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

Fangqin Wang, Guixue Xia, Xuqian Lang, Xuanxuan Wang, Zixian Bao, Zeenat Shah, Xiaojie

Cheng, Ming Kong, Chao Feng, Ya Liu * and Xiguang Chen *

* Corresponding authors: Ya Liu, E-mail: yaliu@ouc.edu.cn;

Xiguang Chen, E-mail: xgchen@ouc.edu.cn

College of Marine Life Science

Ocean University of China

5[#] Yushan Road, Qingdao, 266003, China

Tel/Fax.: 86-0532-82032586

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.

Keywords: Hydrophobic group, Thermo-responsive, Nanoparticles, Graft density, Drug delivery

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 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],

(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 ¹H NMR spectroscopy

¹H NMR spectra (400 MHz) of the CS, HBC and DAHBCs in D₂O 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 the Eq. (1) and Eq. (2):

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

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

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). The NPs aggregate dispersions (1 mg/mL) were prepared by the procedure as 2.4.1.

2.4.3. Size distribution and zeta potential.

Mean particle diameter, polydispersity index (PDI) and zeta potential (ZP) of NPs dispersions were determined using a dynamic laser light scattering technique (DLS) (Zetasizer ZEN3600, Malvern 95 Instruments Ltd, UK) at 25 °C. The concentration of the NPs was kept constant at 1 mg/mL in PBS (0.02 M, pH 7.4) solution. The data were analyzed with a Zetasizer software (version 7.1).

2.5 Hemocompatibility

2.5.1 Hemolysis test

Different concentrations (0.0625 mg/mL - 1 mg/mL) of DAHBCs NPs dissolved in normal saline were incubated at 37 °C for 30 min. Thereafter, the erythrocyte stock dispersions (100 µL) were added into DAHBCs NPs suspensions (3 mL) and incubated at 37 °C for 0.5 - 1 h, respectively. Then reaction mixtures were centrifuged and the supernatants were dissolved in a hydrochloric acid and ethanol mixture, following an additional centrifugation [26]. The absorbance of the supernatant was determined at 398 nm by UV-VIS spectrophotometer (UV-1200 MAPADA, China). The hemolysis rates (HRs %) were calculated with distilled water as positive control (100% lysis) and normal saline as the negative control (0% lysis) by Eq. (3).

$$HR (\%) = (D_s - D_n) / (D_p - D_n) \times 100 \quad (3)$$

Where D_s , D_n , and D_p were the absorbance of the sample, the normal saline and the distilled water, respectively. The experiments were run in triplicate.

2.5.2 Protein adsorption

When the foreign material was injected into the blood, the material surface may adsorb plasma proteins resulting in aggregates appearing in the blood [27, 28]. The adsorption of serum proteins to DAHBCs NPs was studied at pH 7.4. DAHBCs NPs suspensions (100 μ g/mL) were incubated in fresh serum (10%, 1 mL) for 0.5 h at 37 °C, then separated by ultracentrifugation at 12,000 rpm for 10 min at 4 °C. Protein-adsorbed DAHBCs NPs were washed three times using distilled water, then separated by ultracentrifugation (12,000 rpm, 10 min) to remove excess proteins. The proteins were desorbed from the DAHBCs NPs surface in a protein solubilizing solution (PBS containing 1% SDS) by sonication and applied to SDS-PAGE. The SDS-PAGE was performed on an acrylamide gel consisting of 12% separating gel and 5% stacking gel, and visualized by staining with Coomassie Brilliant Blue R-250 [29]. In addition, the content of adsorbed proteins was determined using a BCA protein assay kit.

2.6. Thermo-responsive behavior of DAHBCs NPs

The thermo-responsive behavior of DAHBCs NPs was determined according to the morphological changes in response to temperature. Typically, DAHBCs NPs dispersions were incubated with water at a temperature above their LCST. Afterwards, morphological changes were observed using a TEM (JEM-1200EX JEOL100 Ltd, Japan).

