Colloids and Surfaces B: Biointerfaces 146 (2016) 707–715

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

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

Antitumor gemcitabine conjugated micelles from amphiphilic comb-like random copolymers

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ARTICLE INFO

Article history:
Received 9 April 2016
Received in revised form 8 July 2016
Accepted 11 July 2016
Available online 12 July 2016

Keywords:
Comb-like copolymers
Nanomicelles
Gemcitabine
PEG
Antitumor activity

ABSTRACT

Gemcitabine is an important pyrimidine antimetabolite that inhibits cellular DNA synthesis. However, the therapeutic efficacy and clinical benefit of gemcitabine are severely compromised due to its rapid plasma metabolism and low selectivity towards tumor tissues. To overcome these limitations, we prepared novel PEGylated gemcitabine-contained comb-like copolymers poly(monomethoxyl PEG_{350} methylacrylate –*co*- 5′-O-vinyladipyl- gemcitabine) (poly($mPEG_{350}$MA-*co*-VAG) and (poly($mPEG_{1000}$MA-*co*-VAG), which could self-assemble into micelles and displayed enhanced antitumor activity. The copolymers and the formed micelles were well characterized for their structure, critical aggregation concentration (CAC), morphology, cellular uptake, cell cytotoxicity, and controlled drug release. Cellular uptake and *in vitro* cytotoxicity assays against human lung cancerous cells (A549) demonstrated that these micelles could be effectively internalized and induced cell apoptosis. These micelles efficiently inhibited tumor growth when injected intravenously into A549 cell derived xenograft tumor bearing Balb/C nude mice using a dose of 10 mg/kg in terms of reduced tumor volume compared to free gemcitabine. In conclusion, PEGylated micelles could protect gemcitabine from rapid plasma metabolism, provided a sustained release and showed enhanced antitumor activity, thus have the potential to be used as novel anticancer drug delivery vehicle.

1. Introduction

Over the last decade, the design and fabrication of nano-sized drug delivery systems such as polymeric micelles, nanogels, liposomes and prodrug have been attracting more and more attentions [1–5]. Compared with traditional low-molecular-weight therapeutic agents, these drug delivery systems show many advantages such as the increased drug solubility, higher stability and good biocompatibility, controllable drug release profiles, enhanced therapeutic efficiency and reduced side effects. Among these different delivery systems reported, polymer-based micelles have emerged recently as promising carriers for delivering anticancer agents to tumor tissues [6–8]. However, most of them are formed from amphiphilic block copolymer, which are prepared usually through complicated routes [9,10], and these micelles are often used only as nanocarriers for drug encapsulation. The aim of this article is to construct novel antitumor micelles for drug delivery from comb-like amphiphilic random copolymers containing conjugated drug and PEG side chains, which has rarely been reported.

Gemcitabine (2′,2′-difluorodeoxycytidine, GEM) is a small molecular anticancer drug used as a first-line treatment against a variety of solid tumors by inhibiting DNA synthesis [11–13]. However, its clinical application is often limited due to its short *in vivo* half-life (8–17 min) [14]. Moreover, it is not easy for hydrophilic gemcitabine to passively cross the plasma membrane to transport into the cancer cells [15]. Thus, a high dose of gemcitabine is often administered to achieve a therapeutic drug concentration *in vivo* [14]. However, high doses of low molecular weight anticancer drugs often cause non-specific tissue toxicity such as hepatoxicity and renal toxicity in patients due to uncontrolled and random dissemination of the drugs in the body [16]. To overcome these limitations of drug pharmacokinetics, many therapeutic strategies have been attempted to improve the stability and efficacy of gemcitabine [17], including the encapsulation of gemcitabine in liposomes of different formulations [13], the entrapment in polymeric micelles [18,19], the attachment of gemcitabine to lipidic or nonlipidic derivatives [20], squalenoylation technique [5,21], polymer-gemcitabine

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http://dx.doi.org/10.1016/j.colsurfb.2016.07.027
0927-7765/

conjugates [22,23] and gemcitabine-initiated polymerization[24], and combination therapy of polymer-gemcitabine with polymer-paclitaxel or polymer-doxorubicin [22,23].

