong BC. Peroxisome proliferator-activated receptor-gamma contributes to the inhibitory effects of Embelin on colon carcinogenesis. Cancer Res. 2009; 69:4776–4783. [PubMed: 19458067]

(21). Heo JY, Kim HJ, Kim SM, Park KR, Park SY, Kim SW, Nam D, Jang HJ, Lee SG, Ahn KS, Kim SH, Shim BS, Choi SH, Ahn KS. Embelin suppresses STAT3 signaling, proliferation, and survival of multiple myeloma via the protein tyrosine phosphatase PTEN. Cancer Lett. 2011; 308:71–80. [PubMed: 21565443]

(22). Nikolovska-Coleska Z, Xu L, Hu Z, Tomita Y, Li P, Roller PP, Wang R, Fang X, Guo R, Zhang M, Lippman ME, Yang D, Wang S. Discovery of embelin as a cell-permeable, small-molecular weight inhibitor of XIAP through structure-based computational screening of a traditional herbal medicine three-dimensional structure database. J. Med. Chem. 2004; 47:2430–2440. [PubMed: 15115387]

(23). Tamm I, Kornblau SM, Segall H, Krajewski S, Welsh K, Kitada S, Scudiero DA, Tudor G, Qui YH, Monks A, Andreeff M, Reed JC. Expression and prognostic significance of IAP-family genes in human cancers and myeloid leukemias. Clin. Cancer Res. 2000; 6:1796–1803. [PubMed: 10815900]

(24). Holcik M, Gibson H, Korneluk RG. XIAP: apoptotic brake and promising therapeutic target. Apoptosis. 2001; 6:253–261. [PubMed: 11445667]

(25). Ahn KS, Sethi G, Aggarwal BB. Embelin, an inhibitor of X chromosome-linked inhibitor-of-apoptosis protein, blocks nuclear factor-kappaB (NF-kappaB) signaling pathway leading to suppression of NF-kappaB-regulated antiapoptotic and metastatic gene products. Mol. Pharmacol. 2007; 71:209–219. [PubMed: 17028156]

(26). La SB, Okano T, Kataoka K. Preparation and characterization of the micelle-forming polymeric drug indomethacin-incorporated poly(ethylene oxide)-poly(beta-benzyl L-aspartate) block copolymer micelles. J. Pharm. Sci. 1996; 85:85–90. [PubMed: 8926590]

(27). Chen J, Nikolovska-Coleska Z, Wang G, Qiu S, Wang S. Design, synthesis, and characterization of new embelin derivatives as potent inhibitors of X-linked inhibitor of apoptosis protein. Bioorg. Med. Chem. Lett. 2006; 16:5805–5808. [PubMed: 16962773]

(28). Kabanov AV, Nazarova IR, Astafieva IV, Batrakova EV, Alakhov VY, Yaroslavov AA, Kabanov VA. Micelle formation and solubilization of fluorescent probes in poly(oxyethylene-b-oxypropylene-b-oxyethylene) solutions. Macromolecules. 1995; 28:2303–2314.

(29). Reul R, Nguyen J, Kissel T. Amine-modified hyperbranched polyesters as non-toxic, biodegradable gene delivery systems. Biomaterials. 2009; 30:5815–5824. [PubMed: 19615740]

(30). Li G, Liu J, Pang Y, Wang R, Mao L, Yan D, Zhu X, Sun J. Polymeric micelles with water-insoluble drug as hydrophobic moiety for drug delivery. Biomacromolecules. 2011; 12:2016–2026. [PubMed: 21568262]

(31). 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; 99:1004–1015. [PubMed: 17596572]

(32). Kumar G,K, Dhamotharan R, Kulkarni NM, Honnegowda S, Murugesan S. Embelin ameliorates dextran sodium sulfate-induced colitis in mice. Int. Immunopharmacol. 2011; 11:724–731. [PubMed: 21296695]

(33). Luo J, Xiao K, Li Y, Lee JS, Shi L, Tan YH, Xing L, Cheng RH, Liu GY, Lam KS. Well-defined, size-tunable, multifunctional micelles for efficient paclitaxel delivery for cancer treatment. Bioconjug. Chem. 2010; 21:1216–1224. [PubMed: 20536174]

(34). Cabral H, Matsumoto Y, Mizuno K, Chen Q, Murakami M, Kimura M, Terada Y, Kano MR, Miyazono K, Uesaka M, Nishiyama N, Kataoka K. Accumulation of sub-100 nm polymeric micelles in poorly permeable tumours depends on size. Nat. Nanotechnol. 2011; 6:815–823. [PubMed: 22020122]

