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Block Copolymer Micelles with Acid-labile Ortho Ester Side-chains: Synthesis, Characterization, and Enhanced Drug Delivery to Human Glioma Cells

Rupei Tang[1,2], **Weihang Ji**[1], **David Panus**[1], **R. Noelle Palumbo**[1], and **Chun Wang**[1,*]

[1]Department of Biomedical Engineering, University of Minnesota, 7-105 Hasselmo Hall, 312 Church Street S. E., Minneapolis, MN 55455

[2]School of Medicine and Pharmaceutics, Jiangnan University, 1800 Lihu Road, Wuxi 214122, P. R. China

Abstract

A new type of block copolymer micelles for pH-triggered delivery of poorly water-soluble anticancer drugs has been synthesized and characterized. The micelles were formed by the self-assembly of an amphiphilic diblock copolymer consisting of a hydrophilic poly(ethylene glycol) (PEG) block and a hydrophobic polymethacrylate block (PEYM) bearing acid-labile ortho ester side-chains. The diblock copolymer was synthesized by atom transfer radical polymerization (ATRP) from a PEG macro-initiator to obtain well-defined polymer chain-length. The PEG-*b*-PEYM micelles assumed a stable core-shell structure in aqueous buffer at physiological pH with a low critical micelle concentration as determined by proton NMR and pyrene fluorescence spectroscopy. The hydrolysis of the ortho ester side-chain at physiological pH was minimal yet much accelerated at mildly acidic pHs. Doxorubicin (Dox) was successfully loaded into the micelles at pH 7.4 and was released at much higher rate in response to slight acidification to pH 5. Interestingly, the release of Dox at pH 5 followed apparently a biphasic profile, consisting of an initial fast phase of several hours followed by a sustained release period of several days. Dox loaded in the micelles was rapidly taken up by human glioma (T98G) cells *in vitro*, accumulating in the endolysosome and subsequently in the nucleus in a few hours, in contrast to the very low uptake of free drug at the same dose. The dose-dependent cytotoxicity of the Dox-loaded micelles was determined by the MTT assay and compared with that of the free Dox. While the empty micelles themselves were not toxic, the IC_{50} values of the Dox-loaded micelles were approximately ten-times (by 24 hours) and three-times (by 48 hours) lower than the free drug. The much enhanced potency in killing the multi-drug-resistant human glioma cells by Dox loaded in the micelles could be attributed to high intracellular drug concentration and the subsequent pH-triggered drug release. These results establish the PEG-*b*-PEYM block copolymer with acid-labile ortho ester side-chains as a novel and effective pH-responsive nano-carrier for enhancing the delivery of drugs to cancer cells.

*Corresponding author. Telephone: 612-626-3990; Fax: 612-626-6583; wangx504@umn.edu.

Supporting Information Available. ^{1}H and ^{13}C-NMR spectra of the methacrylate monomer EYM, UV spectra of Dox before and after alkaline neutralization treatment, quantification of nuclear localization of Dox in T98G cells, cytotoxicity of empty micelles in dendritic cells.

Keywords

Block copolymer; micelles; ortho ester; anticancer drug delivery; glioma

1. Introduction

Many potent anticancer drugs are hydrophobic with poor solubility in water. The delivery of such molecules aiming to enhance their bioavailability has been a tremendous challenge. Core-shell-type micelles formed by amphiphilic block copolymers are potentially excellent carriers for hydrophobic drugs, because they have the capacity of loading such drugs in their hydrophobic core [1-4]. A number of block copolymers has been reported in the past – most of them utilized polyethylene glycol (PEG) to form the hydrophilic shell of micelles [5]. There has been a greater variety of choices for the hydrophobic core-forming polymer block, of which some of the most studied included poly(α-hydroxy esters) [6] (such as polylactide [7], polyglycolide [8], poly(ε-caprolactone) [9]), polyether [10], hydrotrophic polymers [11], poly(amino acids) [12]. The incorporation of poorly water-soluble drugs into these micelles can be realized by both physical (i.e. partitioning/solubilization) and chemical (i.e. covalent conjugation) means. In addition to much improved drug loading, block copolymer micelles enter tumor cells through endocytosis, achieving high intracellular drug concentration and overcoming multi-drug resistance by bypassing efflux pumps in the cell membrane [10,13]. Furthermore, with sizes in the nanometer range, long-circulating block copolymer micelles can accumulate preferentially in solid tumor tissue due to the enhanced permeability and retention (EPR) effect [14]. Finally, it is often possible to incorporate targeting moieties to target micelles specifically to tumor tissue [15].

Controlling the release of anticancer drugs from block copolymer micelles using pH as a trigger has been an attractive approach to enhance tumor-killing efficacy and to minimize harmful side-effects. The existence of mildly acidic microenvironments both in the interstitium of solid tumors and in the endolysosome of tumor cells [16] provides the rationale for designing elaborate pH-responsive block copolymer micelles that would stabilize anticancer drugs at physiological pH and unload the drugs in a pH range of 5 and 6. There are several general approaches to such polymer systems that undergo chemical transitions around the critical pH range of 5 to 6. One approach relies on “titratable” or “protonizable” chemical groups such as amines and carboxylic acids as part of the core-forming polymer block [17-21]. Using amines with different chemical structures and different pKa values, it is possible to tune the chemical transition behavior of the polymeric micelles, which is accompanied by changes in solubility and release of drugs. Another approach is to introduce acid-labile chemical bonds (such as hydrazone) as linkers to attach drugs covalently to the core-forming polymer block [22-29]. Drug release is accomplished when the linker is cleaved at acidic pH. A third approach is to incorporate acid-labile bonds into the main-chain of the core-forming polymer block, such as polyacetal [30] and poly(β-amino esters) [31,32], which hydrolyzes rapidly at acidic pH, leading to micelle dissolution and drug release.

Recent studies have shown that core-forming polymer blocks with acid-labile side-chains can endow block copolymer micelles with pH-sensitivity that is readily tuned and controlled. For example, trimethoxybenzylidene acetals have been built into the side-chains of PEG-*b*-poly(aspartic acid) [33] and PEG-*b*-polycarbonate [34]. These polymers formed micelles with low critical micelle concentration (CMC) and small sub-100-nm size, and accelerated acetal hydrolysis at mildly acidic pH triggered the release of a hydrophobic fluorescent dye (Nile Red). We [35] and others [36-39] have reported on PEG-*b*-polymethacrylamides containing acid-labile ortho ester side-chains and characterized in

detail the pH-sensitive side-chain hydrolysis and its influence on the physico-chemical properties of the block copolymer micelles. Although it has been proposed that such amphiphilic block copolymers with acid-labile side-chains may be promising nano-carriers for hydrophobic anticancer drugs, the incorporation and release of actual drugs from such block copolymer micelles and the efficacy of killing cancer cells has not been demonstrated.