2.7. Encapsulation efficiency and drug loading efficiency

CUR-loaded DAHBCs NPs were prepared by a dialysis method mentioned above. Typically, different amounts of CUR were dissolved together with DAHBCs in DMSO, then the solutions were added dropwise into PBS (0.02 M, pH 7.4) under continuous stirring, followed by dialyzing against PBS for 24 h before CUR-loaded NPs were collected [30]. The entrapped CUR in various DAHBCs NPs was

determined by a Fluorescence Microplate Reader (PerkinElmer, Boston, MA, USA). The weight of CUR loaded in NPs was determined by a standard calibration curve. The encapsulation efficiency (EE%) and drug loading capacity (LC%) of CUR were calculated by Eq. (4) and Eq. (5), respectively.

$$EE (\%) = Drug_{(w)} / Drug_{(t)} \times 100 \quad (4)$$

$$LC (\%) = Drug_{(w)} / L_{(t)} \times 100 \quad (5)$$

Where $Drug_{(w)}$ was the amount of CUR loaded in NPs; $Drug_{(t)}$ was the total amount of CUR; $L_{(t)}$ was the weight of CUR-loaded NPs.

2.8. Thermal-responsive drug release

Thermal-responsive drug release studies of CUR from DAHBCs NPs were carried out at 31 °C (below body temperature), 37 °C (body temperature) and 43 °C (upper the LCST) by a dialysis method [31]. The CUR-loaded DAHBCs NPs dispersions were quickly transferred into a dialysis tube, immersed in 50 mL PBS (0.02 M, pH 7.4) and then gently stirred at 31 °C, 37 °C, 43 °C in separate water baths, respectively. At a specific time interval, 5 mL of the dialysis mediums were taken out for fluorescence measurement and replenished with the same volume of fresh medium. All the measurements were performed in the dark. The CUR concentrations were quantified using a standard CUR calibration curve. The cumulative drug release (Q%) was calculated according to the Eq. (6):

$$Q (\%) = (C_n \times V + \sum_{i=0}^{n-1} C_i \times V_i) / (CUR_t \times EE\%) \times 100 \quad (6)$$

Where C_n was the concentration of CUR at time T_n , V was the total volume of release medium, V_i was the sampling volume at T_i , C_i was the CUR concentration at T_i (both V_0 and C_0 were equal to zero), and CUR_t was the total amount of CUR used for preparation of the original mixture, $EE\%$ was the encapsulation efficiency of CUR loaded into the DAHBCs NPs.

2.9 Cytotoxicity analysis

MTT assay was applied to evaluate the cytotoxicity of the DAHBCs NPs in

Caco-2 and L929 cells. Caco-2 and L929 cells were cultured in DMEM supplemented with 10% fetal bovine serum (Gibco, USA) at 37 °C in 5% CO₂. Then cells were placed in 96-well plates at a density of 5×10^4 . Thereafter, the mediums were replaced with the different concentrations of DAHBCs NPs **suspensions**. Then cells were subjected to MTT assay at predetermined time. Afterword, DMSO was added to each well to dissolve the formazan crystals produced by the living cells. Cells without DAHBCs NPs treatment were as control [26]. The absorbance of the solution was measured on a Bio-Rad model 550 microplate reader at 490 nm. The cell viability (%) was calculated according to the Eq. (7):

$$\text{Cell viability (\%)} = (OD_{\text{sample}} - OD_{\text{blank}}) / (OD_{\text{control}} - OD_{\text{blank}}) \times 100 \quad (7)$$

Where, OD_{control} and OD_{sample} were the absorbance at 490 nm in the absence and in the presence of sample treatment, respectively. OD_{blank} was the absorbance at 490 nm without cells. The cytotoxicity test was performed in replicates of five wells. Experiments were performed in triplicate.

2.10. Thermal-responsive cell growth inhibition

Cell growth inhibition of the CUR-loaded DAHBCs NPs was evaluated with Caco-2 cells using MTT as described above, with little modification. Briefly, the medium was replaced with different concentrations (0.0313 mg/mL - 0.5 mg/mL) of CUR-loaded DAHBCs NPs. The cells were cultured at 37 °C and 43 °C in 5% CO₂ respectively. Cells cultured at 37 °C without CUR-loaded DAHBCs NPs treatment were as the control. Then the cells were applied to MTT at predetermined time.

2.11. Statistical analysis

Statistical analysis was performed using one-way ANOVA, results were expressed as means $\pm$ SD (standard deviation). Asterisks (*) denote the statistical significance for * $P < 0.05$. Croisillon (#) denote the statistical significance for # $P < 0.01$. All experiments were performed at least three times. Calculations were done using the software SPSS 13.0.