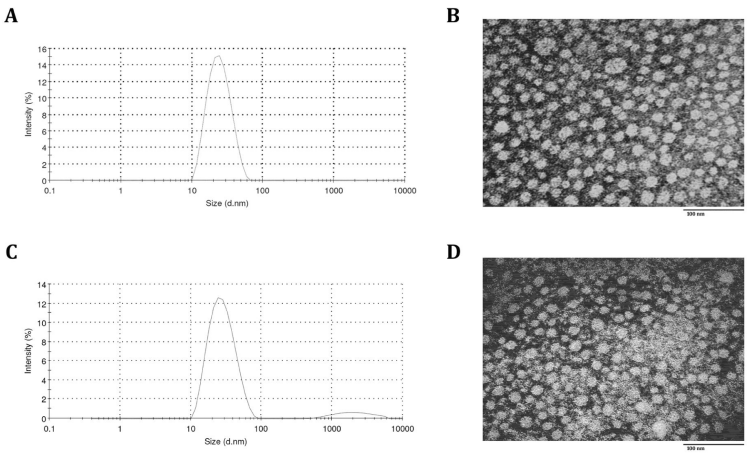



**Figure 1.**

Particle Size distribution of $PEG_{3.5K}$-$EB_2$ (A) and PTX-loaded $PEG_{3.5K}$-$EB_2$ (C); TEM images of self-assembled micelles of $PEG_{3.5K}$-$EB_2$ (B) and PTX-loaded $PEG_{3.5K}$-$EB_2$ (D). The spherical micelles with the diameter of around 20 nm were observed. The drug loading level was 1 mg/ml (PTX) in $PEG_{3.5K}$-$EB_2$.

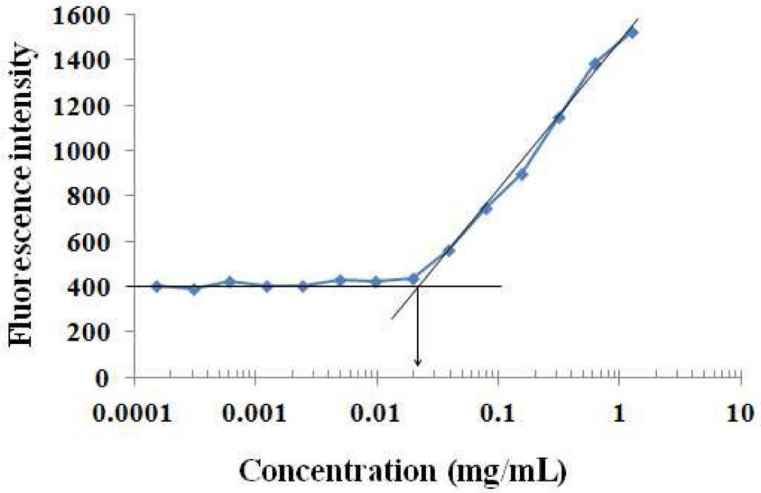



**Figure 2.**

CMC measurement of the $PEG_{3.5K}$-$EB_2$ micelles using pyrene as a hydrophobic f1uorescence probe. The f1uorescence 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 $PEG_{3.5K}$-$EB_2$ micelles [pyrene] = $6\times 10^{-7}$ mol/L. Values reported are the means ± SD for triplicate samples.

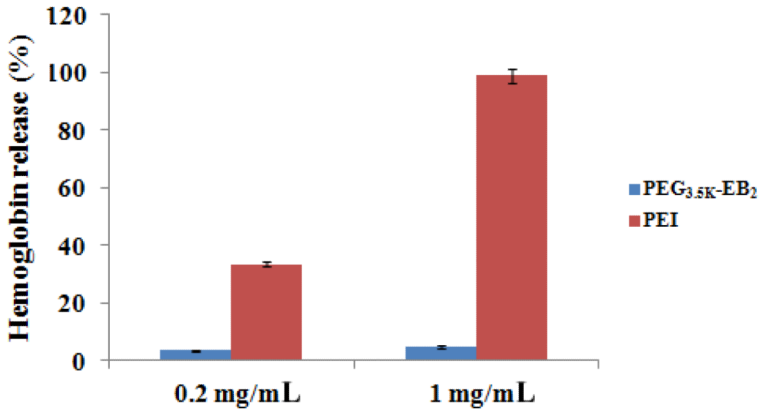



**Figure 3.**

*In vitro* hemolysis assay of $PEG_{3.5k}$-$EB_2$ compared with PEI. Both $PEG_{3.5k}$-$EB_2$ and PEI with two different concentrations (0.2, 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 measured spectrophometrically (λ=540 nm) according to the release of hemoglobin in process (2% Triton X-100 and DPBS were used as a positive and negative control, respectively). Values reported are the means ± SD for triplicate samples.