Here we describe the synthesis and characterization of a new amphiphilic block copolymer consisting of PEG and a polymethacrylate derivative, poly(2-ethoxytetrahydrofuran-2-yloxyethyl methacrylate) (PEYM), bearing acid-labile ortho ester side-chains, which formed pH-sensitive core-shell-type micelles in water. Doxorubicin (Dox) was loaded into the hydrophobic core of the micelles, and the pH-sensitive release of Dox was demonstrated. The uptake of Dox-loaded micelles into a line of multi-drug resistant human glioma cells *in vitro* was examined using flow cytometry and confocal fluorescence microscopy. The cytotoxicity against the human glioma cells by Dox-loaded micelles was determined and found to be much enhanced over that of the free drug.

2. Materials and methods

2.1 Chemicals

Monomethoxy-PEG (average M_n of 5000) was purchased from Polysciences and was used after vacuum drying at 80°C for 2 h. Tetrahydrofuran (THF) and toluene (Aldrich) were dried by refluxing over sodium and benzophenone and distilled. Acetonitrile, dichloromethane, and methanol were dried by distillation over CaH_2. 2,2,2-trifluoro-*N*-(2-methoxy-[1,3]-dioxolan-4-ylmethyl) acetamide and PEG macro-initiator for ATRP were synthesized as described elsewhere [35]. Copper (I) bromide (CuBr) and 1,1,4,7,7-pentamethyldiethylenetriamine (PMDETA), and doxorubicin hydrochloride were purchased from Sigma.

2.2 Synthesis of 2,2-diethoxytetrahydrofuran (Compound 1)

Triethyoxonium tetrafluoborate (25.00 g, 0.13 mol) was dissolved in γ-butyrolactone (11.33 g, 0.13 mol) under argon, and set for 72 h. To the mixture was added dichloromethane (20 mL), stirred, cooled to 0°C, and sodium ethoxide solution (49 mL, 0.13 mol, 21 wt% in ethyl alcohol) was added dropwise. The mixture was stirred at room temperature, added to 200 mL of saturated aqueous Na_2CO_3 solution, extracted by ethyl ether, dried over $MgSO_4$, and distilled under vacuum to yield 13.94 g (66%) of 2,2-diethoxytetrahydrofuran as colorless oil. ^{1}H NMR (300MHz, $CDCl_3$): δ (ppm) 1.09-1.21 (m, 6H, CH_3), 1.82-1.87 (m, 2H, CH_2), 2.30-2.35 (t, 2H, CH_2), 3.35-3.40 (m, 4H, OCH_2CH$_3$), 4.02-4.09 (q, 2H, OCH_2). ^{13}C NMR ($CDCl_3$, δ ppm): 14.25, 15.19, 25.13, 31.13, 60.27, 66.13, 69.45, 173.58.

2.3 Synthesis of 2-(2′-hydroxyethoxy)-2-ethoxytetrahydrofuran (Compound 2)

A mixture of compound 1 (7.60 g, 47.44 mmol), ethylene glycol (11.78 g, 189.79 mmol), and *p*-toluene sulfonic acid (*p*TSA; a trace amount) was heated at 130°C until no volatile component was distilled. After cooling to room temperature, the residue was dissolved in ethyl acetate (250 mL), washed with aqueous Na_2CO_3 solution and brine, dried over $MgSO_4$, and evaporated to yield 6.94 g (83%) of 2-(2·-hydroxyethoxy)-2-ethoxytetrahydrofuran as colorless oil. ^{1}H NMR (300MHz, $CDCl_3$): δ (ppm) 1.15-1.20 (t, 3H, CH_3), 1.89-1.94 (m, 2H, CH_2), 2.42-2.47 (t, 2H, CH_2), 3.42-3.61 (m, 4H, OCH_2), 3.69-3.82 (m, 2H, CH_2OH), 4.19-4.23 (t, 2H, OCH_2). ^{13}C NMR ($CDCl_3$, δ ppm): 15.22, 25.15, 30.97, 31.42, 61.07, 62.16, 63.43, 66.01, 69.17, 72.43, 173.92.

2.4 Synthesis of 2-ethoxytetrahydrofuran-2-yloxyethyl methacrylate (monomer EYM)

Compound 2 (4.33 g, 24.58 mmol) and *N*,*N*-diisopropylethylamine (DIPEA) (6.53 g, 49.15 mmol) were dissolved in dichloromethane (100 mL), and cooled to −20°C under argon. Methacryloyl chloride (5.65 g, 27.03 mmol) in 20 mL of dichloromethane was then added dropwise, kept stirring for 2 h, and slowly warmed to room temperature. The mixture was vigorously stirred overnight, and 150 mL of dichloromethane was added. The mixture was washed with aqueous Na_2CO_3 solution and brine, dried over $MgSO_4$, and evaporated. The residue was purified with column chromatography (silica gel, hexane/ethyl acetate (1/5) as eluent) to yield 4.23 g (70%) of 2-ethoxytetrahydrofuran-2-yloxyethyl methacrylate as yellowish oil. ^{1}H NMR (300MHz, $CDCl_3$): δ (ppm) 1.17-1.21 (t, 3H, CH_3), 1.91-1.96 (m, 5H, CH_2, CH_3), 2.42-2.47 (t, 2H, CH_2), 3.43-3.48 (m, 4H, O-CH_2), 4.33-4.36 (m, 4H, O-CH_2), 5.60-6.14 (d, 2H, C=CH_2). ^{13}C NMR ($CDCl_3$, δ ppm): 15.23, 18.34, 25.11, 31.01, 62.06, 62.52, 69.38, 126.13, 136.01, 167.21, 173.40. ESI-MS Calcd for ($C_{12}H_{20}O_5$), 244.3; found m/z, 245.2 ($M + H^+$), 267.2 ($M + Na^+$).

2.5 Synthesis and characterization of poly(ethylene glycol)-block-poly(2-ethoxytetrahydrofuran-2-yloxyethyl methacrylate) (PEG-b-PEYM)

The diblock copolymers (PEG-*b*-PEYM) were synthesized by ATRP as follows. A glass two-neck flask was charged with PEG macro-initiator, monomer EYM, CuBr, and PMDETA, at three molar ratios of 1:(30,50,100):1:1. The system was degassed three times. Degassed toluene was then added and the solution was stirred at 90°C overnight. After opening to the air to stop the ATRP and cooling to room temperature, the reaction solution was passed through an aluminum oxide column to remove the copper catalyst, and the crude product was obtained by precipitating in hexane. The resulting solid was then dissolved in dichloromethane, filtered, and precipitated again in hexane. The polymers were dried overnight in vacuum at room temperature. The ^{1}H and ^{13}C-NMR spectra of the block polymers were recorded on a Varian Unity spectrometer (300 MHz), and chemical shifts were recorded in ppm. High-resolution electrospray-ionization (ESI) mass spectrum of the EYM monomer was obtained on a Bruker MicroTOF-Q mass spectrometer. The molecular weight and polydispersity of the block copolymers were determined using a gel permeation chromatography (GPC) system equipped with a Waters 590 HPLC pump, a Waters 2410 differential refractometer, and three Waters Styragel HR1, HR2, and HR3 columns calibrated with narrowly-dispersed polystyrene standards. THF was used as eluent at a flow rate of 1.0 mL/min at 35°C. All sample solutions were filtered through a 0.45-μm filter before injecting into the GPC system. The number average molecular weights of three block polymers were determined by GPC to be 1.08, 1.37, and 1.61×10^4 with the polydispersity indices of 1.21, 1.27, and 1.17, respectively. The numbers average degrees of polymerization of the PEYM block were calculated to be 23, 35, and 45. Therefore, these diblock copolymers are denoted as PEG-*b*-$PEYM_{23}$, PEG-*b*-$PEYM_{35}$, and PEG-*b*-$PEYM_{45}$. Micelles formed by PEG-*b*-$PEYM_{45}$ were characterized in details in the following sections.