3. Results and discussion

3.1 Characterization of DAHBCs

3.1.1 ¹H NMR spectra of the DAHBCs

A series of DAHBCs with different DS of hydrophobic DOCA groups were synthesized by conjugating DOCA to the amino groups of HBC based on a carbodiimide reaction according to the route as shown in Figure 1A. DAHBC was synthesized through double grafting of hydroxybutyl group and DOCA groups onto chitosan. Different grafting degrees of DOCA were achieved through adjusting DOCA feeding amounts as listed in Table 1. Compared with the ¹H NMR spectrum of CS (Figure 1B), the new signals at 0.6 - 1.7 ppm (peaks 4, 5) in the ¹H NMR spectra of HBC were attributed to the appearance of -CH₂- and -CH₃ in the hydroxybutyl group. The signals of DOCA in DAHBCs at 2.0 - 3.6 (peaks 1, 2, 3) ppm corresponded to the protons of -CH₃ groups at position 18, 19 and 21 of the steroid skeleton of DOCA, respectively [32, 33]. The result of ¹H NMR spectra indicated that series of DAHBCs were successfully synthesized.

3.1.2 Elemental analysis

Percentages of each element (C, H, O and N) and known structural formula were used to calculate the DS of DOCA and hydroxybutyl groups. The DS of hydroxybutyl groups was calculated to be 105.84 ± 1.24 per 100 anhydroglucose units of chitosan. The DS of the DOCA was summarized in Table 1. The DS of DOCA on DAHBCs was positively correlated with the feeding ratios (w/w) of DOCA to HBC. With the feeding ratios of DOCA to HBC increasing from 0.1:1 to 0.2:1, the DS of DOCA significantly increased from 2.45% to 5.80%, while the feeding ratios rose from 0.2:1 to 0.8:1, the DS only changed lightly (from 5.80% to 6.82%), indicating the saturation of substitution.

Figure 1. (A) Synthetic route of the deoxycholic acid decorated hydroxybutyl chitosan (DAHBC); (B) ^1H NMR spectra of CS, HBC and DAHBCs.

3.1.3 Properties of DAHBCs in aqueous solution.

DAHBCs were soluble in PBS (0.02 M, pH 7.4) at 4 °C. The solubility of the DAHBCs decreased with an increased DS of DOCA. All the DAHBCs solutions were visually transparent at 4 °C, while the aqueous solutions became cloudy along with a rising temperature. Figure 2A showed the temperature dependence of transmittance. The LCST was defined as the transmittance showing a 50% decrease in optical transmittance (summarized in Table 1). It was noticed that the initial transmittance of the DAHBCs in aqueous solution remained 80% until about 30 °C, as the temperature continued to increase, the transmittance decreased sharply to ~ 45% and then remained stable within a range of 10 °C. Lower DS of DOCA led to a higher LCST. A reasonable explanation was that the replacement of amino hydrogens by more

hydrophobic DOCA groups (less capable of formation of hydrogen bonds with water) considerably decreased the LCST of aqueous solutions of DAHBCs. H-bonds between the DAHBCs and water molecules were **significant** for understanding the behavior of DAHBC in PBS. The number of H-bonds was generally decreased with the increase of temperature and the amount of hydrophobic DOCA groups. One would expect that the relative hydration may also vary with temperature, and microenvironmental heterogeneity in the DAHBCs polymer aqueous solutions **above** their LCST may exist. Therefore, DAHBCs achieved thermal sensitivity and their LCST decreased with increasing DS of DOCA [34, 35].

Figure 2. (A) Low critical solution temperature of series DAHBCs in PBS (0.02 M, pH 7.4) upon heating processes; (B) Hemolysis rates (HR %) of DAHBCs NPs. The results were expressed as mean $\pm$ SD (n = 3). (C) Separation of serum desorbed from DAHBCs NPs surface using SDS-PAGE electrophoresis. Lane M: **marker**; Lane 0: 10% serum before adsorption; Lane 1-5, serum adsorbed on DAHBC1-DAHBC5 NPs.

Table 1. Characterization of different DAHBCs, (n = 3).

Polymer	DOCA:HBC (W/W)	DS of DOCA (%)	LCST (°C)	CAC (mg/mL)
DAHBC1	0.8:1	6.82 ± 0.64	35.4 ± 0.2	0.016
DAHBC2	0.4:1	6.13 ± 0.29	36.3 ± 0.3	0.019
DAHBC3	0.2:1	5.80 ± 0.48	38.3 ± 0.4	0.027
DAHBC4	0.15:1	3.38 ± 0.15	39.5 ± 0.1	0.048
DAHBC5	0.1:1	2.45 ± 0.76	42.1 ± 0.3	0.057

