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Influence of the graft density of hydrophobic groups on thermo-responsive nanoparticles for anti-cancer drugs delivery

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ABSTRACT

A series of deoxycholate-chitosan-hydroxybutyl (DAHBCs) with different degrees of substitution (DS) of hydrophobic deoxycholate (DOCA) were successfully synthesized. The lower critical solution temperature (LCST) of various DAHBCs could be adjusted from 35.4 °C to 42.1 °C by controlling the graft density of DOCA. DAHBCs could self-assemble into nanoparticles (NPs) which gradually evolved from irregular aggregates to spherical particles with the decrease of the DS of DOCA groups. The size of DAHBCs NPs ranged from 100 nm to 250 nm and their zeta potential varied between 3.85 and 12.37 mV. Hemolysis tests and protein adsorption assay exhibited DAHBCs NPs had few adverse effects on the blood components even at a concentration as high as 1 mg/mL. DAHBCs NPs showed high curcumin (CUR) encapsulation efficiency up to 80%. CUR-loaded DAHBCs NPs displayed thermal-dependent drug release profiles, and the release rate of CUR (~75%) was significantly ($p < 0.05$) accelerated at a temperature above the LCST compared with that (~40%) below the LCST. Cytotoxicity analysis identified no toxicity associated with DAHBCs NPs at a concentration up to 0.5 mg/mL. However, when the cells were incubated with the CUR-loaded NPs, their growth was significantly inhibited at 43 °C (>LCST), demonstrating the thermal-responsive release of encapsulated cargoes from the NPs. With the capacity to control the LCST of DAHBCs NPs at specific temperatures, it could be speculated that DAHBCs NPs might serve as a promising thermo-responsive nanoplatform for the delivery of antitumor drugs.

1. Introduction

Cancer has become a serious threat to the human health at present. Many of the cytotoxic antitumor drugs have been in clinical use for many decades [1,2]. The small size and poor solubility of antitumor drugs lead to fast renal clearance and poor bioavailability by the typical injection route of delivery, which results in limited accumulation of antitumor agents in tumors and poor clinical therapeutic efficacy [3,4]. Nanoparticles (NPs) delivery systems have gained increasing attention in the cancer chemotherapy [5]. They are expected to overcome the disadvantages of conventional chemotherapy thanks to their unique properties including enhanced solubility, pharmacokinetics of loaded hydrophobic drugs, improved therapeutic efficacy, lower toxicity and strong retention in tumors [6,7].

Stimuli-responsive NPs are of more scientific and technological importance in recent years due to their ability to sense and respond to external stimuli (*e.g.* temperature, chemicals, light, pH, electric or magnetic field) [8–12]. Among them, thermo-responsive NPs can exhibit sharp changes in morphology in response to a small or modest change in temperature, and this property could be utilized for the preparation of delivery vehicles of antitumor drugs [9,11]. Up to now, numerous thermo-responsive NPs-based delivery systems have been developed including poly(*N*-isopropylacrylamide-co-acrylamide),poly(*N*-isopropylacrylamide)-block-poly(L-lysine), polyaniline NPs and poly (*N*-isopropyl acrylamide-co-methacrylic acid-co-ethyl methacrylate) based NPs [13–16]. However, these synthetic materials may cause serious side effects at excessive doses. To improve the performance of the anticancer drug delivery systems, biocompatible and biodegradable thermo-sensitive NPs are desired as drug delivery platforms for tumor therapy.

Chitosan (CS), a natural linear polysaccharide, has attracted much attention as a drug delivery vector due to its special properties, such as ease of modification, biodegradability and low immunogenicity [17]. Demands on CS derivatives with desirable

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properties are increased for efficient and safe drug delivery. In our previous studies, hydroxybutyl grafted chitosan (HBC) was developed as a novel thermo-responsive hydrogel which has been utilized in tissue engineering [18], post-operative treatment and therapeutics delivery [19]. Yang et al. constructed an amphiphilic polymer *via* hydrophobically modified HBC with deoxycholic acid (DOCA), which could self-assemble into NPs as delivery vehicles of therapeutic agents. The NPs exhibited thermo-sensitive drug release and non-toxicity to mouse embryo fibroblasts, which proved great potential as drug carriers [20]. However, the influence of the grafting density of hydrophobic DOCA groups on thermo-responsive NPs remains unknown.

Hence, we investigated the influence of the grafting density of hydrophobic DOCA groups on thermo-responsive NPs as a nanoplatform for anticancer drugs delivery (Scheme 1). Morphologies, hemocompatibility and thermo-responsive behaviors of DAHBCs NPs were investigated to reflect the influence of the grafting density of DOCA groups. We also detected the encapsulation efficiency and drug loading efficiency of DAHBCs NPs under different feeding ratios of curcumin (CUR) to optimize the weight ratios of CUR/DAHBC. In addition, the influence of temperature on drug release behaviors and *in vitro* cell growth inhibition was evaluated.

2. Materials and methods

2.1. Materials

Chitosan was purchased from Haili Biotechnology Co. Ltd (Shandong, China) with deacetylation degree of 85% and average molecular weight (MW) of 1050 kDa. Deoxycholate (DOCA), 1, 2-Butene oxide, lauryl sulfate sodium salt (SDS), curcumin (CUR), pyrene, *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), *N*-hydroxysuccinimide (NHS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Sigma-Aldrich (St. Louis, USA). Other chemicals mentioned above were analytical grade and used without further purification. All the solvents were of chromatographic grade.

