Journal of Applied Polymer Science

Synthesis and *In Vitro* Cytotoxicity of Poly(ethylene glycol)-Epothilone B Conjugates

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ABSTRACT: Natural epothilone B (EPOB) is currently in clinical trials for treatment of advanced cancers. In this study, two poly(ethylene glycol) (PEG)–EPOB conjugates were synthesized with carbodiimide chemistry with linear PEG Methoxy-PEG-Carboxymethy(mPEG-COOH) with different molecular weights (5 and 20 kDa). The products were confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy and ^{1}H-NMR, which showed that PEGylation only took place at the 7-OH site of EPOB. The solubilities of PEG5K–EPOB the conjugate of mPEG-COOH (MW 5,000) and epothilone B and PEG20K–EPOB the conjugate of mPEG-COOH (MW 20,000) and epothilone B were determined to be 4.93×10^{-2} and 1.58×10^{-2} mmol/mL; this showed improvements of 35 and 11 times, respectively, over that of free EPOB (1.4×10^{-3} mmol/mL). Moreover, the conjugates were more stable than that of free EPOB in plasma. The cytotoxicity of conjugates was evaluated on human breast cancer MCF-7 cells with an 3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di- phenytetrazoliumromide(MTT) based assay. The half maximal inhibitory concentration of a substance(IC50) values of EPOB, PEG5K–EPOB, and PEG20K–EPOB were 6.0×10^{-4}, 0.57, and 8.4×10^{-3} μM, respectively. © 2014 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2014**, *131*, 41123.

KEYWORDS: applications; drug-delivery systems; processing and synthesis

Received 21 January 2014; accepted 6 June 2014

DOI: 10.1002/app.41123

INTRODUCTION

Epothilones are a novel class of natural cytotoxic compounds originally isolated from the myxobacterium *Sorangium cellulosum* in the early 1990s.[1,2] Natural epothilones are characterized by the letters A–F. Their chemical structure consists of a 16-membered macrocyclic ring system with a common side chain that differs in its oxidation status. Epothilones induce the hyperstabilization of microtubules similar to the that of taxanes; this leads to cell cycle arrest and apoptosis.[3] The most advanced compound is ixabepilone, a semisynthetic epothilone B (EPOB) derivative, which was approved by the FDA in 2007 for the treatment of advanced breast cancer either as single agent or in combination with capecitabine. The next in development is natural EPOB, which is currently in phase III clinical trials for the treatment of advanced ovarian cancer.[4] However, because of its poor solubility, adjuvants must be used to confer water solubility.[5,6]

Poly(ethylene glycol) (PEG) is a synthetic polymer comprised of repeating ethylene oxide subunits with a molecular weight (MW) of 44 Da; it is nontoxic, nonimmunogenic, nonantigenic, and amphiphilic.[7] PEGylation was first reported by Davies and Abuchowski in the 1970s for albumin and catalase modification. Since then, the procedure of PEGylation has been broadened and developed thereafter tremendously.[8,9] PEG conjugates of conventional drugs can increase the solubility of hydrophobic drugs, including taxol and camptothecin, and increase the oral bioavailability of poorly soluble and poorly bioavailable drugs. At the same time, PEGylation can cause drug-selective accumulation in tumors with the enhanced permeability and retention (EPR) effect[10,11] and increase the circulation half-life of rapidly eliminated compounds.[12–18]

Because of these advantages over the free form of the drug, in this study, EPOB was covalently linked to CH_3O-PEG-COOH with different MWs (5 and 20 kDa) to improve the water solubility of EPOB. The PEG–EPOBs were characterized by ^{1}H-NMR spectroscopy and matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF-MS). The stabilities of the conjugates in buffer solution at different pHs and plasma were investigated. The cytotoxicity of PEG–EPOB was evaluated on human breast cancer MCF-7 cells *in vitro*.

