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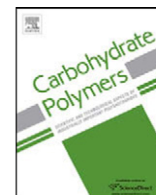

Preparation and characterization of self-assembled nanoparticles of 6-*O*-cholesterol-modified chitosan for drug delivery

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6-*O*-Cholesterol modified chitosan (*O*-CHCS) conjugates with succinyl linkages were synthesized through a protection-graft-deprotection method with phthaloylchitosan as an intermediate. *O*-CHCS conjugates were characterized by Fourier transform infrared spectroscopy (FTIR) and proton nuclear magnetic resonance (^{1}H NMR), and the degrees of substitution (DS) of the cholesterol moiety determined by elemental analysis were 1.7%, 4.0% or 5.9%. *O*-CHCS self-assembled nanoparticles prepared by the dialysis method displayed the classic "core–shell" structures and their sizes were in the range of 100–240 nm. All-trans retinoic acid (ATRA), as a model drug, was physically entrapped inside *O*-CHCS self-assembled nanoparticles by the dialysis method. With increasing initial levels of the drug, the drug loading content increased, but the encapsulation efficiency and the particle size decreased. The release profiles *in vitro* demonstrated that ATRA showed slow sustained released over 72 h, which indicated that *O*-CHCS self-assembled nanoparticles had the potential to be used as a carrier for hydrophobic drugs.

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1. Introduction

Self-assembly of block copolymers or hydrophobically modified polymers is of growing interest with respect to biotechnology and pharmaceuticals. Amphiphilic block or graft copolymer can spontaneously form nano-sized micelle-like aggregates with an inner hydrophobic core and an outer shell of hydrophilic groups in aqueous solution (Liu, Pramoda, Yang, Chow, & He, 2004; Zhang, Ping, Zhang, & Shen, 2003). The hydrophobic core serves as a reservoir for hydrophobic drugs and the hydrophilic part serves as an interface between the bulk aqueous phase and the hydrophobic domain. Therefore, many investigations have been performed over the past decades to design and synthesize various amphiphilic polymers, especially based on natural polysaccharides (Akiyoshi et al., 1998; Kim et al., 2008; Leonard, Boisseson, Hubert, Dalencon, & Dellacherie, 2004; Na, Park, Kim, & Bae, 2000; Park et al., 2004).

Chitosan is the second most abundant, renewable natural polysaccharide after cellulose, which is composed of β-(1,4)-2-amino-2-deoxy-D-glucopyranose residues with little or no N-acetyl-D-glucosamine units (Yang et al., 2008). It is a cationic polymer with outstanding biological properties such as biocompatibility, biodegradability, non-toxicity, bioadhesiveness and antimicrobial activity. Chitosan is soluble in aqueous acidic solutions but cannot form micelles in water (Opanasopit, Ngawhirunpat, Rojanarata, Choochottiros, & Chirachanchai, 2007). In recent years, therefore, much attention has been paid to hydrophobically modified chitosan as the drug (Hu, Ren, Yuan, Du, & Zeng, 2006; Wang, Liu, Jiang, & Zhang, 2007) or gene (Lee, Kwon, Kim, Jo, & Jeong, 1998) carriers. Nevertheless, in the literature chitosan modification through amino groups is predominant. Chemical modifications of this type may change the fundamental skeleton of chitosan especially with high degree of substitution, and the modified chitosan loses the original physicochemical and biochemical activities (Sugimoto, Morimoto, Sashiwa, Saimoto, & Shigemasa, 1998). On the other hand, modification of chitosan through hydroxyl groups may have an advantage because there may be less influence on the fundamental skeleton (Gorochovceva & Makuška, 2004) and it preserves free amino groups of chitosan which play an important role in biological activity and cationic polymer properties (Liu, Li, Fang, & Chen, 2005).

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Cholesterol is an indispensable structural building block in cells and plays an important role in many body functions. It is often used to hydrophobically modify biomaterials because of its rigidity and highly hydrophobic sterol skeleton (Akiyoshi, Deguchi, Moriguchi, Yamaguchi, & Sunamoto, 1993; Akiyoshi et al., 1998; Yuan, Li, & Yuan, 2006). Previously, our group has reported that self-assembled nanoparticles of *N*-cholesterol-modified chitosan (*N*-CHCS) were irregularly spherical in shape with a bumpy surface due to the chain rigidity of chitosan arising from various hydrogen bonds such as O-3 ··· O-5 (intramolecular) and N-2 ··· O-6 (intermolecular) (Wang et al., 2007a; Wang, Liu, Weng, & Zhang, 2007). In order to weaken these hydrogen bonds, retain the fundamental skeleton and free amino groups of chitosan, in this study, 6-*O*-cholesterol modified chitosan (*O*-CHCS) conjugates with succinyl linkages were synthesized through a protection-graft-deprotection method with phthaloylchitosan (PHCS) as an intermediate. Here PHCS, phthaloyl group of which could be easily deprotected to generate free amino groups, was prepared as a key intermediate for the further grafting reaction (Kurita, Shimada, Nishiyama, Shimojoh, & Nishimura, 1998; Makuška & Gorochovceva, 2006; Yoksan, Akashi, Biramontri, & Chirachanchai, 2001). Furthermore, *O*-CHCS self-assembled nanoparticles were prepared by the dialysis method and all-trans retinoic acid (ATRA) was chosen as a model drug to estimate the potential of *O*-CHCS nanoparticles as a novel carrier for hydrophobic drugs. As an active metabolite of retinol, ATRA has been shown to exert anti-tumor activities against a number of cancer cells and tissues. However, ATRA has poor aqueous solubility, short half-lives in blood and serious side effects such as retinoid resistance, hypertriglyceridemia and headache extremely (Jeong et al., 2004; Jeong et al., 2006). Therefore, ATRA was loaded into *O*-CHCS self-assembled nanoparticles in order to sustain its release, enhance its therapeutic index and reduce its toxicity.

