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Block copolymer micelles with acid-labile ortho ester side-chains: Synthesis, characterization, and enhanced drug delivery to human glioma cells

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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 a 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 h) and three-times (by 48 h) 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.

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 have 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], and 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 endo-lysosome 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 to 6. There are several

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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 have 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 (300 MHz, $CDCl_3$): 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 (300 MHz, $CDCl_3$): 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 stirred 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 (300 MHz, $CDCl_3$): 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