EXPERIMENTAL

Materials

EPOB was isolated from the fermentation extract of the myxobacterium *S. cellulosum*. mPEG–COOH 5K and mPEG–COOH 20K were purchase from Kaizheng Biotech Developing, Ltd. (Beijing, China). Dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (DMAP), and dichloromethane were obtained from Sinopharm

(China). Methanol (chromatographic pure) was purchased from Fisher Scientific (Waltham, MA). SephadexLH-20 resin was obtained from GE. All other reagents were analytical grade.

Synthesis of the PEG–EPOB Conjugates

mPEG–COOH 5K (200.0 mg, 0.04 mmol) was dissolved in 2 mL of anhydrous dichloromethane. The solution was cooled to 0°C with stirring; this was followed by the addition of DCC (16.48 mg, 0.08 mmol), 4-dimethylamiopryidine (5.04 mg, 0.04 mmol), and EPOB (40.56 mg, 0.08 mmol) under N_2. The temperature was maintained at 0°C for 2 h and then allowed to warm to room temperature with continued stirring overnight. The reaction product was filtered to remove precipitated dicyclohexylurea, which was the byproduct of the reaction of DCC with mPEG–COOH, and the solution was dropped into 200 mL of a 1:1 mixture of diethyl ether/methanol and kept at 4°C for 12 h. The residue was obtained by filtration and washed with a cold 1:1 mixture of diethyl ether/methanol three times. The residue was dried *in vacuo* and purified by Sephadex LH-20 gel filtration chromatography eluted with chloroform/methanol = 1:1. The fractions were analyzed by thin-layer chromatography (TLC; chloroform/methanol = 10/1) with an iodine assay. The fractions containing PEG–EPOB were pooled together and evaporated *in vacuo* to dry for further purification through a silica-gel chromatographic column with a gradient of chloroform/methanol (40/1 v/v, two-column volume; 30/1 v/v, two-column volume; 20/1 v/v, two-column volume; 10/1 v/v, two-column volume) as the eluent. The purified PEG–EPOB-containing fractions were pooled and dried *in vacuo* to obtain PEG5K–EPOB (97.2 mg, yield = 45%).

PEG20K–EPOB was prepared with a similar procedure to that listed previously. mPEG–COOH (20,000 kDa, 800 mg, 0.04 mol) was used to obtain the final purified product (307.6 mg, yield = 37.5%).

Structural Confirmation

^{1}H-NMR spectroscopy was performed in $CDCl_3$ with JNM-ECA-400 (JEOL, Japan).

Mass spectroscopy was performed with an Autoflex III mass spectrometer (Bruker, Germany).

Solubility of the PEG–EPOB Conjugates

The solubilities of the conjugates were determined by reverse-phase chromatography with a Waters Acquity Ultra Performance Liquid Chromatography(UPLC) system (Waters, Milford, MA) equipped with a binary pump, an auto sampler, a degasser, and a diode-array detector in the range 190–400 nm. A chromatographic column (UPLC BEH C18, 2.1 × 100 mm^2, 1.7 μm) was used and eluted with a linear gradient of A (methanol) and B (pure water) under the following conditions: 0–1 min, 55% A; 1–2.5 min, 65–80% A; 2.5–4 min, 80–100%; and 4–4.5 min, 100–55%. The flow rate was 0.4 mL/min, and the column temperature was kept at 35. The injection volume was 10 μL.

Drug Loading of the PEG–EPOB Conjugates

The drug loading on the PEG–EPOB was determined with an ultraviolet–visible spectrophotometer (Unico, China). The PEG–EPOB conjugate was dissolved in methanol and analyzed at 249 nm. The free EPOB was used as a standard for making a standard curve. The actual drug loading (EPOB mass) in the compound was determined by UV/compound mass determination.[19,20] The theoretical drug loading was equal to the EPOB MW divided by the PEG–EPOB conjugate MW.