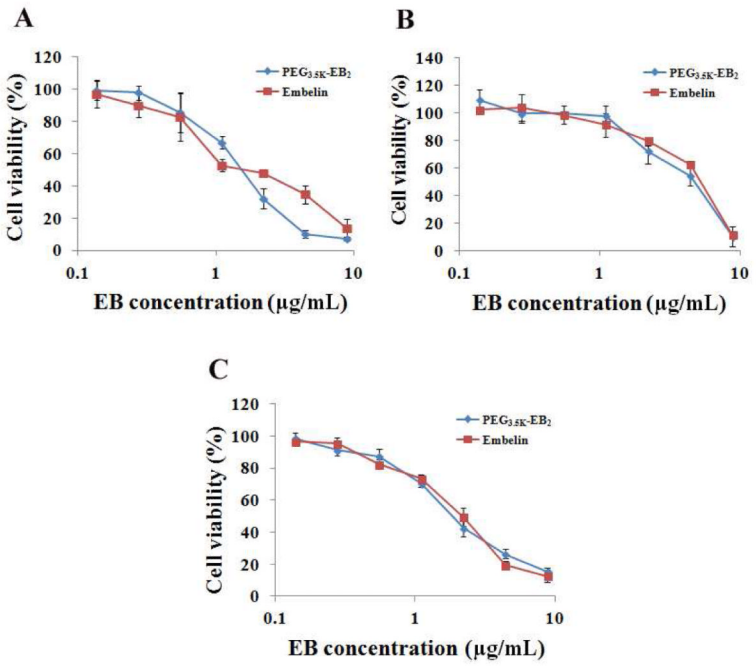



**Figure 4.**

Cytotoxicity of free EB and $PEG_{3.5K}$-$EB_2$ against the 4Tl mouse breast cancer cell line (A) and two androgen-independent human prostate cancer cell lines PC-3 (B) and DU145 (C). Cells were treated by free EB or $PEG_{3.5k}$-$EB_2$ for 72 h under the equivalent concentrations ofEB. Cytotoxicity was detennined by MTT assay.

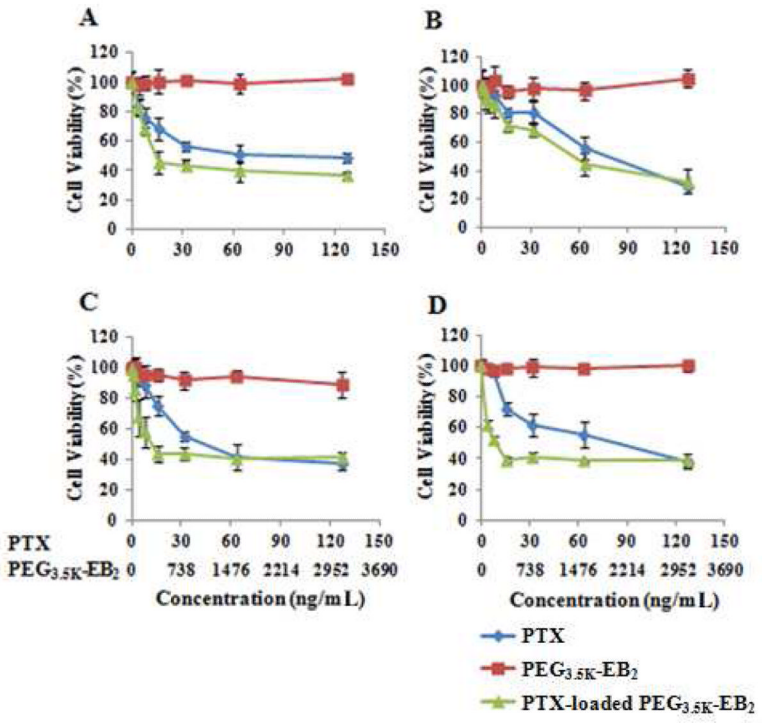
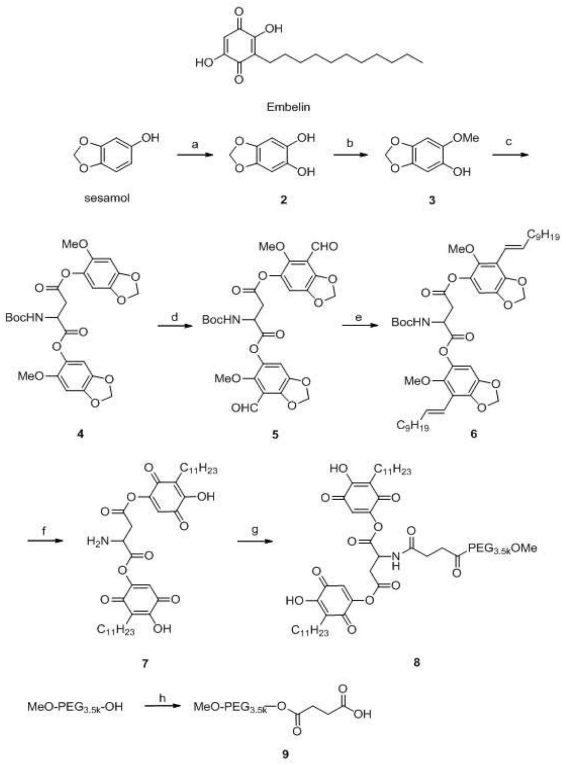