2.6 Preparation of polymeric micelles

The diblock copolymer PEG-*b*-$PEYM_{45}$ (10 mg) was dissolved in DMSO (1 mL), placed in a semi-permeable dialysis membrane (MW cut-off: 3500), and dialyzed against water with 0.1% triethylamine for 24 h. The polymeric micelles obtained were then lyophilized. Before characterization experiments, the dried micelle solids were directly added to phosphate buffer (20 mM, pH 7.4) and phosphate-citrate buffer (20 mM, pH 6, 5, and 4), vortexed, incubated at room temperature for 15 min, and filtered through PTFE membrane filters with 0.45-μm pores. Micelles were also dissolved in deuterated PBS (20 mM, pH 7.4) and the ^{1}H-NMR spectra were recorded.

2.7 Determination of the critical micelle concentration (CMC)

The CMC of the PEG-*b*-PEYM$_{45}$ micelles was determined using pyrene as a fluorescence probe. A pyrene solution in acetone (1.8×10^{-4} mol/L) was added to 3.0 mL of polymer solution of different concentrations ranging from 1×10^{-5} to 1.0 mg/mL in 20 mM phosphate buffer (pH 7.4) and the final pyrene concentration was 6×10^{-7} M. The samples were incubated for 15 h at room temperature in the dark. The excitation spectra were recorded with a Quantamaster Fluorimeter (PTI London, Ontario) at room temperature from 300 to 350 nm with the emission wavelength at 390 nm and emission and excitation slit widths of 2 nm. The intensity ratio of pyrene at 337 and 334 nm was plotted against the polymer concentration to determine the CMC.

2.8 Dynamic light scattering (DLS)

The average hydrodynamic diameter of the PEG-*b*-PEYM$_{45}$ micelles (in 1-mg/mL solution) and the dependence of micelle size on pH (7.4, 6, 5, 4) and incubation time at 25°C were measured using a ZetaPlus dynamic light scattering detector (Brookhaven Instruments Corporation, Holtsville, NY; 27 mW laser; 658 nm incident beam, 90° scattering angle).

2.9 Cryogenic transmission electron microscopy (cryo-TEM)

A droplet of micelle solution (1 mg/mL in 20 mM phosphate buffer, pH 7.4, or in 20 mM acetate buffer, pH 4, incubated for 24 h) was placed on a carbon-coated, lacey film-supported, micro-perforated TEM copper grid. The excess solution was blotted with a piece of filter paper. The sample grids were then quickly plunged into liquid nitrogen and kept there until imaging. The TEM samples were examined on a JEOL 1210 transmission election microscope operating at 120 kV. Vitrified specimens were mounted on a cryogenic sample holder (Gatan 626). Adequate phase contrast was obtained at a nominal underfocus of 3-15 μm. Images were recorded with a Gatan 724 multiscan digital camera.

2.10 Determination of the rate of micelle side-chain hydrolysis

PEG-*b*-PEYM$_{45}$ solutions (2 mg/mL) were prepared in phosphate buffer (20 mM, pH 7.4) and sodium acetate buffers (20 mM, pH 6, 5, and 4). The amount of ethanol released as a result of ortho ester side-chain hydrolysis was quantified at various time points over 48 h using an alcohol dehydrogenase assay kit following the instructions from the vendor (BioChain, Hayward, CA).

2.11 Preparation of micelles loaded with doxorubicin

Doxorubicin hydrochloride (Dox-HCl, 10 mg) was dissolved in DMSO (1 mL) containing four molar equivalent of triethylamine (0.01 mL), and stirred overnight. To this alkaline Dox solution was added 20 mg of PEG-*b*-PEYM$_{45}$ in 1 mL of DMSO followed by aqueous 20 mM phosphate buffer (pH 7.4) added dropwise under vigorous stirring. The resulting solution was continuously stirred for another 4 to 6 h, and was then dialyzed against 500 mL of de-ionized water containing a trace amount of triethylamine for 24 h using Spectra/Por dialysis membrane (MW cut-off: 3500). The solution was filtered through a 0.45-μm filter and freeze–dried to obtain micelles loaded with Dox (“micelle-Dox”). The same procedure was used to prepare micelles loaded with coumarin-6. To determine the loading content of Dox, Dox-loaded micelles were dissolved in DMSO and the absorbance at 480 nm was recorded using a Bio-Tek Synergy HT plate reader. The amount of Dox loaded in micelles was calculated according to a standard curve obtained using DMSO solutions of free Dox of known quantities.

2.12 Cellular uptake of micelles loaded with coumarin-6

The uptake of coumarin-6-loaded micelles in T98G human glioma cells was analyzed using flow cytometry and confocal fluorescence microscopy. T98G cells (American Type Cell Culture, ATCC) were maintained in DMEM media (Gibco) with 10% FBS and penicillin/streptomycin (100 units/mL) and cultured at 37°C in 5% CO_2. The cells were plated at 1.5×10^5 cells/well in six-well plates overnight before polymer micelles were added to a final concentration of 0.2 mg/mL in 2 mL. For flow cytometry analysis the cells were cultured with micelles for 1, 3, and 24 h at 37°C, washed thrice with cold PBS, removed from the wells with trypsin-EDTA, and analyzed using a Becton Dickson FACSCalibur flow cytometer and Flowjo software. Coumarin-6 was excited with a 488-nm laser and emission detected with a 585-nm filter. The cells were also stained with Alexa Fluor 594-labeled concanavalin A (Molecular Probes) to visualize cell membrane, and analyzed using a Bio-Rad MRC 1000 laser on an upright microscope equipped with a Nikon Optiphot camera. Coumarin-6 was excited at 488 nm and emission detected with a 522-nm DF32 band-pass filter. Labeled concanavalin A was excited at 568 nm and detected with a 605-nm DF32 band-pass filter. Image analysis was conducted using Bio-Rad Laser Sharp 3.0 and Image J softwares.