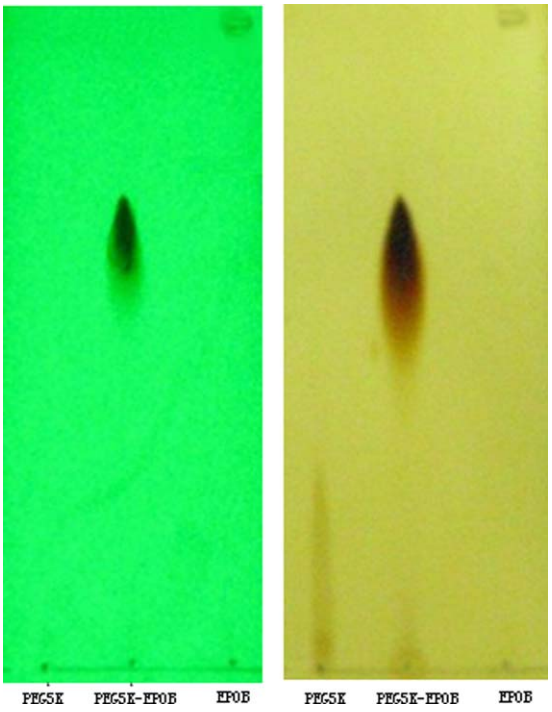

Figure 1. TLC of PEG5K–EPOB, PEG5K, and EPOB. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Stability of the Conjugates in Buffer Solution and Plasma

PEG5K–EPOB (3.7×10^{-4} m*M*) was dissolved in phosphate buffer at pH 4.0 and 7.4, and the solutions were kept at 37°C for 4 days to evaluate the stability. Samples of 200 μL were withdrawn at 1, 2, 4, 8, 24, 48, 72, and 96 h; filtered through a 0.2-μm membrane; and analyzed with UPLC as mentioned previously.

EPOB and PEG5K–EPOB (3.7×10^{-4} mmol) was dissolved in plasma at 37°C for 12 h to evaluate their stabilities in plasma. Samples of 200 μL were withdrawn at 0, 1, 2, 3, 4, 6, and 12 h; precipitated with 400 μL of CH_3CN; centrifuged at 12,000 rpm for 5 min; and filtered through a 0.2-μm membrane filter. The filtrate was analyzed by UPLC as mentioned previously.

Cytotoxicity Against Human Breast Cancer MCF-7 Cells *In Vitro*

MCF-7 cells (4×10^4) were seeded into each well of a 24-well cell culture plate. After 24 h, the culture medium was replaced with fresh medium containing various concentrations of PEG5K–EPOB, PEG20K–EPOB, and free EPOB. The cells were incubated under standard conditions for a further 72 h. A trypan blue assay was performed to determine the cell viability. Cytotoxicity data were expressed with IC50 values which represent the concentration of an inhibitor that is required for 50% inhibition of things like an enzyme, a cell, a cell receptor or a microorganism.

RESULTS AND DISCUSSION

PEG–EPOB Conjugate Synthesis and Structural Confirmation

The conjugates, PEG5K–EPOB and PEG20K–EPOB, were successfully synthesized by one-step esterification with DCC as a