The aggregation behavior of DAHBCs in aqueous media was determined in the presence of pyrene as a fluorescence probe [36]. If hydrophobic micro-domains of DAHBCs were formed in an aqueous solution, pyrene molecules preferably located inside or closed to the hydrophobic micro-domains rather than the aqueous phase, resulting in different photophysical characteristics [37]. Intensity ratio (I_{373}/I_{384}) was low when pyrene was mixed with hydrophilic environment, whereas it increased substantially when pyrene was located into hydrophobic micro-domain [38]. The fluorescence emission spectra of pyrene incorporated into self-aggregates of DAHBCs were obtained in various concentrations. The CAC values of the DAHBCs were in the range of 0.016 mg/mL (DAHBC1) to 0.057 mg/mL (DAHBC5) (Table 1, Figure S1, Supporting Information), which decreased with the increase of DOCA substitution. This finding indicated that DAHBCs could form self-assembled structures with a hydrophobic inner core at ambient temperature in PBS (0.02 M, pH 7.4), and aggregation tendency was expected to increase with the increase of the DOCA groups substitution.

3.2 Hemocompatibility

The hemolysis rates (HRs) of human fresh blood with DAHBCs NPs were shown in Figure 2B. The hemolytic activity was clearly concentration-dependent. After incubation for 1 h, the HR increased from 0.91% to 3.68% when the concentration of DAHBCs NPs increased from 0.0625 mg/mL to 1 mg/mL. The HRs

in the test were all below 5.0%, which indicated the good blood compatibility of DAHBCs NPs [39]. When blood is in contact with a foreign material surface, the adsorption of plasma proteins occurs [27]. To examine the specific interactions between protein and the DAHBCs NPs, adsorption of serum was evaluated using SDS-PAGE and BCA protein assay kit. As shown in Figure 2C and Table S1, Supporting Information, compared with the belt of 10% serum before adsorption (lane 0), only 2.1% - 4.8% proteins adsorption were detected on DAHBCs NPs. The DS of DOCA had little effect on the protein adsorption rates [40]. Hemolysis test and protein adsorption assay exhibited few adverse effects of DAHBCs NPs on the blood components.

3.3. Characterization of DAHBC NPs

The morphology of DAHBCs NPs was observed via TEM. As shown in Figure 3A, DAHBC3, DAHBC4, DAHBC5 could self-assemble into solid-like spherical NPs. The diameter of DAHBC3 was smaller as compared with that of the DAHBC4 and DAHBC5. DAHBC2 with the higher DS of DOCA groups began to form more nanorods, and further increase in DS of DOCA groups caused irregular aggregate formation of DAHBC1. The average diameters of the DAHBCs NPs were measured by DLS, and the results were summarized in Table 2. Zeta potentials of NPs were 3.85 - 12.37 mV. The diameters of all the NPs in the well swollen state in PBS (0.02 M, pH 7.4) solution at room temperature were about 100 - 250 nm. Diameters of DAHBCs NPs increased from ~104 nm of DAHBC3 to ~181 nm of DAHBC5. The average diameter of the DAHBC2 nanorods was ~ 251 nm. DAHBC1 had a similar diameter with DAHBC3. The average diameters of the nano-assemblies were larger than those observed by TEM. The shrinkage of nanoparticles might be attributed to the loss of water during the dehydration process for TEM observation.

DAHBC consisted of (i) a hydrophilic hydroxybutyl moiety and (ii) a hydrophobic DOCA groups. Thus, DAHBC3, DAHBC4 and DAHBC5 could self-assemble to form spherical NPs depending on the hydrophilic/hydrophobic balance [41]. With an increased DS of DOCA, the hydrophobic DOCA core of NPs became more compact, so the size of DAHBC3 was smaller than that of DAHBC5.

Hydrophilic/hydrophobic balance was destroyed with further increase of the DS of DOCA, leading to formation of nanorods or irregular aggregates. The LCST of DAHBC3, DAHBC4 and DAHBC5 was higher than the body temperature, which was conducive to controlling the drug release in mild hyperthermia environment. Based on the LCST and TEM patterns, the DAHBC3, DAHBC4 and DAHBC5 were selected for further studies.

Figure 3. (A) Typical TEM images of DAHBCs NPs; (B) TEM images of DAHBC5 NPs at special time point, 0 h: before incubation at 43 °C, 0.25 h: after incubation at 43 °C for 0.25 h, 24 h: after incubation at 43 °C for 24 h; (C) The size variation of DAHBC5 NPs after incubation at 43 °C for 0.25 h, 0.5 h, 1 h, 2 h, 4 h, 8 h and 24 h. The results were expressed as mean $\pm$ SD (n = 3).