2.13 Dox release from micelles and pH-dependence

Dox-loaded PEG-*b*-$PEYM_{45}$ micelle solution (4 mg/mL, 1 mL, in PBS pH 7.4) was placed in disposable dialyzers (Spectra/Por, MW cut-off: 3500), and immersed in 2 L of buffers with pH 7.4 and 5. At 1-h intervals, triplicate samples (100 μL each) of the micelle solution inside the dialyzer were removed, and the UV absorbance was recorded at 480 nm using a Bio-Tek Synergy HT plate reader. The sample solutions were then returned to the dialyzer, and the dialysate was replaced with 2 L of fresh buffer. The amount of Dox released was determined by the decrease in absorbance of the solution inside the dialyzers.

2.14 Cellular uptake of micelles loaded with doxorubicin

The uptake of micelle-Dox by T98G human glioma cells was analyzed by confocal fluorescence microscopy and was compared with that of the free Dox. T98G cells were plated on two 4-well chamber slides (Lab-Tek) at a density of 60,000 cells per well in low-glucose DMEM media containing 10% FBS (both from Gibco) and were cultured for 24 h. After washing the cells with PBS, fresh media with 10% FBS was added. In separate chamber slides, micelle-Dox and free Dox were added to the cells at a final Dox concentration of 0.15 μg/mL and allowed to incubate at 37°C, 5% CO_2, and 99% humidity for 1, 4, and 11 h. Prior to imaging, cell media was removed and cells were washed twice with cold PBS and fixed using BD Cytofix (BD Biosciences) at 4°C for 10 min. The cells were then mounted using VectaShield (Vector Laboratories) mounting media containing DAPI to label the cell nucleus. Images of cellular uptake of micelle-Dox as well as free Dox were captured with an Olympus FV1000 IX2 inverted confocal microscope using a 40×/1.30NA Olympus oil objective and FV10-ASW imaging software (Olympus). Dox was excited using a 543 nm wavelength nitrogen laser with a 555-655 nm band-pass emission filter. A nitrogen laser with a wavelength of 405 nm was used to excite DAPI and emission was detected using a 405-505 nm band-pass filter. Captured images were then merged in Image-J software with the median z-slice image used for representation.

2.15 Cytotoxicity assay

The cytotoxicity of free drug, micelles, and Dox-loaded micelles were evaluated by an MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. T98G glioma cells were seeded into 96-well plates at 6000 cells/well, cultured overnight, and incubated with free drug, empty micelles, and Dox-loaded micelles of various concentrations for 24, and 48

h in T98G glioma cell media (DMEM low-glucose, 10% fetal bovine serum, 10 mM HEPES, 100 U/mL penicillin/streptomycin) at 5% CO_2 and 37°C. MTT in PBS (5 mg/mL, 20 μL) was added to each well reaching a final concentration of 0.5 mg/mL. After 4 hours un-reacted MTT was removed by aspiration. The formazan crystals were dissolved in 100 μL DMSO and the absorbance was measured at 570 nm using a Bio-Tek Synergy HT plate reader. Cell viability was calculated by [Absorbance of cells exposed to micelles or drug] / [Absorbance of cells cultured without micelles or drug] in percentage.

3. Results

3.1 Block copolymer synthesis

The synthetic pathway of poly(ethylene glycol)-*block*-poly(2-ethoxytetrahydrofuran-2-yloxyethyl methacrylate) (PEG-*b*-PEYM) bearing acid-labile ortho ester side-chains is shown in Scheme 1. The methacrylate monomer, EYM, containing a five-membered ortho ester ring, was synthesized in three steps. First, compound 1 containing the ortho ester ring was obtained from reacting γ-butyrolactone with triethyoxonium tetrafluoborate and sodium ethoxide solution. Trans-esterification with excess ethylene glycol catalyzed by *p*-TSA resulted in the generation of compound 2, whose single hydroxyl group was then efficiently converted to methacrylate by reacting with methacryloyl chloride in the presence of DIPEA. The correct chemical structure of the final product (Scheme 1) was confirmed by ^{1}H and ^{13}C-NMR spectroscopy (Supporting Information, Fig. S1).

PEG-*b*-PEYM block copolymers were synthesized by ATRP (Scheme 1) using a PEG macro-initiator (M_n 5,000). The polymerization of the methacrylate monomer EYM was carried out in toluene using PDMETA as ligand. Different amount of EYM in the feed resulted in PEYM blocks of different length. The results of polymerization and molecular weight analysis by GPC and NMR are summarized in Table 1. All the block copolymers were synthesized with good yield and appeared as single peaks in the GPC chromatograms with narrow molecular weight distribution. The polydispersity index (PDI) ranges from 1.17 to 1.27, suggesting that controlled polymerization by ATRP was successful. The ^{1}H-NMR spectrum of the block copolymers was acquired in $CDCl_3$, a good solvent, which contained signals from protons of the PEG and PEYM blocks (Fig. 1A). The average M_n of the block copolymers were determined by integrating the area of peaks characteristic of the PEG protons (peak “a”) and of the PEYM protons (peaks “d” and “h”), which agree well with the average M_n values determined by GPC using $CHCl_3$ as eluant and polystyrene as standard (Table 1). The average numbers of repeating unit of the PEYM block were estimated to be 23, 35, and 45, for the three block copolymers, which are thus denoted as PEG-*b*-$PEYM_{23}$, PEG-*b*-$PEYM_{35}$, and PEG-*b*-$PEYM_{45}$, respectively.

3.2 Formation of core-shell-type micelles

Based on the chemical structure, the amphiphilic PEG-*b*-PEYM block copolymers are expected to self-assemble into core-shell-type micelles in aqueous solvents. Indeed, micelles were formed in alkaline water through a dialysis process [3]. The core-shell structure of the micelles is established based on evidence from NMR study and the fluorescence spectroscopy of pyrene – both methods have been previously applied to other block copolymer micelle systems. First, NMR experiment on micelles dissolved in deuterated chloroform, a good solvent for both the PEG and PEYM blocks, showed clear signals of protons of both blocks (Fig. 1A, peak “a”), whereas micelles in deuterated water showed clear signals only for the PEG block and much diminished signals for the PEYM block (Fig. 1B, peaks “b” through “j”). This suggests that the PEG block is the fully hydrated corona and the PEYM block, with its hydrophobic ortho ester methacrylate structure, forms the dehydrated micellar core. Second, the excitation spectra of pyrene in PEG-*b*-$PEYM_{45}$

aqueous solutions of increasing concentrations showed a red-shift of the low-energy band from 334 to 337 nm, indicating that the hydrophobic pyrene molecule partitioned from the aqueous environment to the hydrophobic core of the micelles (Fig. 2A). Furthermore, the intensity ratio of I_{337}/I_{334} was dependent on the logarithm of the block copolymer concentration, and the critical micelle concentration (CMC) of PEG-*b*-PEYM$_{45}$ was determined to be 4.28×10^{-3} g/L, a value that is comparable to other known polymeric micelles such as PEG-*b*-PLA [41]. Finally, the size of the PEG-*b*-PEYM$_{45}$ micelles in PBS (pH 7.4) was determined by dynamic light scattering. The experiment was completed using freshly prepared micelle solution in a time frame that the ortho ester side-chain hydrolysis was negligible. The micelles exist as a single size distribution with an average diameter of 74 nm and a polydispersity of 0.226 (Fig. 3A). Cryo-TEM shows that the micelles are discrete and spherical in shape at pH 7.4 (Fig. 3B).

