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Preparation and self-assembly of a dual-functional copolymer for cancer therapy

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ABSTRACT

The pH-responsive amphiphilic copolymer poly(SDMA-co-OEGMA) (PSO) was prepared from the pH-sensitive hydrophobic monomer 2-styryl-1,3-dioxan-5-yl methacrylate (SDMA) and the hydrophilic monomer oligo(ethylene glycol) methyl ether methacrylate (OEGMA) by radical polymerization. Polymeric aggregates with about 130 nm diameter were obtained by the self-assembly of PSO in neutral aqueous solution. The critical aggregation concentration of the copolymer was determined to be 6.5 mg/L (1.2×10^{-7} M). Cinnamic aldehyde (CA) small molecules are broken away from PSO side chains after the hydrolysis of acid-labile cyclic acetal in cultured A375 human melanoma cells and further suppress the proliferation of this kind of tumor cells. Furthermore, the PSO aggregate was demonstrated to be a drug carrier for encapsulating Nile Red as model drug in *in vitro* testing. Based on the pH-responsive characteristic, the Nile Red molecules loaded in self-assembly process could be released from the aggregate inside cultured B16 mouse melanoma cells.

1. Introduction

In the past few decades, nanostructures as carriers for drugs and imaging agents have been investigated extensively [1–7]. Among these, considerable research has been focused on the development of polymeric micelles/aggregates, of which the functionality can be modified simply by changing the chemical structures of the polymers, and many types of drugs can be loaded into the drug carriers. Nanocarriers such as poly(ethylene glycol) (PEG)- and poly(ethylene oxide)-modified systems have long circulating times and can preferentially accumulate in the tumor sites, through the leaky tumor neovasculature, by the enhanced permeability and retention (EPR) effect which is known as passive targeting [8,9]. To further improve delivery efficiency and cancer cell specificity, stimuli responsive delivery systems have attracted considerable attention. These systems are sensitive to variations in biological and environmental signals such as pH [10–14], glutathione [15], specific enzymes [16] and temperature [17]. Since physiological pH is essentially neutral (7.0–7.4) while it is mildly acidic in tumor cells and in their intracellular compartments, such as endosomes and lysosomes, it is necessary to develop acid responsive drug carriers for the purpose of controlled drug release. To obtain pH-sensitive drug delivery systems, acid-labile covalent bonds like hydrazone [18,19], orthoester [20] and acetal [21,22], are positioned in the main chain, side chain, or at the terminal of the core-forming blocks. Except for several works on conjugating doxorubincin to polymers via acid-labile covalent bonds [23,24], few works pay attention to the released chemical and its influence on the target after acid-labile bonds breaking.

Cinnamic aldehyde (CA) has been reported to be bioactive and exhibit potent antitumor activity both *in vitro* and *in vivo* [25–28]. Sondra et al. have demonstrated that the cinnamon-derived dietary Michael acceptor CA impairs melanoma cell proliferation and tumor growth. CA is the only α, β-unsaturated aldehyde that is FDA-approved for use in foods and is given the "generally recognized as safe" status by the Flavor and Extract Manufacturers' Association in the United States. However, it is easily oxidized and poorly soluble in water, which restricts its application in cancer therapy. To our knowledge, there have been no studies on protecting CA by introducing it into an amphiphilic polymer and analyzing its suppression of melanoma cell proliferation.

In this paper, we have designed a hydrophobic unit (SDMA) by attaching CA to the methacrylate ester monomer via an acid-labile bond. By copolymerizing the pH-sensitive hydrophobic monomer with biocompatible hydrophilic monomer OEGMA, we obtained an amphiphilic copolymer poly(OEGMA-*co*-SDMA) containing pH-sensitive acetal group. The copolymer can form micellar structure in water with a narrow size distribution, comprising acid-labile acetal as a hydrophobic inner core and biocompatible PEG as the hydrophilic outermost shell. At pH 7.4 (i.e., physiological blood pH), the nanoparticles are quite stable, while at slightly acidic pH (i.e., the pH in endosomes/lysosomes of tumor cells), the nanoparticles can release CA by hydrolysis of acetal group in PSO. Owing to the anti-proliferative activity of CA against cultured A375 human melanoma cell lines [29], the PSO-based aggregates could further