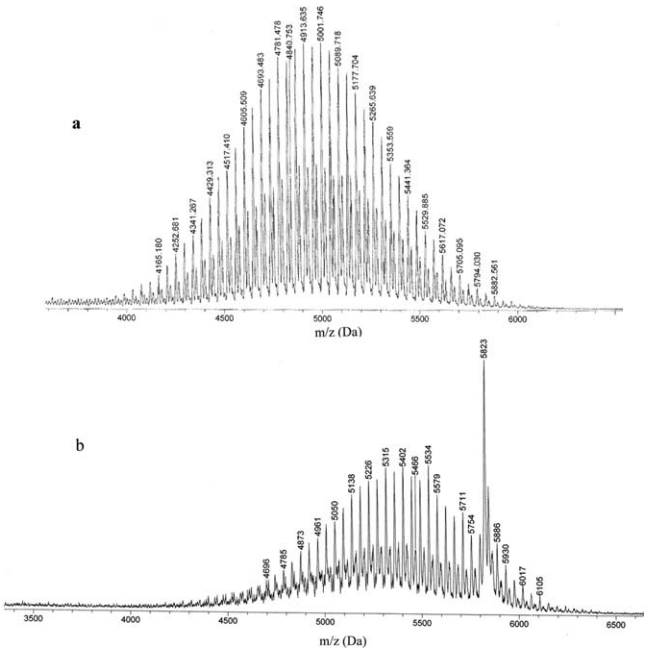

Figure 2. MAIDI-TOF-MS spectra of PEG5K and PEG5K–EPOB. Each mass peak of PEG5K–EPOB increased 489 Da compared with free PEG; this indicated that one PEG molecule conjugated exactly one EPOB molecule. PEG–EPOB(5402) = PEG–5K(4913) + EPOB(507) − H_2O (18). m/z means the ratio of the mass and charge number of fragment. mass charge ratio. As single - charged ions, m/z means the mass of fragment.

condensing agent and DMAP as a catalyst.[17,21] The product was recrystallized to remove most of small-molecular-weight impurities and then purified with size exclusion chromatography (Sephadex LH-20) to remove residual free EPOB. The final pure product was obtained with silica-gel column chromatography by a gradient eluent to remove the residual PEG, as shown by TLC (Figure 1).

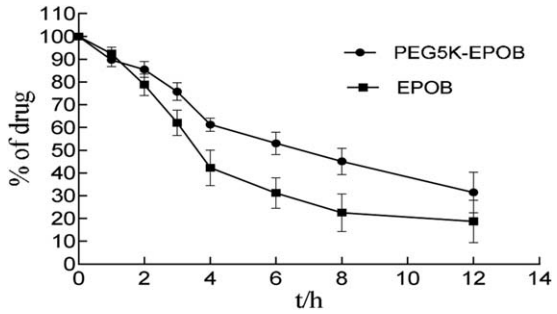

Figure 4. Stability of the PEG5K–EPOB and EPOB in plasma at 37°C. The amount of compound was characterized by the ratios of the compound's peak area at a specified time to that at the initial time. The mean values plus or minus the standard deviation from three independent experiments are presented. "t" means the time from the sample was placed in the solution to the sampling point.

The structures of the conjugates were confirmed by mass spectroscopy and ^{1}H-NMR. As shown in the MALDI-TOF-MS spectra of PEG5K–EPOB (Figure 2), each mass peak increased 489 Da compared with that of free PEG; this indicated that one PEG molecule conjugated exactly one EPOB molecule. The ^{1}H-NMR spectra of PEG5K–EPOB (Figure 3) showed that the chemical shift signal for H-7 was downshifted to δ 5.73 compared to H-7 of EPOB at δ 3.77; this demonstrated that the acylation took place selectively at the C-7-OH position of EPOB, whereas the introduction of mPEG–COOH at the C-3-OH position of EPOB was sterically hindered.

Passarella et al.[21] synthesized a novel series of dimeric epothilone A derivatives in the presence of DCC and DMAP. Esterification at the C-7-OH (rather than the C-3-OH) group of epothilone A was demonstrated by NMR spectroscopy. In this