3.3 Hydrolysis of the acid-labile ortho ester side-chains

It is anticipated that the ortho ester bond on the side-chain of the PEYM block would be readily cleaved by hydrolysis at accelerating rate under acidic pH, given the well-known hydrolytic reactivity of the ortho ester established through extensive investigation of the biodegradable poly(ortho esters) by Heller and co-workers [42-44]. The five-membered cyclic ortho ester reported here is the same structure as what was used in the main-chain of the first-generation poly(ortho esters) known as “POE I” [42]. Based on these previous studies, the pathway of hydrolysis of PEG-*b*-PEYM is depicted in Scheme 2. The ortho ester ring undergoes exocyclic cleavage, generating butylactone (which quickly opens to yield hydroxyl butyric acid), ethanol, and PEG-*b*-poly(hydroxyethyl methacrylate) (PEG-*b*-PHEMA). The effect of ortho ester side-chain hydrolysis on the size and morphology of the block copolymer micelles is apparent according to the results of dynamic light scattering measurement and cryo-TEM. After hydrolysis at pH 4 for 24 h, the distribution of micelle size broadened and the average diameter increased to over 100 nm (Fig. 3A). As a result, both large (100 to 200 nm or more) and very small particles (20 to 40 nm) have appeared (Fig. 3). These particles are presumably aggregates of the degradation product, PEG-*b*-PHEMA. Despite its highly hydrophilic nature, water-solubility of PHEMA is known to be dependent on its chain-length: only short oligomers of HEMA below 20 repeating units are soluble [45]. In our case, the average length of the PHEMA block is 45 repeating units, which should render the polymer insoluble. The supramolecular structure of the PEG-*b*-PHEMA aggregates is likely to be disordered due to the well-known hydrogen-bonding capacity of the PEG and PHEMA chains with water.

The kinetics and pH-dependence of ortho ester side-chain hydrolysis are determined by quantification of the degradation product, ethanol, released from degrading PEG-*b*-PEYM micelles in aqueous buffers of different pH. The results are summarized in Fig. 4. The micelles were very stable at pH 7.4 with less than 10% degradation in 2 days. As pH of the solvent decreased to 6, 5, and 4, the hydrolysis accelerated as expected. At the typical lysosomal pH (~5), hydrolysis was essentially complete in no more than 5 hours, suggesting that it may be feasible to use these micelles for delivering drugs into the lysosome.

3.4 Cellular uptake of coumarin-6-loaded micelles by human glioma cells

To determine the uptake of the block copolymer micelles by cancer cells, micelles of PEG-*b*-PEYM$_{45}$ were prepared using dialysis and a hydrophobic fluorescent dye, coumarin-6, was incorporated into the micelle core. The labeled micelles were then incubated with human glioma cells (T98G) in cell culture media containing serum. The uptake of micelles by the tumor cells was quantified by flow cytometry and confocal fluorescence microscopy. The high intensity of coumarin-6 fluorescence signal associated with the cells suggests that the uptake was highly efficient and almost complete after 1 h of incubation (Fig. 5A). A

typical confocal fluorescence image taken at the 4th hour shows abundant internalization of the coumarin-6-labeled micelles, most of which presumably in the endolysosomes, but also with substantial amount of diffusive staining that could be attributed to endosomal-pH-triggered release of courarin-6 (Fig. 5B). These observations are consistent with what has been reported previously that PEGylated polymeric micelles of sub-100-nm in size can be taken up efficiently by cells *in vitro* [46]. The route of uptake is presumably through endocytosis [47], although the exact pathway for this particular type of micelles is not yet clear.

3.5 Release of doxorubicin from micelles triggered by acidic pH

To explore the possibility of using the PEG-*b*-PEYM micelles for intracellular delivery of hydrophobic drugs, we chose doxorubicin for proof-of-principle. Doxorubicin (Dox) is a drug widely used clinically for treating many forms of cancer, and it is often used as a hydrophobic model compound to demonstrate utility of various drug delivery systems including block copolymer micelles and pH-sensitive polymer-drug conjugates. We prepared water-insoluble Dox amine from commercial water-soluble Dox hydrochloride (Dox-HCl), and encapsulated Dox into the core of micelles through dialysis. The amount of drug loading, determined by dissolving Dox-loaded micelles ("micelle-Dox") in DMSO and measuring absorbance of Dox at 480 nm, was 2.6% by weight. To demonstrate acid-triggered drug release, a known amount of micelle-Dox was incubated in aqueous buffer of physiological pH (7.4) and endosomal pH (5) at room temperature. The remaining micelle-Dox was sampled at various time points and the amount of Dox quantified by fluorometry, so as to calculate the cumulative amount of Dox that was released. For comparison, the release of free Dox (HCl) from the same dialysis tubing was also determined at pH 7.4 and 5. As seen in Fig. 6, free Dox diffused quickly through the dialysis membrane with almost 90% released in a few hours. The release profile for free Dox was the same for pH 7.4 and 5. On the other hand, the release of Dox from micelles showed distinct pH-dependence. At pH 7.4 there was an initial burst release of approximately 10% in the first hour, most likely due to hydration of lyophilized micelles, and the subsequent release remained minimal for several days (Fig. 6). There was less than 20% Dox released at pH 7.4 even after 6 days. At pH 5, however, the release of Dox was much accelerated through the initial 4 hours with 50% Dox released, and continued at a slower but steady pace till complete release at day 6 (Fig. 6).

3.6 Uptake of doxorubicin-loaded micelles by human glioma cells

The PEG-*b*-PEYM micelles loaded with Dox were incubated with the T98G human glioma cells and the internalization of micelle-Dox was observed using confocal fluorescence microscopy. Due to the concern that the toxicity of Dox might alter the endocytic behavior of the cells, a low, nontoxic dose (0.15 μg/mL) of Dox was used for the uptake experiment. Free Dox dissolved in DMSO or water-soluble Dox-HCl was also tested at the same Dox concentration. Images of Fig. 7 showing the fluorescence of Dox (red) and cell nuclei (blue) were captured after 1, 4, and 11 hours after adding the free Dox or micelle-Dox to the cells. It is clearly seen that the uptake of micelle-Dox occurred quickly even after 1 h and continued to increase with time (Fig. 7). By the 4^{th} hour, the Dox concentration in the cytoplasm was already very high, and some drug have localized in the nucleus (in purple). The Dox signal in the cytoplasm appears diffusive rather than punctate, suggesting that the majority of the internalized drug may have escaped the endosomes. In contrast, detectable uptake of the free drugs was only seen by the 11^{th} hour (Fig. 7). Dox added via DMSO has limited solubility in the aqueous cell culture media, and therefore, there was some drug in the cytoplasm and very few in the nucleus. The water-soluble Dox-HCl appeared to accumulate exclusively in the nucleus, although the amount of drug there was very low.

