Polymer 49 (2008) 4769–4775

Contents lists available at ScienceDirect

Polymer

journal homepage: www.elsevier.com/locate/polymer

Preparation, characterization and controlled release of liver-targeting nanoparticles from the amphiphilic random copolymer

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ARTICLE INFO

Article history:
Received 30 April 2008
Received in revised form 1 August 2008
Accepted 2 September 2008
Available online 13 September 2008

Keywords:
Liver-targeting
Nanoparticle
Random copolymer

ABSTRACT

Liver-targeting ribavirin-conjugating nanoparticles were successfully constructed via self-assembly of the lactose-functionalized amphiphilic random copolymer, which was facilely prepared by a two-step chemoenzymatic synthetic route. Aggregation morphology of the resulting self-assemblies observed by transmission electron microscopy (TEM) and scanning electron microscopy (SEM) was regularly spherical shape, and hydrodynamic diameter determined by dynamic light scattering (DLS) was 174 ± 27 nm. Critical aggregation concentration (CAC) was measured by fluorescence probe technology using pyrene as the hydrophobic molecule, and the CAC value was about 0.1 mg/L. Cell cytotoxicity tests performed by MTT assay showed that the nanoparticles had effective growth-inhibitory activity in hepG2 human hepatoma cells. Moreover, ribavirin could be slowly released from the copolymer with pseudo zero-order kinetics in different incubation media. The targeting nanoparticles self-assembled from amphiphilic random copolymers could be used as novel potential drug delivery vehicles.

1. Introduction

In recent years, design and synthesis of polymeric micelles or nanoparticles suitable for biomedical applications have been an intense field of research, among which, the most challenging issues are how to prolong the circulation of the micelles or nanoparticles in blood and enhance their targeting functionalities to the specific site of interest [1,2]. A reliable strategy for achieving the long blood circulation is to design polymeric micelles or nanoparticles containing covalently bound drug. Therefore, polymeric micelles or nanoparticles generated through the directed-assembly of drug-containing amphiphilic polymers have emerged as a promising new class of polymer therapeutics [3–5]. The introduction of targeting ligands such as antibodies, sugars, folic acid, or RGD on the surface of the micelles or nanoparticles can effectively realize the specific recognition of these micelles or nanoparticles to certain site of interest, and thus enhances their targeting ability [6–10]. Bertin et al. prepared core–shell polymeric nanoparticles, which allowed for the surface conjugation of DNA and tumor-targeting antibodies, by self-assembly of amphiphilic block copolymers containing small-molecule drug segments and tosylated hexaethylene glycol segments [11]. Bae et al. designed folate-functionalized adriamycin-containing polymeric micelles self-assembled from a amphiphilic block copolymer, folate–poly(ethylene glycol)–poly(aspartate hydrazone adriamycin) [12].

Hepatitis C virus (HCV) infection is the leading cause of sporadic, post-transfusion, non-A, non-B hepatitis [13]. An estimated 170 million people worldwide are thought to be infected with hepatitis C virus and up to 80% of infected individuals turn chronic infection [14–16]. Chronic HCV is the single most common indication for orthotopic liver transplantation worldwide, and long term chronic HCV infection can lead to liver cirrhosis and to hepatocellular carcinoma [17–19]. However, treatment of HCV infection remains problematic. Currently, the recommended therapy is a combination of interferon alpha (IFN-α) or pegylated IFN-α and nucleotide analogue ribavirin, which has only a success rate of $<60\%$ [20–22]. Furthermore, this combination treatment is limited by severe side effects. The major toxicity is a dose-dependent hemolytic anemia that is caused by high ribavirin accumulation in red blood cells, which results in a ribavirin dose reduction or premature cessation of therapy [23]. Constructing liver-targeting ribavirin-conjugating polymeric micelles or nanoparticles is a strategy for improving drug efficacy and reducing systemic side effects.

