International Journal of Pharmaceutics 441 (2013) 291–298

Contents lists available at SciVerse ScienceDirect

International Journal of Pharmaceutics

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

Pharmaceutical Nanotechnology

Glucose-functionalized multidrug-conjugating nanoparticles based on amphiphilic terpolymer with enhanced anti-tumorous cell cytotoxicity

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ARTICLE INFO

Article history:
Received 18 April 2012
Received in revised form 22 October 2012
Accepted 18 November 2012
Available online 27 November 2012

Keywords:
Glucose-functionalized
Terpolymer
Cytarabine
Fluorodeoxyuridine
Cytotoxicity

ABSTRACT

It is well known that combination therapy can significantly enhance the cytotoxicity and bypass some resistance mechanisms. However, the different solubility and pharmacokinetics of drugs limit the applications of combination therapy. In this study, novel glucose-functionalized polymeric micelle nanoparticles containing multidrugs were successfully fabricated and characterized. Two chemotherapeutic agents, cytarabine (Ara-C) and fluorodeoxyuridine (FUDR), were conjugated to a glucose-functionalized amphiphilic random terpolymer to create a novel nanocarrier for the delivery of multiple drugs simultaneously with an identical pharmacokinetic profile. The incorporation of D-glucose markedly increased the dispersity and biocompatibility of the novel polymeric micelles. *In vitro* drug release studies showed the two anticancer agents could be simultaneously released from multidrug-conjugating nanoparticles. Cellular uptake assay observed by confocal laser scanning microscopy and cytotoxicity tests performed by MTT assay against hepG2 human hepatoma cells indicated that glucose-functionalized multidrug-conjugating nanoparticles could be effectively internalized by HepG2 cells and showed much more effective growth-inhibitory activity than two single-drug-conjugating polymer aggregates or free drugs. This finding, therefore, illustrated that the D-glucose functionalized nanoparticles could be used as a novel potential multidrug delivery vehicle.

1. Introduction

In the past few decades, a large number of nanoparticle delivery systems have been developed for cancer therapy (Duncan, 2006; Lammers et al., 2008; Satchi-Fainaro et al., 2006). Among them, polymeric micelles from amphiphilic block copolymers have excellent biocompatibility, high drug-loading content, and markedly improved biodistribution, making them one of the most promising drug carriers (Lavasanifar et al., 2002; Nishiyama and Kataoka, 2006). Due to their changed pharmacokinetics (hyperpermeability of angiogenic tumor vasculature), polymer-based drug delivery systems have distinct advantages compared to conventional chemotherapy such as the prolongation of the circulation time in blood, decreased toxicity and side effect of bound drug, enhanced permeability and retention (EPR) effect (Duncan, 2006; Li and Wallace, 2008; Matsumura and Maeda, 1986; Vasey et al., 1999).

So far, most investigations about polymeric micelles and nanoparticles have been focusing on amphiphilic block copolymer (Bae et al., 2005; Lee et al., 2010), which is widely used for drug delivery. However, the preparation of the drug conjugating block copolymers is not easy and often requires nontrivial conditions such as extremely pure reagents, stringently dry environment and rigorously clean reactors (Li et al., 2003). In contrast to amphiphilic block copolymers, amphiphilic homopolymers and random copolymers can be easily prepared under ordinary reaction conditions (Basu et al., 2004; Liu et al., 2005; Savariar et al., 2006; Tian et al., 2008). Ma et al. (2010) synthesized a novel biodegradable random copolymer poly (lactide-co-glycolide)-D-a-tocopheryl polyethylene glycol 1000 succinate (PLGA-TPGS). The docetaxel-loaded nanoparticles had significant cytotoxicity against Hela cells. However, works about the functional micelles forming from drug conjugating amphiphilic random copolymers for drug delivery are rarely reported.