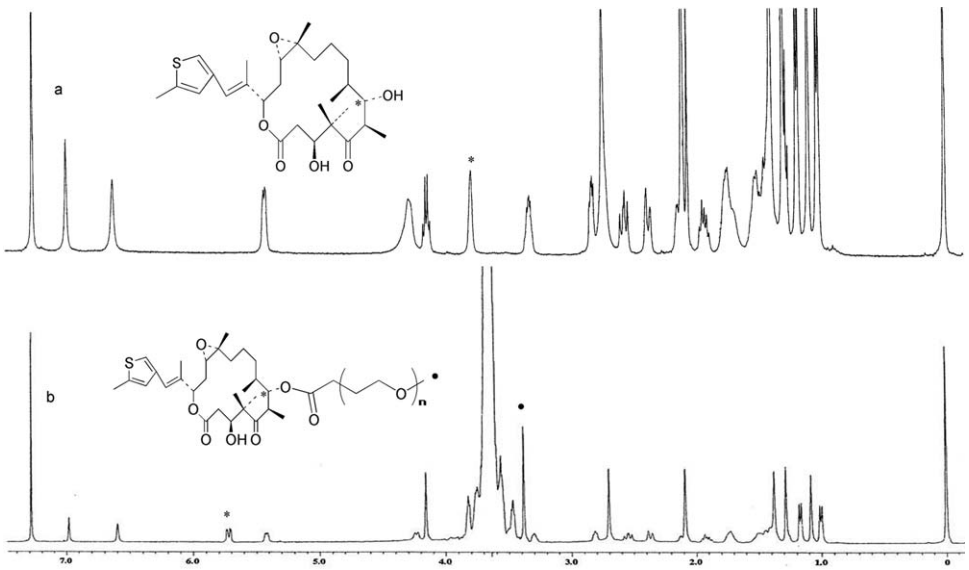

Figure 3. ^{1}H-NMR of (a) EPOB and (b) PEG5k–EPOB in $CDCl_3$. Chemical shift signal of PEG5K–EPOB for H-7 was downshifted to δ 5.73 compared to the H-7 of EPOB at δ 3.77; this demonstrated that acylation took place selectively at the C-7-OH position of EPOB.

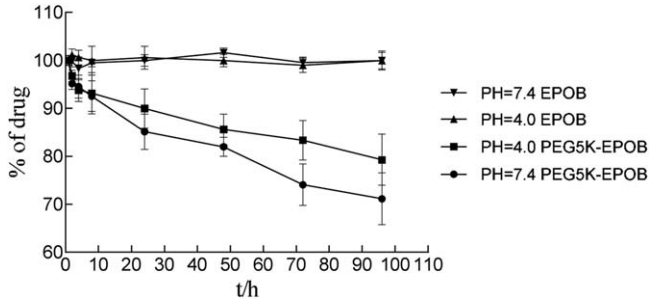

Figure 5. Stability of the PEG5K–EPOB and EPOB in phosphate buffer at pH 4.0 and 7.4. The amount of compound was characterized by the ratio of compound's peak area at a specified time to that the initial time. The mean values plus or minus the standard deviation from three independent experiments are presented.

study, the similar reaction conditions implied that PEG was likely to be attached to the C-7-OH group of EPOB because the chemical structure of EPOB was similar to that of epothilone A.

Solubility of the PEG–EPOB Conjugates

The water solubility of the conjugates was determined by reverse-phase UPLC chromatography. The determination of PEG5K–EPOB showed a good linear relationship in the range 1.04–15.3 μg related Coefficent(r). The determination of PEG20K–EPOB showed a good linear relationship in the range 5. 00–50.00 μg ($r = 0.9995$). The solubilities of PEG5K–EPOB and PEG20K–EPOB were determined to be 4.93×10^{-2} and 1.58×10^{-2} mmol/mL, respectively, whereas the solubility of free EPOB was only 1.4×10^{-3} mmol/mL. PEGylation improved the water solubility of EPOB over 35 to 11 times.

There are a host of formulations and drug design strategies that have evolved to overcome solubility limitations. Surfactants, cosolvents, soluble complicated agents, and solid-state manipulation have been used in formulation strategies available for nonelectrolytes, whereas salt formation is routinely used for ionizable drugs. Compared to this, PEG conjugates, as one polymer therapeutic, cannot only increase the solubility but can also be accumulated passively at the tumor site to reduce the systemic toxicity due to the EPR effect.[10,11]

Drug Loading of the PEG–EPOB Conjugates

The drug loading of the PEG–EPOB conjugates was determined by ultraviolet–visible spectrophotometry. EPOB, with a UV absorbance at 249 nm, showed a good linear relationship in the range 0.01–0.028 mg/mL ($r = 0.9991$). The drug loadings of PEG5K–EPOB and PEG20K–EPOB were determined to be 7.19 and 1.82%, respectively, which were lower than the theoretical drug loadings of 9.23 and 2.47%, respectively.

