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Folate-conjugated amphiphilic hyperbranched block copolymers based on Boltorn® H40, poly(L-lactide) and poly(ethylene glycol) for tumor-targeted drug delivery

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ABSTRACT

Folate-conjugated amphiphilic hyperbranched block copolymer (H40–PLA-*b*-MPEG/PEG–FA) with a dendritic Boltorn® H40 core, a hydrophobic poly(L-lactide) (PLA) inner shell and a hydrophilic methoxy poly(ethylene glycol) (MPEG) and folate-conjugated poly(ethylene glycol) (PEG–FA) outer shell was synthesized as a carrier for tumor-targeted drug delivery. The block copolymer was characterized using ^{1}H NMR and gel permeation chromatography (GPC) analysis. Due to its core–shell structure, this block polymer forms unimolecular micelles in aqueous solutions. The micellar properties of H40–PLA-*b*-MPEG/PEG–FA block copolymer were extensively studied by dynamic light scattering (DLS), fluorescence spectroscopy, and transmission electron microscopy (TEM). An anticancer drug, doxorubicin in the free base form (DOX) was encapsulated into H40–PLA-*b*-MPEG/PEG–FA micelles. The DOX-loaded micelles provided an initial burst release (up to 4 h) followed by a sustained release of the entrapped DOX over a period of about 40 h. Cellular uptake of the DOX-loaded H40–PLA-*b*-MPEG/PEG–FA micelles was found to be higher than that of the DOX-loaded H40–PLA-*b*-MPEG micelles because of the folate-receptor-mediated endocytosis, thereby providing higher cytotoxicity against the 4T1 mouse mammary carcinoma cell line. *In vitro* degradation studies revealed that the H40–PLA-*b*-MPEG/PEG–FA block copolymer hydrolytically degraded into polymer fragments within six weeks. These results indicated that the micelles prepared from the H40–PLA-*b*-MPEG/PEG–FA block copolymer have great potential as tumor-targeted drug delivery nanocarriers.

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1. Introduction

Micelles formed from amphiphilic block copolymers have recently attracted significant attention in various medical and biological fields. In particular, polymeric micelles have been developed as drug delivery systems as well as carriers for various contrasting agents in diagnostic imaging applications [1–3]. With the amphiphilic structure of the polymeric micelles, hydrophobic pharmaceutical compounds are solubilized within the hydrophobic cores, whereas the shell maintains a hydration barrier that protects the integrity of each micelle. As a result, polymeric micelles can be used as efficient containers for reagents with poor solubility and/or low stability in physiological environments [4]. However, the formation of polymeric micelles is thermodynamically favorable only above the critical micelle concentration (CMC) of the amphiphilic molecules. When the concentration drops below the CMC, the micellar structure becomes unstable and dissociates into free chains. Such thermodynamic instability of the micelles below the CMC is one of the major concerns for their *in vivo* drug delivery application. Once the micelles are introduced into the bloodstream, they are subjected to severe dilution and become thermodynamically unstable when below the CMC. The disruption of micellar structures leads to the burst release of entrapped drugs, which may cause serious toxicity problems due to the potentially large fluctuations in drug concentrations [5].

The problem associated with the self-assembled multimolecular polymeric micelles can be potentially overcome by developing an amphiphilic hyperbranched block copolymer that has a hydrophobic inner block and a hydrophilic outer block. When the number of arms is sufficiently high, due to its unique chemical structure and amphiphilic nature, a single amphiphilic