Poly (ethylene glycol) (PEG) is a water soluble polymer with excellent biocompatibility. And it is frequently used in numerous biomedical applications [25], as it is FDA approved for systemic human use. The most usual strategy to achieve "PEGylated gemcitabine" is the non-covalent incorporation of gemcitabine into PEGylated liposomes[13], micelles [26] or nano-vesicles [27]. And the "PEGylated gemcitabine" has shown to improve the stability and its pharmacokinetic profile, and also displayed passive tumor targeting due to leakiness of angiogenic tumor blood vessels by the well-known Enhanced Permeability and Retention (EPR) effect [28]. However, the drug loading ratio of these PEGylated gemcitabine is usually very low, for example only 4.14% in nano-vesicles [27]. And it always needs the modification of gemcitabine with hydrophobic functional groups [26] in order to enhance the adsorption of gemcitabine in the hydrophobic core of PEGylated nanocarriers, which certainly increases the complexity of synthetic steps. Another strategy, is conjugating gemcitabine to the PEGylated copolymer directly *via* covalent interaction [11]. As the molecular weight of PEG part is always much larger than gemcitabine, the payload of gemcitabine is also low [29] and difficult to be adjusted.

In the present study we developed a new "PEGylated gemcitabine" strategy, namely a novel amphiphilic comb-like random copolymer with abundant PEG and Gemcitabine side chains, which could self-assemble into PEGylated prodrug micelles with gemcitabine as the bound anti-tumor drug. The well-defined structure of the random copolymers was analyzed by IR, NMR, and gel permeation chromatography (GPC). And their critical aggregation concentration (CAC), morphology and size distribution were measured by fluorescence probe technology, transmission electron microscopy (TEM) and dynamic light scattering (DLS), respectively. The *in vitro* drug release behavior, *in vitro* cytotoxicity, cellular uptake assay of the micelles, as well as *in vivo* antitumor efficiency, pharmacokinetic studies and *in vivo* imaging in A549 cell derived xenograft tumor model after intravenous administration, were also studied systematically. These results clearly verify these novel amphiphilic comb-like random copolymer micelles with PEG and gemcitabine side chains are promising for delivering gemcitabine to A549 tumors with enhanced antitumor activity.

2. Experimental

2.1. Materials and methods

Lipase immobilized on acrylic resin from *Candida antarctica* (CAL-B, 10,000 U/g) was purchased from Sigma. Gemcitabine (GEM) was purchased from Jinkang Medical Technology Co., Ltd (Lianyungang, China). Methoxy-poly(ethylene glycol) ($mPEG_{350}$ and $mPEG_{1000}$) and methacryloyl chloride were purchased from Aladdin. Roswell Park Memorial Institute (RPMI) 1640 incubation medium and fetal calf serum were purchased from Genom Bio Med Technology Co. Ltd. (Hangzhou, China). All other chemicals were of analytical grade.

IR was measured in the form of a KBr disk using a Nicolet Nexus FTIR 670 spectrophotometer. ^{1}H NMR spectra were measured by a Bruker AMX–500 MHz FT-NMR spectrometer using DMSO-d_6 as the solvent and tetramethylsilane (TMS) as an internal reference. Polymer molecular weights were measured by GPC equipped with a refractive-index detector (Waters2410) and Waters Styragel® HR columns. The GPC columns were standardized with PMMA, and DMF was used as the mobile phase. TEM on a JEM-1230 transmission electron microscope at an accelerating voltage of 80 kV was used to investigate the morphology of the micelles formed from the copolymer. DLS was carried out on a Nanoseries (Malvern, UK) zeta sizer with 90° collecting optics to determine the hydrodynamic size (DH) and the size distribution (PDI) of the micelles. Analytical HPLC was performed using an Agilent 1100 series with a reversed-phase Agilent TC-C18 column (4.6 mm × 250 mm) and a UV detector (268 nm). The mobile phase consisted of deionized water (HPLC grade) and methanol (HPLC grade) 70:30 v/v, at a flow rate 1 mL/min.