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suppress proliferation of cultured A375 human melanoma cells after endocytosis and hydrolysis of aggregates. Furthermore, the PSO-based aggregates could act as drug carriers to load model drugs (Nile Red) into A375 human melanoma cells or B16 mouse melanoma cells and then be released by the acidic stimuli in tumor cells. The self-assembly of aggregates, pH-triggered release of CA and its inhibiting effect to A375 human melanoma cells and pH-dependent release of the model drug have all been investigated.

2. Experimental section

2.1. Materials

CA, oligo(ethylene glycol) methyl ether methacrylate (OEGMA) (average MW ~300, 475, and 950), glycerol, sulfuric acid, triethylamine were all purchased from Shanghai Chemical Reagent Co., Ltd., as analytical reagents and used without further purification. Azobisisobutyronitrile (AIBN) (chemically pure, Shanghai Chemical Reagent Co., Ltd.) was recrystallized from anhydrous methanol. Methacryloyl chloride was produced from Haimen Best Fine Chemical Industry Co., Ltd., and used after redistillation. Tetrahydrofuran (THF) was purified by reduced pressure distillation. 1,4-Dioxane was distilled over Na wire. All other reagents were commercially available and used as received.

2.2. Instruments and methods

^{1}H NMR spectra were measured by a Bruker 400 MHz NMR spectrometer using deuterated dimethylsulfoxide (DMSO-d_6) and deuterated water (D_2O) as solvents at ambient temperature. The aggregates before and after acetal hydrolysis in D_2O were studied by ^{1}H NMR. The aggregates were prepared by the addition of 2.0 mL of D_2O to 20 mg copolymer (10 mg/mL) followed by stirring for 1 h to form the aggregates solutions. The resulting aggregates solutions (10 mg/mL) were divided into two aliquots. One was used for ^{1}H NMR analysis. The other was treated with one drop of concentrated hydrochloride acid for 4 h and then analyzed by ^{1}H NMR. Molecular weight (Mn) and polydispersity (Mw/Mn) relative to PMMA were measured on a gel permeation chromatography using a Waters 1515 pump and differential refractometer. DMF was used as the mobile phase at a flow rate of 1.0 mL/min. Room temperature emission and excitation spectra were recorded using an Edinburgh-920 fluorescence spectrophotometer. Melting point determinations were performed on a microscopic (20×10) melting point apparatus. The samples for transmission electron microscopy (TEM) observations were prepared by placing a drop of the aggregates solution (0.2 mg/mL) on copper grids, which were coated with thin films of Formvar and then carbon. TEM images were obtained using a TecnaiG220 electron microscope at an acceleration voltage of 200 kV. The size of the aggregates was determined using dynamic light scattering (DLS), which was carried out at 25 °C using the Zetasizer Nana-ZS from Malvern Instruments equipped with a 633 nm He–Ne laser by back-scattering detection. Steady-state fluorescence spectra were recorded using a FLS920 spectrofluorometer (Edinburgh Co., UK) with an excitation wavelength of 335 nm with a pyrene probe. The intensity ratio of the third band (383 nm) to the first band (372 nm) of the pyrene emission spectrum (I_3/I_1) was used to determine the polarity of the pyrene environment.

2.3. Preparation of (E)-2-styryl-1,3-dioxan-5-ol (SDOO)

SDOO was synthesized according to a method reported in the literature [30] (Scheme 1) and gave a yield of 18% and m.p. of 121 °C. ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 3.46 (s, 1H), 3.90 (dd, 4 H), 4.90 (d, 1H), 5.16 (m, 1H), 6.20 (q, 1H), 6.70 (d, 1H), 7.28 (t, 1H), 7.35 (t, 2H), 7.46 (d, 2H). Elem. Anal. Calcd for C12H14O3: C, 69.88; H, 6.84. Found: C, 69.38; H, 6.687. TOF-MS (EI): calcd for C12H14O3: 206.0943, found: 206.0941.

