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Synthesis and characterization of biotin modified cholesteryl pullulan as a novel anticancer drug carrier

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ABSTRACT

A series of biotin modified cholesteryl pullulan (Bio-CHSP) conjugates with different degrees of substitution (DS) of biotin moiety were synthesized and characterized by Fourier transform infrared (FT-IR), proton nuclear magnetic resonance (^{1}H NMR) and X-ray diffraction (XRD). Bio-CHSP conjugates were amphiphilic in nature and their self-aggregation behavior in aqueous media was evaluated by the fluorescence probe technique. Bio-CHSP self-aggregated nanoparticles (Bio-CHSP NPs) were prepared and analyzed by dynamic light scattering (DLS), zeta potential and transmission electron microscopy (TEM) technologies. These novel nanoparticles were almost spherical in shape, and their size, ranging from 178.8 to 100.0 nm. The safety of Bio-CHSP NPs was studied through single dose toxicity test in mice, and the result showed that Bio-CHSP NPs were well tolerated at the intravenous dose of 200 mg/kg in mice. Moreover, as a model anticancer drug, mitoxantrone loaded Bio-CHSP NPs were also prepared and characterized in this study.

1. Introduction

Polymeric micelles self-aggregated from biocompatible and biodegradable polymers have attracted much attention due to their potential application in drug delivery (de Las Heras Alarcon, Pennadam, & Alexander, 2005; Li, Wang, Wang, Wang, & Jiang, 2013; Savić, Eisenberg, & Maysinger, 2006; Tong & Cheng, 2007). Polymeric amphiphiles consisting of hydrophilic and hydrophobic segments can form nanometer scale self-aggregates with a hydrophobic core and a hydrophilic shell due to the intramolecular and/or intermolecular interactions of hydrophobic segments in the aqueous media (Akiyoshi, Deguchi, Moriguchi, Yamaguchi, & Sunamoto, 1993; Kuroda, Fujimoto, Sunamoto, & Akiyoshi, 2002). Highly hydrated outer shells of the polymeric micelles can inhibit intermicellar aggregation of their hydrophobic inner cores. Thus, the self-aggregated nanoparticles are suitable for trapping hydrophobic drugs or some biomacromolecules such as proteins and genes to act as artificial molecular chaperones to improve their stability, control their release and intensify their bioactivity (Akiyoshi, Sasaki, & Sunamoto, 1999; Hirakura et al., 2010; Nomura, Ikeda, Yamaguchi, Aoyama, & Akiyoshi, 2003). Moreover, polymeric nanoparticles with targeting ligands, are promising candidates for cancer therapy, leading to a better therapeutic efficiency as well as reduced side effects (Blasi, Giovagnoli, Schoubben, Ricci, & Rossi, 2007; Nie, Xing, Kim, & Simons, 2007; Pulkkinen et al., 2008).

Recent studies revealed that biotin receptors were over expressed on numerous tumors characterized by rapid dividing and aggressive growth (Li, Lam, et al., 2013; Patel, Vadlapatla, Shah, & Mitra, 2012; Russell-Jones, McTavish, McEwan, Rice, & Nowotnik, 2004). For this reason, polymers that were bound with biotin tended to be uptaken by cancer cells and had higher distribution proportion in malignant tissues, compared to normal tissues (Heo et al., 2012; Kim, Cho, Lee, & Chu, 2007; Su, Chen, Cryns, & Messersmith, 2011; Xiong, Gong, Li, Li, & Guo, 2011). Na et al. (2003) synthesized vitamin H grafted pullulan acetate (BPA) and prepared the self-assembled nanoparticles as a targeted anti-cancer drug delivery system, the loading rhodamine B isothiocyanate (RITC)-labeled BPA nanoparticles exhibited very strong adsorption to the HepG2 cells, while the RITC-labeled PA nanoparticles did not show any significant interaction. Therefore, biotin act as the active tumor targeting ligand for various anti-cancer drugs, has the characteristics of special physicochemical and biological properties, including, low cytotoxicity and absence of antigenicity and immunogenicity, which enhances the intracellular uptake of drug within cancer cells (Bu et al., 2013; Kim et al., 2012).