**Figure 5.**

Cytotoxicity of free PTX, free $PEG_{3.5k}$-$EB_2$, and PTX-loaded $PEG_{3.5k}$-$EB_2$ (1/5, m/m) nanoparticles against the MDA-MB-231 human brest cancer cell line (A), the 4T1 mouse brest cancer cell line (B), two androgen-independent human prostate cancer cell lined PC-3 (C) and DU145 (D). Cells were treated with free or formulated PTX for 72 h and cytotoxicity was determined by MIT assay.




**Scheme 1.**

The synthesis of $PEG_{3.5k}$-$EB_2$. Conditions: (a) water, Fremy's salt, $KH_2PO_4$, 5min; $Na_2S_2O_4$, 30 min; (b) water, MeI, NaOH, 1h; (c) Boc-Aspartic acid, DCC, DMAP, $CH_2Cl_2$ overnight; (d) MeCN, DMF, $POCl_3$; (e) THF, LiHMDS 2M in THE, decyltriphenylphosphonium bromide, 2h; (f) 1) MeOH, $H_2$ Pd/C; 2) MeCN, CAN; 3) dioxane, HCl, 2h; (g) $CH_2Cl_2$, DCC, DMAP, **9**; overnight; (h) succinic anhydride, DMAP, $CH_2Cl_2$, 2 days

## Table 1

DLS analysis of the sizes of free and drug-loaded $PEG_{3.5K}$-$EB_2$ micelles

| micelles | molar ratio | size (nm)[a] | PDI[b] |
|---|---|---|---|
| $PEG_{3.5K}$-$EB_2$ | — | 22.8±0.3 | 0.09 |
| $PEG_{3.5K}$-$EB_2$:PTX[c] | 2.5:1 | 143±17 | 0.23 |
| $PEG_{3.5K}$-$EB_2$:PTX | 5:1 | 58.7±0.5 | 0.32 |
| $PEG_{3.5K}$-$EB_2$:PTX | 7.5:1 | 27.5±0.2 | 0.23 |

[a] Measured by dynamic light scattering particle sizer (Zetasizer)

[b] PDI = polydispersity index

[c] PTX = paclitaxel

PTX concentration in micelle was kept at 1 mg/mL. Blank micelle concentration was 20 mg/mL Values reported are the means ± SD for triplicate samples

## Table 2

Physicochemical characterization of PTX-loaded $PEG_{3.5K}$-$EB_2$ micelles

| $PEG_{3.5K}$-$EB_2$:PTX (m/m) | concentration of PTX in micelles (mg/mL) | DLC[a] (%) | DLE[b] (%) | zeta[c] (mV) |
|---|---|---|---|---|
| 2.5:1 | 1.07 | 7.51 | 79.9 | 1.58±0.37 |
| 5:1 | 1.07 | 3.90 | 96.7 | 1.89±0.08 |
| 7.5:1 | 1.07 | 2.63 | 98.6 | −1.52±0.20 |
| | 2.14 | 2.63 | 97.5 | −1.29±0.19 |
| | 3.21 | 2.63 | 81.3 | −2.64±0.43 |

[a] DLC = drug loading capacity

[b] DLE = drug loading efficiency

[c] Measured by dynamic light scattering particle sizer (Zetasizer) Values reported are the means ± SD for triplicate samples.

**Table 3**

$IC_{50}$ of PTX and PTX-loaded $PEG_{3.5K}$-$EB_2$ (1/5, m/m) after 72 h incubation with different cancer cell lines

| | $IC_{50}$[a] (ng/mL) | | | |
|---|---|---|---|---|
| | **MDA-MB-231** | **4TI** | **PC-3** | **DUI45** |
| PTX-loaded $PEG_{3.5K}$-$EB_2$ | 13.5 | 51 | 12.7 | 8.9 |
| PTX | 65 | 73 | 42.3 | 78 |

[a]The concentration of a drug that is required for 50% inhibition *in vitro*.