3.7 Cytotoxicity in human glioma cells

To assess the effectiveness of PEG-*b*-PEYM micelles as anti-cancer drug carriers, human glioma cells (T98G) were exposed to varying concentrations of micelle-Dox, free Dox (HCl salt), and empty micelles for 24 and 48 h, and the viability of the cells was determined by the MTT assay. The empty micelles without drug lacked any cytotoxicity up to the highest polymer concentration tested (5 mg/mL) after 24 h of incubation. Only at 5 mg/mL after 48 h did the cell viability drop to 70% (Fig. 8A). This suggests that neither the PEG-*b*-PEYM block copolymer itself nor its hydrolytic products affect cell metabolism substantially. The cytotoxicity of the micelle-Dox and free Dox with equivalent drug dosage was compared at 24 h and 48 h (Fig. 8B-C). The IC_{50} dose of free Dox at 24 h was estimated to be >20 μg/mL, whereas the value for micelle-Dox was 2.5 μg/mL – almost 10-fold lower. At 48 h the IC_{50} values of free Dox and micelle-Dox were about 6 μg/mL and 2 μg/mL, respectively. Therefore, at both time points the micelle-Dox was apparently more potent in killing T98G cells than the free drug.

4. Discussion

Ortho esters have been extensively used to construct biodegradable polymer main-chains, resulting in four generations of the POE polymers developed over several decades by Heller and co-workers [42-44], and have been studied and tested in clinical trials for various drug delivery applications including the delivery of small molecules, proteins, and nucleic acids [44,48]. Our report here is the first demonstration, to our knowledge, that an ortho ester incorporated in polymer side-chains can be used to control and enhance drug delivery.

Recently we [35] and the group of Li, Du, and coworkers [36-38] have synthesized diblock copolymers containing PEG and polymethacrylamide with ortho ester side-chains and characterized their physico-chemical properties. In a series of studies Li, Du, and co-workers have reported a number of acid-labile cyclic ortho ester side-chain structures and have shown that the stereochemistry of the ortho ester side-chain influences greatly the solution behavior, in particular, the thermal responsive properties, of the copolymers [36-38]. They have also shown that the polymers undergo hydrolysis at acidic pH, which releases a model compound, Nile Red [37-39]. Our group reported an amphiphilic PEG-*b*-PMYM copolymer, of which the pH-responsive hydrolysis of the ortho ester side-chains could be used to tune the average size and morphology of the copolymer nanoparticles in water [35]. In this work, instead of polymethacrylamide, we used a more hydrophobic polymethacrylate backbone to which the ortho ester group is appended (Scheme 1), in order to maximize the capacity of incorporating water-insoluble drugs into the core of the block copolymer micelles. Indeed, NMR experiments in different solvents (Fig. 1) and pyrene fluorescence assay (Fig. 2) confirmed that the new block copolymer, PEG-*b*-PEYM, did form stable micelles in water with a hydrophilic PEG corona and hydrophobic PEYM core. In conjunction with results from DLS and cryo-TEM (Fig. 3), we have shown that the PEG-*b*-PEYM block copolymers form micelles with a hydrated shell and hydrophobic core, a small size less than 100 nm, and a low CMC, all of which are desirable features of a nanoparticulate carrier for delivering hydrophobic drugs to cancer cells.

The kinetics of ortho ester side-chain hydrolysis is highly dependent on pH with much accelerated hydrolysis occurring at slightly acidic pH (Fig. 4). Similar pH-dependent *in vitro* release profiles of Dox from the micelles are also obtained (Fig. 6), indicating that the release of Dox is largely controlled by the hydrolysis of the ortho ester side-chain at least during the initial few hours, which changes the environment of the micelle core from being quite hydrophobic (PEYM block) to quite hydrophilic (PHEMA block). However, the release of Dox at pH 5 over the course of 6 days appears biphasic. The first phase is rapid release of approximately 50% of total drug content within the first 4 hours, which is

followed by the second phase of sustained release to completion over the next several days. Although the ortho ester side-chain hydrolysis is essentially complete by day 2, gradual release of Dox trapped inside the loose aggregates of the hydrolytic product, the hydrophilic PEG-*b*-PHEMA, continues through day 6. We speculate that the biphasic release property of micelle-Dox at slightly acidic pH could be more desirable than either single-phase burst release or single-phase sustained release for the delivery of anticancer drugs to solid tumors *in vivo*. The PEGylated micelles are expected to have a long circulation time and accumulate in solid tumors due to the EPR effect. Once in the acidic tumor microenvironment or in the acidic endosome of tumor cells, the hydrophobic ortho ester side-chains of PEG-*b*-PEYM would degrade, triggering the first phase of rapid drug release to kill tumor cells. The remainder of the drug trapped in the nano-aggregates of the hydrophilic PEG-*b*-PHEMA might still be taken up by tumor cells, providing a sustained supply of drug for longer lasting tumor suppression. This purported mechanism of action will need to be evaluated in appropriate animal models.