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hyperbranched block copolymer can behave as a micelle composed of a hydrophobic core and hydrophilic shell in an aqueous solution [6,7]. This is in contrast to the classical multimolecular polymeric micelles that require the aggregation of multiple amphiphilic linear molecules to achieve the micellar behavior above the CMC. Boltorn® H40 (H40), a hyperbranched aliphatic polyester, has recently received much attention in designing unimolecular micelles made of amphiphilic hyperbranched block copolymers because of its biodegradability, biocompatibility, globular architectures and chain end functionalities [8]. Moreover, the ability to tailor its numerous end groups offers considerable scope for fine-tuning its drug loading and targeted drug release properties. So far, several amphiphilic hyperbranched block copolymers have been prepared using H40 as the macroinitiator [9,10]. The synthesis of amphiphilic hyperbranched block copolymer based on a H40 core, a hydrophobic poly(ε-caprolactone) inner shell, and a hydrophilic poly (acrylic acid) outer shell has been described for the improved micelle stability and drug loading ability [11]. In another study, water-soluble amphiphilic star block copolymers with a large number of arms were prepared by sequential atom transfer radical polymerization (ATRP) of *n*-butyl methacrylate and poly(ethylene glycol) methyl ether methacrylate using H40 as the macroinitiator [12]. Recently, folate-conjugated amphiphilic hyperbranched polymers were prepared by ring-opening polymerization of ε-caprolactone using H40 as the initiator followed by coupling reactions with PEG–diol and subsequently folate. The resulting products formed nanoparticles in aqueous solutions [13].

Despite continuous and intense effort to discover highly effective oncology drugs, conventional chemotherapeutic agents still exhibit poor specificity in reaching tumor tissue and are often restricted by dose-limiting toxicity [14]. The current focus in the development of cancer therapies is on targeted drug delivery to provide therapeutic concentrations of anticancer agents at the site of action and to spare the normal tissues [15]. Nanoparticulates (NPs) are desirable tumor-targeting vehicles because of a unique property inherent in solid tumors. Due to their rapid growth, many tumors present fenestrated vasculature and poor lymphatic drainage, resulting in an enhanced permeability and retention effect, which allows NPs to accumulate specifically at the tumor site [1,16]. This is often referred to as *passive targeting*. One major limitation with passive tumor-targeting alone is its inability to achieve a sufficiently high level of drug concentration at the tumor site, resulting in low therapeutic efficacy and eliciting adverse systemic effects [17,18]. To further improve delivery efficiency and cancer specificity, a strong emphasis has been placed on developing NPs with *active tumor-targeting* ability. Active targeting can be achieved by functionalizing NPs with targeting ligands such as small molecules (e.g., FA), antibodies, and peptides, which can recognize and bind to specific receptors that are unique to cancer cells. FA is non-immunogenic and has a high affinity for FA-binding proteins that are selectively over-expressed on the surface of many human tumor cells, including ovarian, lung, breast, endometrial, renal, and colon cancer cells [19,20].

In this work, we describe the synthesis and micellar characterization of folate (FA)-conjugated amphiphilic hyperbranched block copolymer (H40–PLA-*b*-MPEG/PEG–FA) based on H40, poly(L-lactide) (PLA), methoxy poly(ethylene glycol) (MPEG) and FA-conjugated poly(ethylene glycol) (PEG–FA) for targeted anticancer drug delivery. Both the core of the copolymer (H40) and the inner hydrophobic block of the arm (PLA) are aliphatic biodegradable polyesters. Since the molecular weight of the PLA and MPEG/PEG–FA blocks can be easily controlled during synthesis, the effective molecular size of the amphiphilic hyperbranched polymer can be tailored and hence the drug loading and release level of the resulting polymeric micelles can be tuned. FA-conjugated micelles can be directed to the cancer cells and subsequently internalized in the target cell *via* receptor-mediated endocytosis [21]. Furthermore, MPEG chains on the micelle surface can protect the nanocarrier from undesired attacks in the biological media, thereby increasing the stability of micelles and prolonging their circulation time in the blood. The structure of the copolymer was characterized by ^{1}H NMR and gel permeation chromatography (GPC) techniques. The micellar properties of the copolymer were determined by dynamic light scattering (DLS), fluorescence and transmission electron microscopic (TEM) analyses. The drug loading and *in vitro* release studies were performed using doxorubicin in the free base form (DOX) as a hydrophobic model anticancer drug at pH 5.3 and 7.4. The cellular uptake and cytotoxicity of the DOX-loaded H40–PLA-*b*-MPEG/PEG–FA copolymer against 4T1 mouse mammary carcinoma cells was assessed using confocal laser scanning microscopy (CLSM), flow cytometry, and the MTT assay. In addition, *in vitro* degradation of the block copolymer at pH 5.3 and 7.4 was analyzed by weight loss determination.