Table 2. Size and zeta potential of different DS of DOCA groups of DAHBCs NPs. (n = 3)

Polymer	Size (nm)	Zeta potential (mV)	PDI
DAHBC1	117.37 ± 2.10	10.10 ± 1.31	0.226 ± 0.03
DAHBC2	251.57 ± 2.04	5.30 ± 0.44	0.424 ± 0.07
DAHBC3	104.67 ± 2.34	12.37 ± 1.05	0.249 ± 0.04
DAHBC4	141.17 ± 1.62	5.16 ± 0.74	0.201 ± 0.01
DAHBC5	181.80 ± 10.11	3.85 ± 0.22	0.125 ± 0.02

3.4 Thermo-responsive properties of DAHBCs NPs

Thermo-responsive properties of the DAHBC3, DAHBC4 and DAHBC5 spherical NPs were investigated according to their changes in morphologies. It could be observed that the DAHBC5 NPs (thermo-responsive properties of the DAHBC3 and DAHBC4 NPs was summarized in Figure S2, Supporting Information) maintained spherical shapes before incubation at 43 °C (Figure 3B, 0 h). However, after incubated at the temperature above their LCST for 10 min, NPs began to shrink and transform into irregular shapes. The size of DAHBCs NPs decreased slightly after incubation for another 1 h, 2 h, 4 h, 8 h and 24 h (Figure 3C). This was most likely that the hydroxybutyl moieties became insoluble because of weakening intermolecular hydrogen bonding interactions in the DAHBC, which led to the switch of hydroxybutyl moieties from hydrophilic groups to hydrophobic groups. Hydrophilic shell of DAHBCs NPs eventually collapsed onto the surface of the hydrophobic core when the temperature was higher than LCST [42, 43].

3.5. Encapsulation efficiency and loading capacity

CUR was selected as a model drug which could be encapsulated into the hydrophobic microdomains of DAHBCs NPs by the dialysis method [20]. The influence of feeding ratios of CUR/DAHBC (w/w) as well as the DS of DOCA on the drug encapsulation process was investigated. As shown in Figure 4A, encapsulation efficiency of DAHBCs NPs significantly decreased from ~ 80% to ~ 20% with the

feeding weight ratios of CUR/DAHBC increasing from 0.1:1 to 0.35:1. However, loading capacity increased significantly from ~ 6% to ~ 12% with the feeding weight ratios of CUR/DAHBC increasing from 0.1:1 to 0.25:1, then loading capacity became stable. The CUR encapsulation efficiency and maximum loading capacity of DAHBC3 NPs were higher than those of DAHBC4 and DAHBC5 NPs. A reasonable explanation for this phenomenon probably was that more DOCA groups generally helped to encapsulate CUR into the hydrophobic microenvironment of DAHBCs NPs. According to the results of encapsulation efficiency and loading capacity, a weight ratio of 0.2:1 (CUR/DAHBC) was chosen to prepare CUR-loaded DAHBCs NPs.

Figure 4. (A) Drug loading capacity of CUR-loaded DAHBCs NPs; (B) Encapsulation efficiency of DAHBCs NPs. The results were expressed as mean $\pm$ SD ($n = 3$, $^3p < 0.05$ among all concentrations, $^4p < 0.05$ among all concentrations, $^5p < 0.05$ among all concentrations, $^{***}p < 0.05$ among DAHBC3, DAHBC4 and DAHBC5); (C, D, E) Thermal-responsive drug release profiles of CUR-loaded DAHBC3, DAHBC4 and DAHBC5 NPs.

3.6 Thermal-responsive drug release

In vitro drug release was monitored at different temperatures (31 °C, 37 °C and 43 °C). As shown in Figure 4C, cumulative CUR release from DAHBC3 NPs was only 45.5% at 31 °C for 24 h and increased slightly to 54.4% after incubated at 37 °C. By contrast, cumulative CUR release from DAHBC3 NPs increased dramatically to 76.9% within 24 h at 43 °C. Cumulative CUR release from DAHBC4 NPs and

DAHBC5 NPs decreased to 72.8% and 62.3% after incubated at 43 °C for 24 h (Figure 4D - E), respectively. These results suggested that DAHBCs NPs had thermal-dependent drug release behaviors.