2.2. Synthesis of deoxycholic acid modified hydroxybutyl chitosan (DAHBC)

HBC was prepared by connecting 1, 2-butane oxidation onto chitosan chain [21]. Briefly, 2 g CS was alkalinized by 14 mL NaOH (50% w:w) for 24 h at room temperature, and additional NaOH solution was removed. Then CS was dispersed in 40 mL isopropanol/water (1:1, v:v) and mixed with 40 mL 1,2-butene oxide. The product was neutralized, dialyzed and lyophilized.

The DAHBCs with various degrees of substitution (DS) of DOCA groups, which were named as DAHBC1, DAHBC2, DAHBC3, DAHBC4 and DAHBC5, were synthesized by DOCA conjugating to the amino groups of HBC based on a carbodiimide reaction [22]. DOCA, EDC and NHS (molar ratio of DOCA:EDC:NHS = 1:1.5:1.5) were dissolved in 10 mL of anhydrous dimethyl sulfoxide (DMSO), and the reaction mixture was stirred at room temperature for 2 h to activate the γ-COOH group of DOCA. HBC dissolved into distilled water at 4 °C, followed by dilution with methanol (volume ratio of water and ethanol was 1:3). Then different amounts of activated DOCA were added dropwise into the HBC solution, weight ratios of DOCA and HBC were 0.1:1, 0.15:1, 0.2:1, 0.4:1 and 0.8:1. Then the resultant mixtures were stirred for 24 h at room temperature. After that, the reactant mixtures were dialyzed (MWCO 8000) against water/methanol mixture (1:4, v:v) for 2 d to remove the organic salt. Finally, the product was dialyzed against distilled water for another 24 h to remove methanol, and the resultant mixtures were dialyzed and lyophilized.

2.3. Characterization of DAHBCs

2.3.1. ^{1}NMR spectroscopy

^{1}H NMR spectra (400 MHz) of the CS, HBC and DAHBCs in D_2O were recorded on a Bruker ARX 400 MHz spectrometer to analyze the chemical structures of CS, HBC and DAHBCs.

2.3.2. The degree of substitution of DOCA

The degree of DS, defined as the number of DOCA groups and hydroxybutyl groups per 100 sugar residues of CS, was measured by elemental analysis according to the method described by previous literatures [23]. Percentages of each element (C, H, O and N) and known structural formula were used to calculate the DS of hydroxybutyl groups and DOCA *via* Eq. (1) and Eq. (2):

$$\text{DS of hydroxybutyl groups} = \left(H\% - m_{H1}/M_{cs}\right) / m_{H2}/M_{HB} \times 100 \quad (1)$$

$$\text{DS of DOCA} = \left(H_1\% - m_{H1}/M_{cs} - m_{H2} \times DS_{HB}/M_{HB}\right) / m_{H3}/M_{DOCA} \times 100 \quad (2)$$

where $H\%$ and $H_1\%$ were the percentages of H in HBC and DAHBCs, respectively. m_{H1}, m_{H2}, m_{H3} were the amount of H in CS units, hydroxybutyl groups and DOCA residues, respectively; M_{cs}, M_{HB}, M_{DOCA} were the molecular weight of CS units, hydroxybutyl groups and DOCA residues, respectively; DS_{HB} was the DS of hydroxybutyl groups.

2.3.3. Lower critical solution temperature (LCST)

LCST of DAHBCs aqueous dispersions in PBS (0.02 M, pH 7.4) was conducted on a UV–vis spectrophotometer (UV-1200 MAPADA, China). The DAHBCs solutions were equilibrated at the special temperatures for 10 min before each measurement was conducted. The change in transmittance as a function of temperature was observed at 500 nm. The PBS without DAHBCs was applied as a reference. The LCST of DAHBCs solutions was defined as the transmittance showing a 50% decrease in optical transmittance [24].

2.3.4. Critical aggregation concentration (CAC)

Critical aggregation concentration (CAC) values of DAHBCs polymer in aqueous solutions were determined by fluorescence measurement using pyrene as a probe. The concentrations of DAHBCs solution varied from 1.0×10^{-4} mg/mL to 1.0 mg/mL. For the measurement of the intensity ratio of the I_{373}/I_{384} in the pyrene emission spectra, the slit openings were set at 1 mm (excitation) and 0.5 mm (emission) [25].

2.4. Formation and characterization of DAHBCs NPs

2.4.1. Preparation of DAHBCs NPs

A dialysis method was used to prepare DAHBCs NPs according to the previously reported procedure [20]. Briefly, DAHBCs were dissolved into DMSO, then the solutions were added dropwise into PBS (0.02 M, pH 7.4) under continuous stirring. Thereafter, the resultant mixtures were dialyzed against PBS for 24 h. The water was replaced hourly for the first 3 h.

2.4.2. Morphology

The morphology of DAHBCs NPs was observed *via* a transmission electron microscope (TEM, JEM-1200EX JEOL100 Ltd, Japan).