2. Materials and methods

2.1. Materials

H40 (64 hydroxyl groups per molecule theoretically and M_n of 2833) was obtained from Perstorp Polyols Inc., USA, and purified with tetrahydrofuran (THF). L-Lactide was purchased from Sigma–Aldrich and recrystallized from ethyl acetate before use. Stannous octanoate ($Sn(Oct)_2$) was purchased from Sigma. Succinic anhydride, 4-dimethylamino pyridine (DMAP), *N*-hydroxy succinimide (NHS) and 1,3-dicyclohexylcarbodiimide (DCC) were purchased from Acros and used without further purification. MPEG (M_w, 2000) was obtained from Fluka. α-Hydroxy-ω-amino poly(ethylene glycol) (HO–PEG–NH_2) with an M_w of 3000 was purchased from Iris Biotech GmbH. FA was obtained from bio-WORLD, USA. Triethylamine (Alfa Aesar) was distilled before use. Dichloromethane was dried over calcium hydride (CaH_2), distilled, and stored in a desiccator prior to use. The model drug, doxorubicin–HCl, was supplied by Tecoland Corporation, USA and used as supplied. All other chemicals used were of analytical reagent grade.

2.2. Synthesis of H40–PLA

H40–PLA was prepared by the ring-opening polymerization of L-lactide using H40 as a macroinitiator and $Sn(Oct)_2$ as a catalyst (Fig. 1). A 250 mL three-necked flask was charged with H40 (240 mg, 6.7 mmol of hydroxyl groups) under an inert atmosphere and placed in an oil bath at 120 °C in order to melt it and to facilitate its mixing with L-lactide. L-Lactide (4.32 g, 30 mmol) was slowly introduced and a catalytic amount ([catalyst]/[monomer] = 1:1000) of $Sn(Oct)_2$ was added. The polymerization reaction mixture was stirred for 24 h, diluted with THF, and precipitated into cold diethyl ether to give a white H40–PLA powder.

2.3. Synthesis of H40–PLA with carboxyl end group (H40–PLA–COOH)

H40–PLA–COOH was prepared by reacting 16.45 g (0.15 mmol) of H40–PLA with 0.47 g (4.7 mmol) of succinic anhydride in the presence of 0.57 g (4.67 mmol) of DMAP as a catalyst (Fig. 1). The reaction was carried out in the mixture of triethylamine (0.55 g) and anhydrous THF (100 mL) for 24 h at room temperature under stirring. Thereafter, the formed product was precipitated with cold diethyl ether and dried under vacuum. The impurities and unreacted materials of the product were removed by dialysis against deionized water using cellulose tubing (molecular weight cut-off, 12 000 Da). After 48 h dialysis, the product was separated out using the freeze-drying method.

2.4. Synthesis of α-hydroxy-ω-FA poly(ethylene glycol) (HO–PEG–FA)

N-Hydroxysuccinimide ester of FA (γ-NHS-FA) was first prepared according to a reported method [22]. FA (1.0 g, 2.3 mmol) was added into a mixture of anhydrous DMSO (40 mL) and triethylamine (0.5 mL) and allowed to dissolve in the stirring mixture under anhydrous conditions and in the dark overnight. Then, the FA solution was mixed with 0.47 g (2.3 mmol) of DCC and 0.26 g (2.3 mmol) of NHS and stirred in the dark for 18 h. The side product, dicyclohexylurea, was precipitated and removed by filtration. DMSO and TEA were evaporated under vacuum. Vacuum-dried γ-NHS-FA was then dissolved into 1.5 mL of the mixture of DMSO and TEA with a volume ratio of 2:1. An equal molar amount of HO–PEG–NH_2 was added to the mixture and the reaction was carried out under an anhydrous condition overnight. The product, HO–PEG–FA, was separated out by vacuum drying.