However, most of the previous studies about polymeric micelles or nanoparticles have been focused on amphiphilic block copolymers, whose synthesis often requires nontrivial conditions [24]. Moreover, the preparation of these block copolymers linking drug and targeting ligands always requires a somewhat complex

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0032-3861/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.polymer.2008.09.006

synthetic route [12]. Therefore, it is significant to seek more facile protocol for the formation of targeting drug-conjugating polymeric micelles or nanoparticles.

Herein, we wish to achieve the formation of liver-targeting ribavirin-linking nanoparticles by self-assembly of the amphiphilic random copolymer, which could be facilely prepared by a two-step chemoenzymatic synthetic route. The aggregation morphologies, size distribution, and critical aggregation concentration of the resulting nanoparticles were, respectively, characterized by transmission electron microscopy, scanning electron microscopy, dynamic light scattering, and fluorescence probe technology. We further evaluated liver-targeting function of the nanoparticles by cell cytotoxicity tests, and investigated in vitro release behaviors of ribavirin in different incubation media by UV–vis spectrophotometer.

2. Experimental

2.1. Materials

Lipase acrylic resin from *Candida antarctica* (E.C. 3.1.1.3, 10,000 U/g, CAL-B) was purchased from Sigma. Alkaline protease from *Bacillus subtilis* (E.C. 3.4.21.14, a crude preparation of the alkaline serine protease, 100 U/mg, Subtilisin) was purchased from Wuxi Enzyme Co. Ltd. (Wuxi, PR China). α,α′-Azobis-(isobutyronitrile) (AIBN) was purchased from Fluka and purified by re-crystallization in ethanol and dried at room temperature under vacuum. Ribavirin (raw drug) was supplied by Xinxiang Tuoxin Biochemical Science & Technology Co. Ltd. (Xinxiang, PR China). 5′-*O*-Vinyladipyl-ribavirin (VAR) was synthesized and purified as described in the paper [25]. 6-*O*-Vinylsebacyl-lactose (VSL) was prepared and purified according to the literature [26]. *N*,*N*-Dimethylformamide (DMF) was HPLC grade. Dimethyl sulfoxide (DMSO), methanol, and all other chemicals were analytical grade.

2.2. Characterization methods

FTIR spectra were recorded on a Nicolet Nexus 670 FTIR spectrophotometer at room temperature in the range of 4000–400 cm^{-1}. ^1H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra measurements were run on a Bruker AMX-500 MHz FT-NMR spectrometer using DMSO-d_6 as the solvent and tetramethylsilane (TMS) as an internal standard. Polymer molecular weights were measured by gel permeation chromatography (GPC). The GPC columns were standardized with near-monodisperse polystyrene from Aldrich in molecular weights ranging from 7.0×10^5 to 1920 Da. DMF was used as the mobile phase at a flow rate of 1.0 mL/min.

2.3. Enzymatic synthesis of vinyl ribavirin derivative and lactose derivative

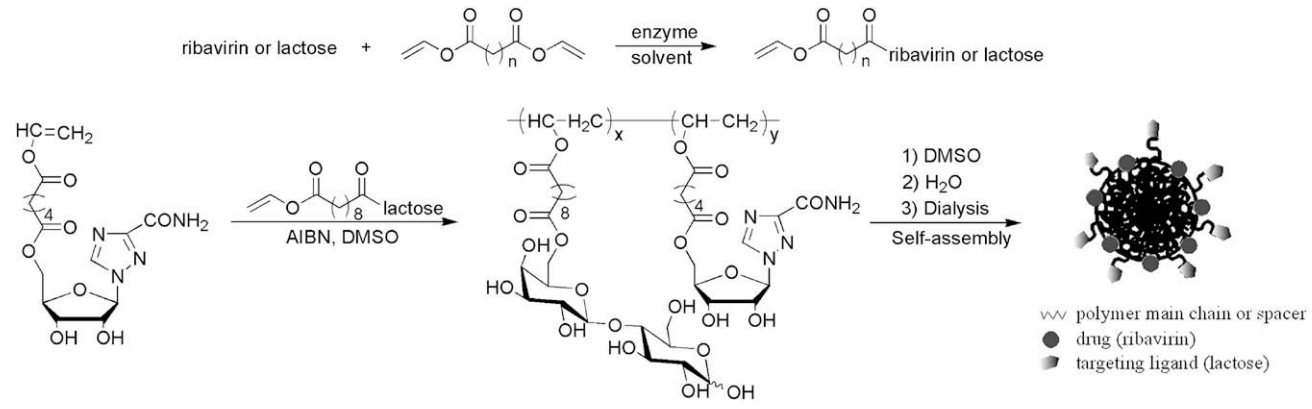
A general procedure: the reaction was initiated by adding CAL-B to anhydrous acetone containing ribavirin and divinyl adipate or adding Subtilisin to pyridine containing lactose and divinyl sebacate [25, 26]. The suspension was then kept at 50 °C and stirred at 250 rpm. The reaction process was monitored using TLC, and reaction was terminated by filtering the enzyme. The product was separated by silica gel chromatography.