Drug combination therapy, especially the multi-agent combinational therapy, has attracted much attention in recent years. Compared with conventional single-agent therapy, multi-agent therapy could evidently reduce the side effects induced by high dose of single drugs, enhance the therapeutic effect and overcome drug resistance mechanisms (Lammers et al., 2009; Shamia et al., 2009). As most anticancer drugs have their own pharmacokinetic profiles, it is significantly important to develop nanocarriers which is able to deliver multiple chemotherapeutics with controlled release characteristics and optimized pharmacokinetic profiles (Vicent et al., 2005). Some pioneer works have reported novel polymer-based drug delivery system conjugating two or more

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http://dx.doi.org/10.1016/j.ijpharm.2012.11.030

low-molecular-weight drugs for treatment of cancers. Bae et al. (2007) developed mixed polymeric micelles assembled from poly (ethylene glycol)-poly (aspartate hydrazide) (PEG-p(Asp-Hyd)) block copolymers conjugating doxorubicin and wortmannin with a well-controlled drug mixing ratio, and investigated the biological activity of the mixed polymeric micelle nanocarriers. Greco et al. (2007, 2005) and Vicent et al. (2005) synthesized a HPMA (*i.e.* N-(2-hydroxypropyl) methacrylamide)-based polymer–drug conjugate carrying both doxorubicin (Dox) and aminoglutethimide (AGM). They demonstrated the markedly enhanced efficiency by *in vitro* cytotoxicity assay compared to HPMA–Dox copolymer (FCE28068), because the synergistic cytotoxic effects of AGM and Dox in conjugated form could decrease the expression of anti-apoptotic protein bcl-2. Lammers et al. (2009) also synthesized a HPMA based polymer–drug conjugate carrying both doxorubicin and gemcitabine, and evaluated its properties both *in vitro* and *in vivo*.

Undoubtedly, the use of nanoparticles which assembled from easily prepared random copolymers as controlled release carriers for multiple anticancer drugs can combine the advantages of both "drug combination therapy" and "nanoparticles forming from random copolymers." However, only limited reports of this kind were published. Cytarabine (Ara-C) and fluorodeoxyuridine (FUDR) are two important clinical anticancer drugs, and it has been verified that the duplex drugs showed synergistic effects against a series of cell lines including leukemia, ovarian and breast cancer cells (Bijnsdorp et al., 2007; Breistøl et al., 1999; Cai et al., 2003; Longley et al., 2003; Menger and Rourk, 1997). Our group has been devoting to the study of self-assembly of drug-loaded amphiphilic random homopolymers and copolymers (Li et al., 2008, 2010; Yin et al., 2010). In the previous research, we have incorporated Ara-C and FUDR into polymeric nanoparticles and investigated their *in vitro* drug delivery characters (Yin et al., 2010). Basing on this result, one interesting question is emerging, namely, whether the synergistic activity of the duplex drugs were retained, even enhanced after their incorporation into the "nanoparticles forming from random copolymers." Herein, as the further research, we developed a glucose-functionalized random terpolymers system containing both Ara-C and FUDR, which was expected to enhance the cytotoxicity after the incorporation of carbohydrate. The glucose-functionalized random terpolymers were easily prepared using chemo-enzymatic strategy and their well-defined structures were characterized by IR, NMR, and gel permeation chromatography (GPC). Critical aggregation concentration (CAC) of nanoparticles was measured by fluorescence probe technology, and their morphology and size distribution were analyzed by TEM and DLS. D-glucose-functionalized copolymer containing single-drug and their aggregates were prepared in the same way as the control. The MTT assay implied that the cytotoxicity of these glucose-functionalized nanoparticles against hepG2 human hepatoma cells was enhanced remarkably compared with the activity of single-drug conjugating nanoparticles and their physically mixed micelles, and also free Ara-C, FUDR and their mixture.

2. Materials and methods

2.1. Materials

Lipase acrylic resin from *Candida antarctica* (CAL-B) (an immobilized preparation of lipase from *C. antarctica* on macroporous acrylic resin, 10,000 U/g) was purchased from Sigma. PSL-C (an immobilized lipase from *Pseudomonas cepacia*, 730 U/g) was obtained from Amano Enzyme Inc., Japan. 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-(iso-butyronitrile) (AIBN) was purchased from Fluka, and purified by re-crystallization in methanol. Cytarabine was purchased from Shanghai Runcheng Biotechnological Co., Ltd. Fluorodeoxyuridine was purchased from Zhejiang Hisun Pharmaceutical Co., Ltd. (Taizhou, PR China). D-Glucose was purchased from Guanghua Chemical Co. Ltd. (Shantou, PR China). Roswell Park Memorial Institute (RPMI) 1640 incubation medium and fetal calf serum were purchased from Genom BioMed Technology Co. Ltd. (Hangzhou, People's Republic of China). Dimethyl sulfoxide (DMSO), dimethylformamide (DMF), tetrahydrofuran (THF), acetone, and all other chemicals were of analytical grade.