Stability of the PEG–EPOB Conjugates in Plasma and Buffer Solution at Different pH Values

As shown in Figure 4, the PEG5K–EPOB conjugates were more stable than free EPOB in plasma. The free EPOB itself could be degraded or metabolized to the analogue in plasma, whereas the PEG5K–EPOB conjugates was retained in this procedure. It was important to study the conjugates' stability profile under physiological conditions (pH 7.4) and tumor-cell conditions (pH 4.0). As shown in Figure 5, the free EPOB was stable in phosphate buffer both at pH 7.4 and 4.0. However, the PEG5K–EPOB conjugate was not stable, especially in phosphate buffer at pH 4.0; this suggested that the conjugate would easily release free EPOB in the lower pH environment of tumor cells.

In this study, conjugates releasing free EPOB based on a simply hydrolytic mechanism with no role of enzymes create an easier and more predictable behavior *in vivo*. Although they are relatively stable under neutral pH values, ester bonds are known to hydrolyze under both acidic and basic conditions. PEGylation caused the drug to have selective accumulation in tumors by the EPR effect. The conjugate released more active drug in the tumor cells, which will be beneficial for the selective treatment of cancer.

The process of PEG20K–EPOB releasing free EPOB was similar to the hydrolysis pattern of PEG5K–EPOB with a breakdown of the ester bond between the PEG and EPOB. As the MW of mPEG increased, the conjugates were more stable because the hydrolysis of the conjugates decreased with an increase in the length of PEG.[22,23] In this study, PEG20K–EPOB was expected to have more stability than PEG5K–EPOB because of the higher MW and longer length. However, the higher MW of PEG20K–EPOB made it difficult to be analyzed by UPLC; the exact stability of PEG20K–EPOB was not determined in our study.

Antiproliferative Activity on MCF-7

In human breast cancer MCF-7 cells, the IC50 values of EPOB, PEG5K–POB, and PEG20K–EPOB were 6.0×10^{-4}, 0.57, and 8.4×10^{-3} μM, respectively (Table I). Conjugates induced a decrease in cytotoxicity in the MCF-7 cell lines when compared to the free drug. This was similar to results obtained from most PEGylation drugs for two reasons:[14] (1) the conjugates entered the cells by the slower endocytosis pathways with respect to the free diffusion of the drug, and (2) the activity of the conjugates was present only after the drug was released from the polymer. The observed cytotoxicity of the conjugates was due to both the released drug in the media and the considerable drug loading.

Epothilones are a potential class of antitumor drug, which have been study tremendously, including total synthesis and structural modification. In 2009, Passarella et al.[21] synthesized a series of epothilone A dimeric compounds. In 2010, Vlahov[24]

Table I. IC50 Values of Different Compounds Against Human Breast Cancer MCF-7 Cells

	Compound		
	PEG5K–EPOB	PEG20K–EPOB	EPOB
IC50 (μM)	0.0006 ± 0.0002	0.5754 ± 0.0591	0.0084 ± 0.0014

synthesized a series of folate–epothilone conjugates. Moreover, one of these compounds (Epofolate, BMS-753493) is in phase I clinical trials for solid tumors. All of the results encourage the structural modification of epothilones.

CONCLUSIONS

In conclusion, this is the first report on the PEGylation of EPOB with DCC coupling chemistry. The solubility of EPOB was improved by PEGylation. The stability of PEG5k–EPOB showed potential advantages of slow-releasing active compounds to sustain activity. The cell growth inhibition assay demonstrated that the conjugates remained active against MCF-7 cell lines, even though the conjugates had higher IC50 values compared with the free drug. However, the *in vitro* assay could not be used to evaluate the advantage for prolonging the half-life of the conjugate *in vivo*. Further work will involve antitumor and pharmacokinetics experiments *in vivo*. We expect that *in vivo* studies could manifest the advantages of PEGylation.

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