The literature reported that drug loaded cholesterol-modified pullulan nanoparticles (CHSP NPs) coated with complexed human serum albumin molecules inhibited the drug release *in vitro* (Tao et al., 2012). CHSP NPs also showed a sustained release carrier for mitoxantrone *in vitro* (Yang et al., 2010). Although CHSP NPs has the excellent property of biocompatibility and shows a sustained

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release carrier for mitoxantrone *in vitro*, it lacks active targeting to tumor tissues. Therefore, in this study, we synthesized biotin grafted CHSP copolymer, which focused on obtaining a tumor-targeted drug delivery carrier. The phisico-chemical characteristics of the Bio-CHSP were confirmed by FT-IR, ^{1}H NMR and XRD, and the biotin DS values were evaluated by ^{1}H NMR. The prepared amphiphilic pullulan composed of biotin and cholesterol grafts formed self-aggregated nanoparticles in aqueous media, and then the acute toxicity of Bio-CHSP NPs was assessed in mice. Furthermore, mitoxantrone (MTO) was chosen as a model drug to assess the potential of Bio-CHSP NPs as a carrier of anticancer drugs. As a dihydroxyanthracenedione anticancer agent, MTO has a wide range of antitumor activity and is used to treat various carcinomas. However, the therapy may cause some serious side effects such as nausea, vomiting, myelotoxicity, anemia, cardiotoxicity and immunosuppression (Hagemeister, Cabanillas, Coleman, Gregory, & Zinzani, 2005; Kroger et al., 2003; Neuhaus, Kieseier, & Hartung, 2006; Wundes, Kraft, Bowen, Gooley, & Nash, 2010; Yang & Morris, 2010; Zingler et al., 2005). Therefore, Bio-CHSP NPs being used as a carrier of MTO was hoped to sustain its release, enhance its therapeutic index and decrease its toxic effects in the future.

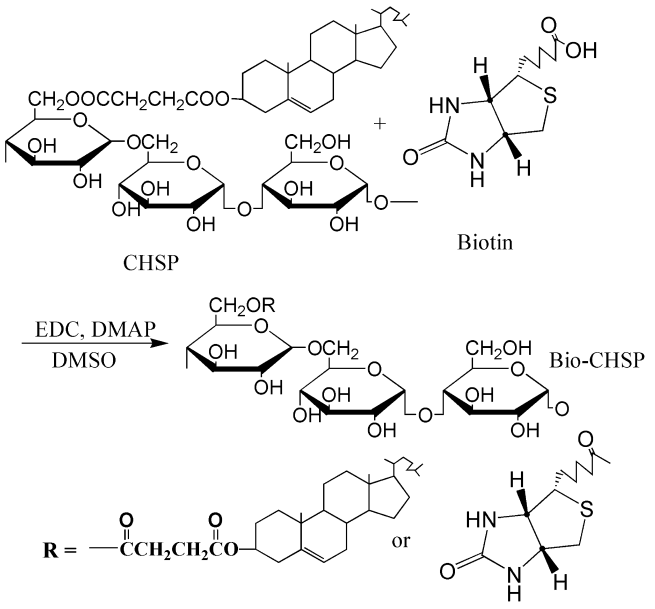

Fig. 1. The synthesis route of Bio-CHSP.

2. Materials and methods

2.1. Materials

Pullulan (Mw 200,000) was purchased from Hayashibara Tokyo (Japan). Biotin was provided by Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China); 4-dimethylaminopyridine (DMAP) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl) were purchased from Sigma Co. (MO, USA). Pyrene was supplied by Aldrich Co. (USA). Mitoxantrone was obtained from Beijing Xinze Science and Technology Co. (Beijing, China). Dialysis bags (Millipore molecular weight cut-off 8–14 KDa USA). Methanol, dichloromethane and ethanol were analytical grade and obtained from Kermel Chemical Reagent (Tianjin, China).