While the PEG-*b*-PEYM micelles could potentially be useful in delivering many anticancer drugs, the choice of Dox as a model drug was made to show proof-of-principle because Dox is a commonly used chemotherapeutic drug against many types of cancer including glioma. It is encouraging to observe that the potency of the micelle-Dox against the T98G cells is much higher than free Dox with much lower IC_{50} values (Fig. 8), given that the T98G cells are some of the most multi-drug-resistant human glioma cells against many drugs including doxorubicin [49-51]. The cell-killing action by the micelle-Dox appears to be more rapid than that of the free Dox, which may be due to the fact that entry by micelle-Dox through endocytosis and drug release into the cytoplasm triggered by endosomal pH are quick and efficient processes. This could also explain why the IC_{50} value of micelle-Dox didn't change much from 24 h to 48 h and suggest that the cytotoxicity of micelle-Dox has already reached maximum by 24 h. The enhanced efficacy against drug-resistant cancer cells by nanocarriers in general compared to free drugs has been attributed to 1) better solubility and bioavailability of poorly water-soluble drugs, 2) altered route of entering cells (endocytosis rather than diffusion), resulting in higher intracellular drug concentration and bypassing membrane efflux pumps, 3) direct inhibition of proteins instrumental in conferring multi-drug resistance (MDR) such as P-glycoprotein (Pgp) and multi-drug resistance-related proteins (MRPs), and 4) overcoming MDR by interfering with efflux pumps through ATP depletion and mitochondrial function [52,53]. These mechanisms have manifested through water-soluble polymer-drug conjugates [54], Pluronic polymers [55], block copolymer micelles [56], lipid nanoparticles consisting of small-molecule surfactants [57,58], and thiolated polymers [59], to name a few. In the case of our PEG-*b*-PEYM micelles, the ability of solubilizing the hydrophobic Dox has been demonstrated. Moreover, the micelle-Dox achieved much higher cellular uptake and high intracellular drug concentration as shown in the confocal fluorescence microscopy images (Fig. 7). Certain amount of Dox has localized in the cell nucleus as early as 1 h (Supporting information, Fig. S3). It is unlikely that the Dox in the nucleus is still bound to the micelles, because the micelles would be too large to enter through the nuclear pores. Therefore, the micelles must have released the free Dox, probably in response to the acidic environment of the endosome, which then diffused and accumulated in the nucleus. These are clearly important reasons for the superior efficiency of killing glioma cells by the PEG-*b*-PEYM micelle-Dox. However, while many nanocarriers of anticancer drug have demonstrated efficient entry into tumor cells, their cytotoxicity is not necessarily greater than the free drug. This is often attributed to the slow rate of drug release from the polymer carriers. Therefore, it is suspected that the fast drug release triggered by the hydrolysis of acid-labile ortho ester side-chains of the PEG-*b*-PEYM micelles may be another significant factor that contributes to its improved cytotoxicity over the free drug. Whether the PEG-*b*-PEYM micelles or their degradation products have any direct or indirect effect on the activity of MDR-related proteins (such as

MRP-1 expressed by the T98G cells [49,50]) or on the metabolism of the glioma cells remains to be explored.

4. Conclusions

We have synthesized and characterized a new amphiphilic diblock copolymer, PEG-*b*-PEYM, with acid-labile ortho ester side-chains. PEG-*b*-PEYM formed micelles in water with the ortho ester side-chains packed in the hydrophobic cores, which became hydrophilic upon rapid ortho ester hydrolysis at slightly acidic pH. Doxorubicin was incorporated into the micelles and was released at accelerated rate under acidic pH. Micelles containing doxorubicin were taken up very efficiently by human glioma cells *in vitro*, resulting in much higher intracellular drug concentration than cells treated with free drug. Importantly, doxorubicin-loaded micelles showed much enhanced cytotoxicity against the drug-resistant human glioma cells than doxorubicin alone. With further improvement, the PEG-*b*-PEYM block copolymer with acid-labile ortho ester side-chains may be a promising type of pH-responsive nanocarrier for delivering poorly water-soluble drugs to solid tumors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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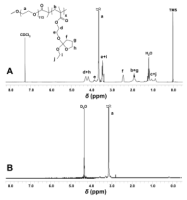


Fig. 1.
The ^{1}H-NMR spectra of PEG-*b*-PEYM copolymer in (A) *d*-chloroform and (B) aqueous *d*-PBS (pH 7.4).

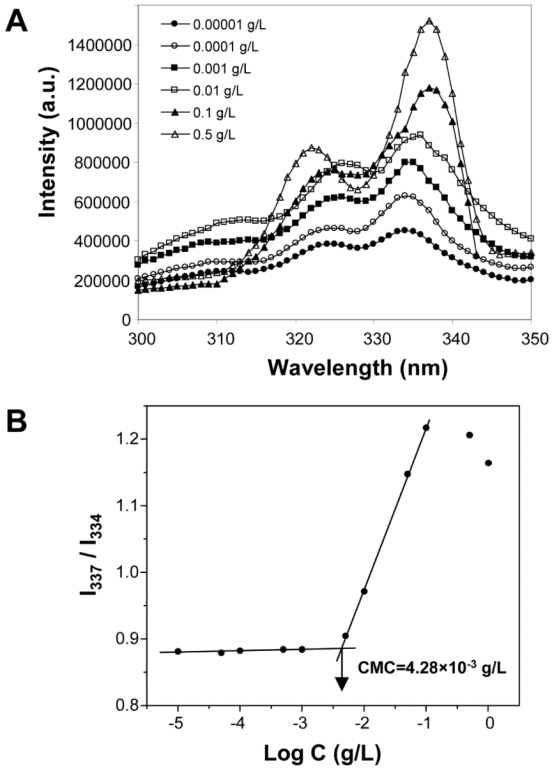


Fig. 2.

(A) Excitation fluorescence spectra of pyrene with PEG-*b*-PEYM$_{45}$ copolymer of different concentrations (0.0001 to 0.5 g/L) in PBS (pH 7.4). The concentration of pyrene was 6.0×10^{-7} M. (B) The dependence of excitation fluorescence intensity ratio of pyrene (I_{337}/I_{334}) on the logarithmic concentration of polymer. The CMC was determined to be 4.28×10^{-3} g/L.

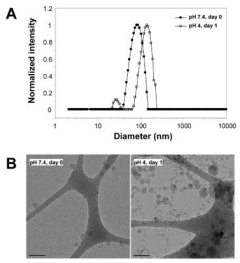


Fig. 3.

Size and morphological changes of the PEG-*b*-PEYM micelles before (pH 7.4, 0 days) and after (pH 4, 1 day) hydrolysis. (A) Particle size distribution determined by dynamic light scattering. (B) Images of cryo-TEM. Scale bar: 200 nm.

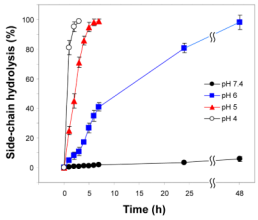


Fig. 4.

Time and pH-dependence of ortho ester side-chain hydrolysis of PEG-*b*-PEYM copolymer determined by quantification of one of the hydrolytic products, ethanol.

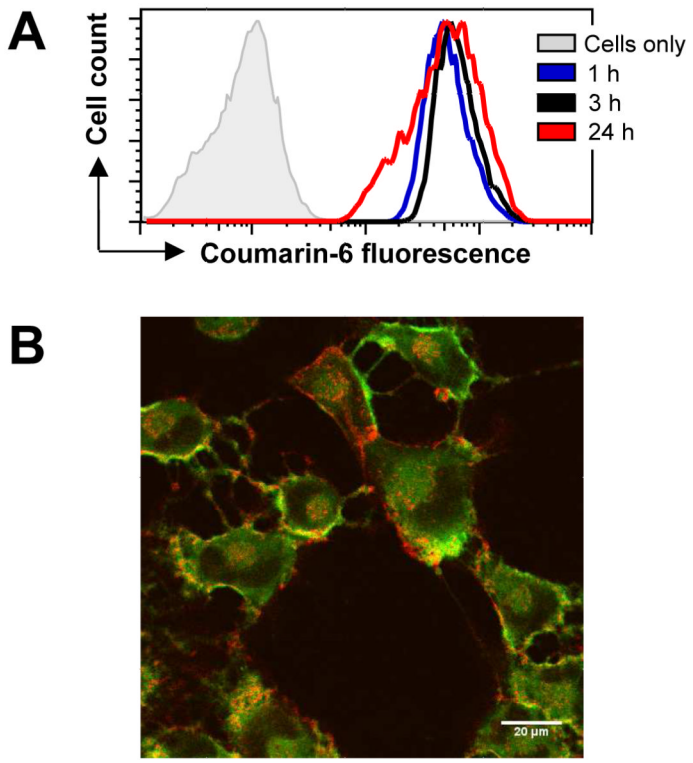


Fig. 5.