2.4. Preparation of (E)-2-styryl-1,3-dioxan-5-yl methacrylate (SDMA)

SDOO (27.4 g, 0.1 mol) and triethylamine (10.1 g, 0.1 mol) were dissolved in 100 mL anhydrous THF and cooled to 0 °C in a water–ice bath. Methacryloyl chloride (10.45 g, 0.1 mol) was added dropwise and the mixture was left to react at room temperature for 12 h. The reaction mixture was filtered, concentrated, and purified by recrystallizing from ethyl alcohol. White crystals were obtained with a yield of 45%. ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 1.92 (s, 3H), 4.08 (d, 4 H), 4.67 (d, 1H), 5.27 (s, 1H), 5.74 (s, 1H), 6.11 (s, 1H), 6.21 (dd, 1H), 6.72 (d, 1H), 7.28 (t, 1H), 7.35(t, 2H), 7.48(d, 2H). Elem. Anal. Calcd for C16H18O4: C, 70.06; H, 6.61. Found: C, 70.06; H, 6.673. TOF-MS (EI): calcd for C16H18O4: 274.1205, found: 274.1206.

2.5. Synthesis of amphiphilic polymer

Five copolymers of poly(SDMA-*co*-OEGMA) (PSO) were prepared by copolymerization of (E)-2-styryl-1,3-dioxan-5-yl methacrylate (SDMA) and OEGMA according to the procedure shown in Scheme 1.

The typical procedure for preparing a copolymer (PSO-475a) with a feed ratio of 1:5 is described below. To a solution of monomer SDMA (54.9 mg, 0.2 mmol) and monomer OEGMA (MW = 475, 475 mg, 1.0 mmol) in 1,4-dioxane (2.0 mL), AIBN (17.8 mg, 5.0 mol% of the total amount of monomer) was added. The resulting solution was degassed by three freeze-evacuate-thaw cycles and heated at 70 °C for 3.5 h under argon. The mixture was added into a large amount of cooled diethyl ether (100 mL). The precipitate was filtered and dried under vacuum at ambient temperature for 24 h to obtain the copolymer PSO (yield: 93%). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 0.6–1.0 (—CH_3); 1.6–2.0 (—CH_2—C(CH_3)—COO—); 3.2–3.4 (CH_3—O—); 3.4–3.6 (—CH_2(OCH_2CH_2—)$_2$—); 4.3–4.7 (—(CH_2O)$_2$CH—); 5.1–5.3 (—CH (CH_2O—)$_2$—); 6.2–6.3(=CHCH(OCH_2)$_2$); 6.7–6.8(=CH—C_6H_5); 7.2–7.6(—C_6H_5).

2.6. Self-assembly of aggregates

The aggregates solutions of the copolymer were prepared by dropwise addition of 10 mL deionized water to the copolymer solution in THF (2 mg/mL) at room temperature and then stirring overnight to completely evaporate the THF solvent. The final concentration of the copolymer was 0.2 mg/mL.

2.7. Cloud point measurement

Turbidimetric measurements of the polymers in 10 mM phosphate buffer (PB, pH = 7.4) solutions were carried out on a Shimadzu 2101 UV–Vis spectrometer in a 1 cm quartz cell at 500 nm. The same buffer without polymer was used as the reference. During the heating and cooling processes, the solutions were equilibrated for 10 min at each temperature prior to measurement unless otherwise mentioned.

2.8. Determination of pH-dependent hydrolysis rate of acetals and CA release from aggregates

Acetal hydrolysis was followed by measuring the UV–Vis spectra at 290 nm for the aggregates solutions of PSO, according to the method of Frechet and co-workers [31–32]. The aggregates solution (2 mg/mL) prepared by the above mentioned method was di-