2. Materials and methods

2.1. Materials

Biomedical grade chitosan (M_w = 100 kDa, degree of deacetylation 87%) was supplied by Jinqiao Biochemical Co., Ltd. (Zhejiang, China). Phthalic anhydride, N-hydroxyl succinimide (NHS) and pyrene were purchased from Sigma–Aldrich (St. Louis, MO). 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl) was supplied by Shanghai Medpep Co., Ltd. (Shanghai, China). All-trans retinoic acid (ATRA) was purchased from Kunming Mingren Chemical Plant (Kunming, China). All other chemical reagents were analytical grade and obtained from commercial sources.

2.2. Phthaloylation of chitosan

Synthesis was carried out according to a previously reported procedure (Kurita, Ikeda, Yoshida, Shimojoh, & Harata, 2002; Rout, Pulapura, & Gross, 1993). Briefly, chitosan (1.0 g, 6.0 mmol) was mixed with excess phthalic anhydride (2.66 g, 18.0 mmol) in 20 mL of N,N-dimethyl formamide (DMF) containing 5% (v/v) water and the mixture was heated in nitrogen at 120 °C with stirring. After reaction for 7 h, the mixture was cooled to room temperature and poured into iced water. The precipitate was filtered and washed with methanol. The degree of substitution (DS) of phthaloyl group was 86% determined by elemental analysis (Heraeus CHN-O-Rapid, Germany).

2.3. Synthesis of cholesterol succinate

Cholesterol succinate (CHS) was prepared as previously described (Wang et al., 2007a). Cholesterol (2.0 g, 5.2 mmol) was added to succinic anhydride (1.56 g, 15.6 mmol) in 12 mL of pyridine. The mixture was stirred for 72 h at 45 °C and then precipitated in the iced dilute hydrochloric acid solution. CHS was obtained by recrystallization in ethyl acetate and ethanol.

2.4. Synthesis of O-CHCS

CHS (0.5 g, 1.0 mmol) in 50 mL of DMF was activated by addition EDC (0.21 g, 1.1 mmol) and NHS (0.12 g, 1.0 mmol) for 2 h. PHCS (0.92 g, 3.3 mmol) was added to the above mixture and the reaction was continued for 72 h at 45 °C. Following this, the mixture was precipitated in ethanol, then filtered and washed with ethanol, tetrahydrofuran (THF) and diethyl ether, respectively to obtain phthaloyl protected graft copolymer (PHCS–CHS).

To a solution of PHCS–CHS (0.5 g, 0.65 mmol) in 5 mL of DMF was added 30 mL of hydrazine hydrate, and the mixtrue was stirred in nitrogen at 90 °C. After 7 h of reaction, the solution was cooled to room temperature and precipitated. Then, the precipitate was collected, washed thoroughly with ethanol and distilled water, and dried in vacuum to obtain *O*-CHCS. The chemical structure of *O*-CHCS was determined by Fourier transform infrared spectroscopy (FTIR, Nicolet Nexus 470-ESP, USA) and proton nuclear magnetic resonance (^{1}H NMR, Varian Inova500, USA) using D_2O/CD_3COOD or DMSO as the solvents. DS of the cholesterol moiety was determined by elemental analysis (Heraeus CHN-O-Rapid, Germany).

2.5. Preparation of O-CHCS self-assembled nanoparticles

O-CHCS self-assembled nanoparticles were prepared by a dialysis method. Briefly, *O*-CHCS was dissolved in 0.1 M acetic acid, and then the solution was dialyzed against physiological saline for 24 h. The dialyzed liquids were exchanged every hour for the first 3 h and each 3 h for the next 21 h (Yuan et al., 2006). The morphology of *O*-CHCS self-assembled nanoparticles was observed using transmission electron microscope (TEM, JEM-100CXII, Japan). The particle size, size distribution and zeta potential of nanoparticles were measured by dynamic laser light scattering (DLLS, Malvern Nano-ZS, UK).

2.6. Measurement of fluorescence spectroscopy

The self-aggregation property of *O*-CHCS and its critical micelle concentration (CMC) were determined using fluorescence spectroscopy with pyrene as a fluorescent probe (Amiji, 1995). The pyrene solutions (3.0×10^{-4} mol/L) in methanol were added into a series of test tubes and evaporated under a stream of nitrogen gas to remove the solvents. Then, various concentrations of *O*-CHCS suspension solutions were added to each test tube, and the final concentration of pyrene was 6.0×10^{-7} mol/L. The mixture solutions were sonicated for 30 min in an ultrasonic bath (KQ-100DY, China). Pyrene emission spectra were recorded using fluorescence spectrophotometer (Shimadzu F-4500, Japan). The probe was excited at 333 nm, and the emission spectra were obtained in the range of 350–450 nm at an integration time of 1.0 s. The slit width for excitation and emission were 10 and 2.5 nm, respectively.

2.7. Preparation of ATRA-loaded O-CHCS self-assembled nanoparticles

ATRA-loaded *O*-CHCS self-assembled nanoparticles were prepared according to the method previously reported (Kim et al., 2006). ATRA dissolved in 1 mL of DMSO was dropped into 10 mL of *O*-CHCS nanoparticle suspension with ultrasonication (Automatic Ultrasonic Processor UH-500A, China) at an output power of 50 W for 2 min, in which the pulse was cycled on and off at 2 s intervals under ice temperature condition. Following this, the mixture solution was dialyzed against deionized water and the medium was replaced several times over a 9 h period. The physicochemical char-

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