Six to eight-week-old male/female mice (20–24 g) were supplied by the Laboratory Animal Center of Hebei Medical University, (Hebei, China). The animals were acclimatized at a temperature of 25 ± 2 °C and a relative humidity of 70% ± 5% under natural light/dark conditions for at least 24 h before dosing. All the animal studies were approved by the center of Hebei Institutional Animal Care and Use Committee and performed in compliance with the Institutional Animal Care and Use Committee (IACUC) guidelines.

2.2. Synthesis of cholesteryl pullulan (CHSP) conjugates

The cholesteryl pullulan, with an average degree of substitution of 5.4 per 100 glucose units, was prepared by reacting pullulan with cholesterol succinate according to a procedure already reported (Yang et al., 2010), and the structure of CHSP is shown in Fig. 1.

2.3. Synthesis and characteristic of biotin modified CHSP (Bio-CHSP)

Cholesteryl pullulan (500 mg) was mixed with different amounts of biotin (753, 452 and 130 mg respectively), the mixture was completely dissolved in 6 mL dried DMSO by stirring, and then EDC (1.2 equiv biotin) and DMAP (0.1 equiv biotin) was added at 45 °C. After reaction for 5d, the mixture was cooled to room temperature and was precipitated in about 100 mL ethanol, filtered and washed with 50 mL dichloromethane, dilute alkali solution (50 mL, pH 11) and 50 mL anhydrous ethanol respectively, the obtained biotin graft polymer (Bio-CHSP) was dried in vacuum.

The chemical structure of Bio-CHSP was determined by FTIR-8400S spectroscopy (KBr pellets) (Shimadzu, Japan), and Avance III 600 MHz NMR spectrometer (Bruker, Switzerland) using DMSO-d_6 as the solvents at 600 MHz, and the characteristic of Bio-CHSP was also recorded with a D8 ADVACE X-ray diffractometer (Bruker, Switzerland)) to get X-ray powder diffraction diagrams. The degree of substitution (DS), defined as the number of the biotin moiety per 100 glucose units of Bio-CHSP, was determined by ^{1}H NMR.

2.4. Preparation of Bio-CHSP NPs

Bio-CHSP self-aggregated nanoparticles were prepared by dialysis method (Jeong et al., 2006; Wang et al., 2008). Briefly, Bio-CHSP (1 mg) was dissolved in 1 mL DMSO. To form nanoparticles, the solution was injected in dialysis bag to against 1000 mL deionized water and the dialyzed liquids were exchanged several times in 9 h followed by sonication using a probe type sonifier (SCIENTZ LCD JY 92-II, Ningbo) at 100 W for 2 min. The sonication step was repeated three times. The ice water bath and pulse (pulse on 2.0 s, pulse off 2.0 s) function were indispensable to protect the sample solution against heating built-up during the sonication. The solution of self-aggregated nanoparticles was filtered through a membrane filter (pore size: 0.45 μm, Millipore) to remove dust and then stored at 4 °C. The dialyzed solution was then analyzed or freeze-dried.

To observe the morphology of Bio-CHSP NPs sample, solutions (1 mg/mL) were dropped onto the carbon-coated 300 mesh copper grids. Then, the grids were air-dried and imaged using a transmission electron microscope (JEM-100C, JEOL, Japan). The particle size and zeta potential were determined by dynamic light scattering (Delsa^{TM} Nano zeta potentiometer, Beckman Coulter, USA).

2.5. Self-aggregation behavior of Bio-CHSP NPs

The self-aggregate property of Bio-CHSP conjugates and their critical aggregation concentration (cac) were estimated by the measurement of fluorescence spectroscopy (SANCO, 970-CRT, China) using pyrene as a hydrophobic probe (Wilhelm et al., 1991). To prepare sample solutions, a known amount of pyrene in methanol was added to each 10 mL test tubes and evaporated under a stream of nitrogen gas to remove the solvent. The amount was adjusted to give a pyrene concentration in the final solution of 6.0×10^{-7} M. Various concentrations (10 mL) of Bio-CHSP solutions were added