Uptake of coumarin-6-loaded PEG-*b*-PEYM$_{45}$ micelle by T98G human glioma cells determined by flow cytometry (A) and confocal fluorescence microscopy (B). Red: Alexa Fluor 594-labed concanavalin A. Green: coumarin-6. Scale bar: 20 μm.

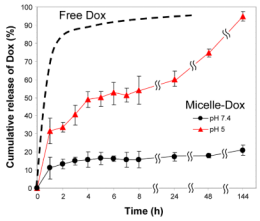


Fig. 6.

Release kinetics of doxorubicin as free drug or loaded in PEG-*b*-PEYM micelles at pH 7.4 and pH 5.

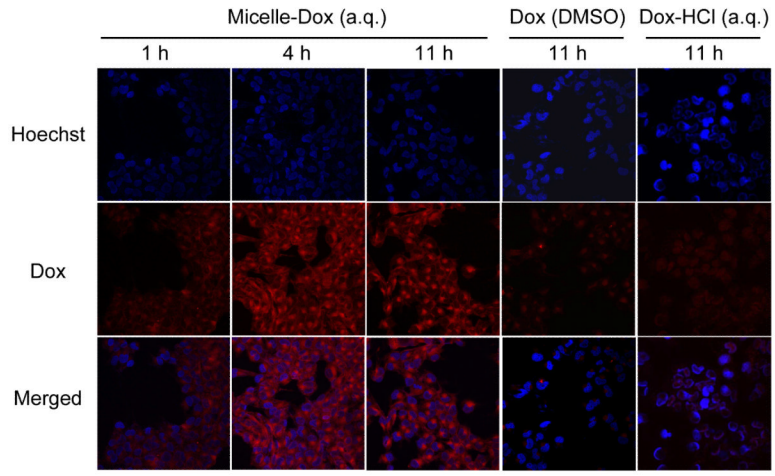


Fig. 7.

Confocal fluorescence microcopy images of doxorubicin uptake by T98G human glioma cells *in vitro*. Dox was either in the form of free drug (Dox-DMSO or Dox-HCL) or loaded in PEG-*b*-PEYM micelles.

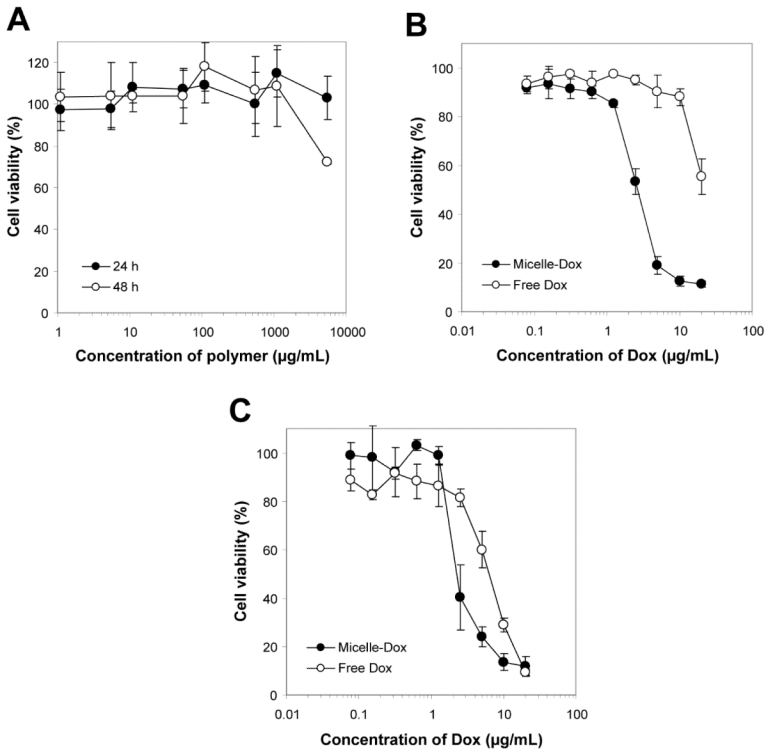
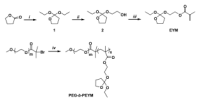


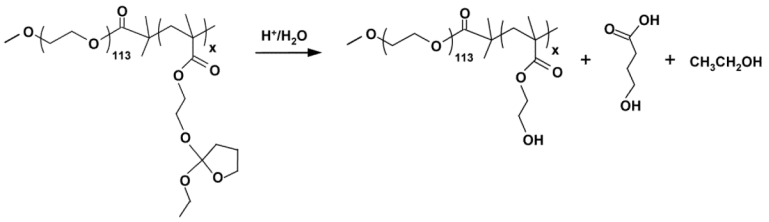
Fig. 8.

Dose-dependent cytotoxicity of empty micelles, free Dox, and micelle-Dox in T98G human glioma cells *in vitro* determined by the MTT assay. Cytotoxicity of empty micelles was determined at 24 and 48 h (A), and the comparison between the free Dox and micelle-Dox was made at 24 h (B) and 48 h (C).



Scheme 1.

Synthesis of poly(ethylene glycol)-*block*-poly(2-ethoxytetrahydrofuran-2-yloxyethyl methacrylate (PEG-*b*-PEYM): (i) $(C_2H_5)_3OBF_4$, CH_3CH_2ONa; (ii) ethylene glycol/*p*-TSA; (iii) methacryloyl chloride, DIPEA/CH_2Cl_2; (iii) CuCl/PMDETA, EYM monomer/toluene.



Scheme 2.
Acid-catalyzed hydrolysis of the PEG-*b*-PEYM copolymer.

Table 1

Polymerization results and molecular weight characterization of polymers

Polymer	Yield (%)	M_n ($\times 10^{-4}$) [a]	M_n ($\times 10^{-4}$) [b]	M_w ($\times 10^{-4}$) [b]	PDI [b]
PEG-*b*-PEYM$_{23}$	68	1.05	1.08	1.31	1.21
PEG-*b*-PEYM$_{35}$	65	1.32	1.37	1.74	1.27
PEG-*b*-PEYM$_{45}$	55	1.56	1.61	1.89	1.17

[a] Determined by ^{1}H-NMR in *d*-chloroform

[b] Determined by GPC in chloroform based on polystyrene standards.