2.2. Synthesis of the comonomers

5′-O-vinylsebacoyl-cytarabine (VSC) and 3′-O-vinyladipoyl-fluorodeoxyuridine (VAF) were prepared by controllable regioselective enzymatic transesterifications. The reactions were initiated by adding CAL-B to anhydrous acetone containing cytarabine and divinyl sebacate, or adding PSL-C to THF containing fluorodeoxyuridine and divinyl adipate. The solutions were then incubated at 50 °C and stirred at 250 rpm. The process of the reactions was monitored by TLC. The reactions were terminated by filtering the enzyme and the product was purified by silica gel chromatography. 6-O-vinylsebacoyl-glucose (VSG) was synthesized in the same way while using subtilisin as the catalyst and pyridine as the solvent.

2.3. Synthesis of poly (3′-O-vinyladipoyl-fluorodeoxyuridine-co-5′-O-vinylsebacoyl -cytarabine-co-6-O-vinylsebacoyl-glucose) (poly (VAF-co-VSC-co-VSG))

VAF (193 mg, 0.5 mmol), VSC (220 mg, 0.5 mmol), VSG (188 mg, 0.5 mmol) and AIBN (30.0 mg) were mixed and dissolved in DMF (0.55 mL) in a sealed flask. The mixture was stirred under nitrogen atmosphere at 70 °C for 24 h. The resulting product was precipitated in acetone and washed with methanol repeatedly to remove the residual monomers, and then dried under vacuum to obtain light yellow product poly (VAF-co-VSC-co-VSG) (156 mg, 26.06%). Mn = 5.05×10^4, Mw/Mn = 2.14. IR (KBr): ν (cm^{-1}) 3438, 1730, 1647, 1258, 1097. ^{1}H NMR (500 MHz; DMSO-d_6; Me_4Si): δ 10.74 (1H, NH of fluorodeoxyuridine), 7.97 (1H, 6-H of fluorodeoxyuridine), 7.45 (1H, 6-H of cytarabine), 7.18 (1H, $-NH_2$ of cytarabine), 6.97 (1H, $-NH_2$ of cytarabine), 6.66, 6.34 (1-OH of D-glucose), 6.06 (1H, 1′-H of fluorodeoxyuridine), 5.66–3.16 (1′-H, 5-H, 2′-OH, 3′-OH, 2′-H, 4′-H, 3′-H, 5′-H, 5′-H of cytarabine; 5′-OH, 3′-H, 4′-H, 5′-H of fluorodeoxyuridine; 1-H, 2-H, 3-H, 4-H, 5-H, 6-H, 2-OH, 3-OH and 4-OH of D-glucose; —CH—O—), 2.27 (2′-H of fluorodeoxyuridine; O=C—CH_2), 1.48–1.20 (—CH_2—).

2.4. Synthesis of poly (3′-O-vinyladipoyl-fluorodeoxyuridine-co-6-O-vinylsebacoyl-glucose) (poly (VAF-co-VSG)) and poly (5′-O-vinylsebacoyl-cytarabine-co-6-O–vinylsebacoyl -glucose) (poly (VSC-co-VSG))

VAF (193 mg, 0.5 mmol) and VSC (220 mg, 0.5 mmol) were respectively dissolved in a sealed flask containing AIBN (20.0 mg) and VSG (188 mg, 0.5 mmol). Mixtures were then degassed and stirred under nitrogen atmosphere at 70 °C for 24 h. The resulting products were precipitated in acetone and washed with methanol repeatedly to remove the residual monomers, and then dried under vacuum to obtain light yellow products poly (VAF-co-VSG) (132 mg, 34.6%). Mn = 2.17×10^4, Mw/Mn = 1.77. IR (KBr): ν (cm^{-1}) 3523, 3175, 1718, 1258, 1095. ^{1}H NMR (500 MHz; DMSO-d_6;