2.4. Synthesis of poly(5′-O-vinyladipyl-ribavirin-co-6-O-vinylsebacyl-lactose)

Poly(5′-*O*-vinyladipyl-ribavirin-*co*-6-*O*-vinylsebacyl-lactose) [poly(VAR-*co*-VSL)] was prepared by adding VAR (398 mg, 1.0 mmol) and VSL (570 mg, 1.0 mmol) into a 10-mL polymerization tube containing DMSO (1.00 mL) and AIBN (20.0 mg). The mixture was degassed by three freeze–thaw cycles, and then stirred under nitrogen at 70 °C for 24 h. The resulting product was repeatedly precipitated in methanol and dried under vacuum to afford a light yellow solid poly(VAR-*co*-VSL) (328 mg, 34%). $M_n = 17{,}000$, $M_w/M_n = 2.65$. IR (KBr): ν (cm^{-1}) 3432, 2932, 2860, 1736, 1686, 1466, 1288, 1176, 1138, 1076. ^1H NMR (500 MHz; DMSO-d_6; Me_4Si) δ 8.83 (5-H of ribavirin), 7.84 (NH_2 of ribavirin), 7.64 (NH_2 of ribavirin), 6.68, 6.38 (1-OH of lactose), 5.94 (1′-H of ribavirin), 5.67 (2′-OH of ribavirin), 5.38 (3′-OH of ribavirin), 5.29–2.75 (CHO; 1-H, 2-H, 3-H, 4-H, 5-H, 6-H, 2-OH, 3-OH, 6-OH, 2′-OH, 3′-OH, 4′-OH, 1′-H, 2′-H, 3′-H, 4′-H, 5′-H and 6′-H of lactose; 2′-H, 3′-H, 4′-H and 5′-H of ribavirin), 2.38–1.23 (CH_2). ^{13}C NMR (125 MHz; DMSO-d_6; Me_4Si) δ 173.4, 173.0, 172.5 (C═O), 160.8 (C-3 of ribavirin), 158.2 (C-6 of ribavirin), 146.2 (C-5 of ribavirin), 104.0 (C-1′ of lactose), 97.2, 92.5 (C-1 of lactose), 91.8 (C-1′ of ribavirin), 82.2 (C-4′ of ribavirin), 81.3, 80.4 (C-4 of lactose), 74.6 (C-2′ of ribavirin), 75.3, 75.2, 75.1, 73.3, 72.8, 71.8, 70.8, 70.2, 68.7, 64.2, 61.0 (C-6, C-5, C-3, C-2, C-6′, C-5′, C-4′, C-3′, C-2′ of lactose), 70.9 (C-3′ of ribavirin), 63.7 (C-5′ of ribavirin), 33.8, 33.4, 29.1, 24.8, 24.2 (CH_2).

2.5. UV measurement of pyrene/poly(VAR-co-VSL)

Self-assembly of the resulting amphiphilic random copolymer poly(VAR-*co*-VSL) was preliminarily proved by UV–vis absorption spectra, which were recorded on an Analytikjena SPECORD 200



Scheme 1. Chemoenzymatic synthesis of lactose-functionalized ribavirin-conjugating amphiphilic random copolymer and preparation of nanoparticles by water addition to copolymer solution in DMSO followed by dialysis against water.