2.2. Synthesis of PEGylated gemcitabine-containing random copolymers

Monomethoxyl PEG_{350} methylacrylate($mPEG_{350}MA$) and monomethoxyl PEG_{1000} methylacrylate ($mPEG_{1000}MA$) were prepared by adding monomethoxyl PEG_{350} (10.5 g, 30 mmol) or monomethoxyl PEG_{1000} (10 g, 10 mmol) into a sealed flask containing CH_2Cl_2 (6 mL) and a certain amount of triethylamine (3 mmol or 1 mmol). The solutions were incubated in ice-bath. Methacryloyl chloride (30 mmol or 10 mmol) was then added dropwise. The process of the reactions was monitored by TLC and the product was purified by silica gel chromatography. 5′-O-vinyladipyl-gemcitabine (VAG) was prepared by controllable regioselective enzymatic transesterification according to our previous research [30].

VAG (104 mg, 0.25 mmol), $mPEG_{350}MA$ (20.9 mg, 0.05 mmol) and AIBN (6 mg) were mixed and dissolved in DMSO (0.2 mL) in a sealed flask. The mixture was vacuumed by pump, and then stirred under nitrogen atmosphere at 70 °C for 24 h. The resulting product was precipitated in acetone and then dried under vacuum to obtain light yellow poly($mPEG_{350}MA$-*co*-VAG) (56 mg, 44.8%). $M_n = 1.72 \times 10^4$, $M_w/M_n = 1.73$. IR (KBr): $\upsilon(cm^{-1})$ 3427, 2878, 1728, 1653, 1451, 1351, 1249, 1107. ^{1}H NMR (400 MHz, DMSO-d_6, δ): 7.76 (1H, 6-H of GEM), 7.63 (2H, 4-NH_2 of GEM), 6.50 (1H, 3′-OH of GEM), 6.22 (1H, 1′-H of GEM), 5.93 (1H, 5-H of GEM), 4.42-4.07 (6H, 3′, 4′, 5′-H of GEM, −COO-CH_2- of PEG), 3.66-3.45 (-CH-O- of main chain; −CH_2-O- of PEG), 3.30 (3H, CH_3-O- of PEG), 2.60-1.31 (-C-CH_3 and −CH_2- of main chain). Poly($mPEG_{1000}MA$-*co*-VAG)(117 mg, 76.2%) was prepared in the same way. $M_n = 5.32 \times 10^4$, $M_w/M_n = 1.80$. IR (KBr): $\upsilon(cm^{-1})$ 3357, 3211, 2911, 2875, 1732, 1651, 1250, 1112. ^{1}H NMR (400 MHz, DMSO-d_6, δ): 7.59 (1H, 6-H of GEM; 2H, 4-NH_2 of GEM), 6.49 (1H, 3′-OH of GEM), 6.23 (1H, 1′-H of GEM), 5.90 (1H, 5-H of GEM), 4.42-4.06 (4H, 2′, 3′, 4′, 5′-H of GEM), 3.57-3.43 (-CH-O- of main chain; −CH_2-O- of PEG), 3.30 (6H, CH_3-O- of PEG), 2.60-1.31 (-C-CH_3 and −CH_2- of main chain).

2.3. Fluorescence measurements and micelles preparation

By using pyrene as the hydrophobic fluorescent probe, the self-assembly ability of the resulting amphiphilic random copolymer was proved by fluorescence spectroscopy. Five aqueous solutions of the PEGylated copolymer with concentrations ranging from 1×10^{-5} to 0.1 g/L were added into the vials containing a final pyrene concentration of 6×10^{-7} M. The samples were incubated for 2 h at 50 °C and then cooled for 12 h at room temperature. The fluorescence spectra were recorded on a Shimadzu RF-5301 PC spectro fluorophotometer with the excitation wavelength of 339 nm and the emission spectra were recorded from 360 to 460 nm. The value of the emission intensity ratio of the third band (I_{384}) to the first band (I_{373}) was analyzed as a function of the copolymer concentration.

Micelles self-assembled from the PEGylated comb-like random copolymer were easily prepared by using a simple dialysis method. Poly($mPEG_{350}MA$-*co*-VAG) was dissolved in DMSO at an initial concentration of 0.1 wt% at room temperature. Then ultrapure water was added dropwise to the solution under stirring until the final