Changes in CUR release behaviors from DAHBCs NPs at different temperatures were attributed to the thermal-induced structural alterations of DAHBCs NPs, which might influence drug diffusion rates. At a temperature below the LCST of DAHBCs (e.g. 31 °C or 37 °C), the hydroxybutyl groups remained hydrophilic and the NPs stayed stable in aqueous solution. The release amount of CUR was only attributed to an inevitable out-diffusion process. The slight increase in CUR release amount with the temperature changing from 31 °C to 37 °C might be attributed to the effect on drug diffusion rate. However, when the temperature of release medium was above the LCST of DAHBCs, the CUR release rate was remarkably accelerated because of shrinkage and deformation of the spherical structured NPs as previously described. Thus, a temperature-responsive release behavior was achieved. The lower LCST of DAHBC3 NPs caused more dramatic morphological changes in response to a mild hyperthermia condition (43 °C) resulting in a higher cumulative release amount.

3.7 *In vitro* cytotoxicity analysis

The cytotoxicity of thermo-responsive DAHBCs NPs was evaluated by using a MTT assay against Caco-2 and L929 cells at pH 7.4, 37 °C. As shown in Figure S3, Supporting Information, the DAHBCs NPs showed low cytotoxicity against Caco-2 and L929 cells at concentrations ranging from 0.0313 mg/mL to 0.5 mg/mL, indicating that DAHBCs NPs had a good compatibility. Thus, it is believed that DAHBCs NPs have the potential to be suitable vehicles for delivery of anticancer drugs.

3.8 Thermal-responsive cell growth inhibition

Inhibition of Caco-2 cell growth by CUR-loaded DAHBCs NPs at various temperatures was also evaluated using a MTT assay. Figure 5 showed the cell viability incubated with CUR-loaded DAHBCs NPs at 37 °C, 43 °C for 24 h and 48 h, respectively. The CUR-loaded DAHBCs NPs at 43 °C exhibited the higher toxicity towards the Caco-2 cells in contrast to that at 37 °C at all concentrations, suggesting

that the cytotoxicity of the CUR-loaded DAHBCs NPs was significantly enhanced when the temperature above their LCST. It could be explained that hydrophilic shell of DAHBCs NPs collapsed onto the surface of the hydrophobic core when the temperature was higher than LCST, which led to CUR release. Thus, cells growth was inhibited, which was in agreement with our previous results. Additionally, the DS of DOCA had little effect on the thermal-responsive cell growth inhibition.

Figure 5. Cells growth inhibition of CUR-loaded DAHBCs NPs at 37 °C and 43 °C . Cells were treated with CUR-loaded DAHBCs NPs at different concentrations. Cells cultured at 37 °C without CUR-loaded DAHBCs NPs treatment were as the control. (A) DAHBC3 NPs; (B) DAHBC4 NPs; (C) DAHBC5 NPs. The results were expressed as mean $\pm$ SD . ($n = 3$, $^{\#}p < 0.01$, $^{*}p < 0.01$ between control and different concentrations of CUR-loaded DAHBCs NPs, $^{**}p < 0.01$ between 43 °C and 37 °C).

4. Conclusions

In summary, we have successfully synthesized a series of thermo-responsive DAHBCs polymer modified with hydrophobic DOCA groups and hydrophilic hydroxybutyl segments for anticancer drugs delivery. ^1H NMR spectra and elemental analysis of various DAHBCs confirmed the successful synthesis of DAHBCs with different DS of hydrophobic DOCA. The LCST values could be easily adjusted from 35.4 °C to 42.1 °C by changing the hydrophobicity/hydrophilicity balance. Hemolysis tests and protein adsorption assays exhibited that DAHBCs NPs had few adverse effects on the blood components at a concentration ≤ 1 mg/mL. DAHBCs could self-assemble into NPs. Morphologies of DAHBCs NPs gradually evolved from irregular aggregates to spherical NPs with the decrease of the DS of DOCA groups. DAHBCs NPs began to shrink after incubation at a temperature higher than the LCST. DAHBCs NPs were able to encapsulate hydrophobic antitumor drugs (CUR). The CUR encapsulation efficiency and loading capacity were influenced by the DS of DOCA as well as initial CUR feeding ratios. Meanwhile, the CUR-loaded NPs exhibited thermo-sensitive drug release and thermal-dependent inhibition of tumor cell growth. More importantly, DAHBCs NPs were non-toxic to Caco-2 cells and normal cells. Overall, these findings suggested that DAHBCs NPs could be a potential vehicle for delivery of anticancer drugs.

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