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Research Paper

Polymeric nanoparticles of cholesterol-modified glycol chitosan for doxorubicin delivery: preparation and in-vitro and in-vivo characterization

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Abstract

Objectives Polymeric nanoparticles have been extensively studied as drug carriers. Chitosan and its derivatives have attracted significant attention in this regard but have limited application because of insolubility in biological solution. In this work, we attempted to utilize cholesterol-modified glycol chitosan (CHGC) self-aggregated nanoparticles to increase aqueous solubility, and to reduce side effects and enhance the antitumour efficacy of the anticancer drug doxorubicin.

Methods CHGC nanoparticles were loaded with doxorubicin by a dialysis method, and their characteristics were determined by transmission electron microscopy examination, light-scattering study, in-vitro drug-release study, pharmacokinetic study in rats and in-vivo antitumour activity in mice.

Key findings The resulting doxorubicin-loaded CHGC nanoparticles (DCNs) formed self-assembled aggregates in aqueous medium. From the observation by transmission electron microscopy, DCNs were almost spherical in shape. The mean diameters of these nanoparticles determined by dynamic light scattering were in the range of 237–336 nm as the doxorubicin-loading content increased from 1.73% to 9.36%. In-vitro data indicated that doxorubicin release from DCNs was much faster in phosphate-buffered saline at pH 5.5 than at pH 6.5 and 7.4, and the release rate was dependent on the loading content of doxorubicin in these nanoparticles. It was observed that DCN-16 (drug loaded content: 9.36%) exhibited prolonged circulation time in rat plasma and showed higher antitumour efficacy against S180-bearing mice than free doxorubicin.

Conclusions These results indicated that CHGC nanoparticles had potential as a carrier for insoluble anticancer drugs in cancer therapy.

Keywords antitumour efficacy; CHGC nanoparticles; doxorubicin

Introduction

Polymeric nanoparticles, self-assemblies of amphiphilic graft or block copolymers, have been extensively studied as drug carriers in recent years.[1,2] These nanoparticles exhibit unique core–shell architecture composed of hydrophobic segments as internal core and hydrophilic segments as surrounding corona in aqueous medium.[3] The hydrophobic core serves as a reservoir for water-insoluble drugs, whereas modification of the hydrophilic shell affects pharmacokinetic behaviour.[4,5] Many studies have demonstrated that these self-assembled nanoparticles containing anticancer agents can reduce unwanted toxic side effects of the drug, prolong circulation time and facilitate extravasation at tumour sites while avoiding renal clearance and non-specific reticuloendothelial uptake, resulting in an increase in the therapeutic index.[6–8] Among these polymeric nanoparticles systems, much attention has been paid to the preparation of biodegradable and nontoxic polymeric amphiphiles. Particularly, chitosan and its derivatives have attracted significant attention due to their specific structure and physicochemical properties, which lead to excellent biocompatibility, biodegradability, biological activity and low immunogenicity.[9–11]

However, the extended applications of chitosan are limited because it is insoluble in biological solution (pH 7.4). This engendered studies to prepare water-soluble chitosan derivatives. Glycol chitosan is a novel chitosan derivative and acts as a carrier of drugs because of its solubility in water at all pH values and its biocompatibility and

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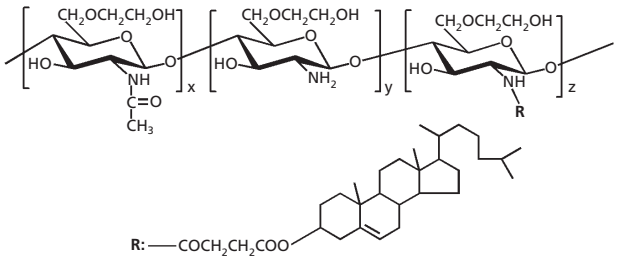

Figure 1 Chemical structure of CHGC (cholesterol-modified glycol chitosan). (x + y + z = 100, z = 6.7.)

biodegradability.[12,13] Hydrophobically modified glycol chitosan derivatives, such as glycol chitosan bearing 5β-cholanic acid, glycol chitosan chemically conjugated doxorubicin, glycol chitosan–deoxycholic acid conjugates and *N*-acetyl histidine-conjugated glycol chitosan, have been focused upon due to their strong amphiphilic nature and their formation of self-assembled nanoparticles in aqueous medium, which is very useful for biotechnological and pharmaceutical applications.[14–16] We have previously reported the synthesis and characteristics of cholesterol-modified glycol chitosan (CHGC, Figure 1).[17] In this study, doxorubicin was chosen as a model drug to assess the potential of CHGC self-aggregated nanoparticles as a carrier for hydrophobic anticancer drugs. Doxorubicin is one of the most powerful and widely used anticancer drugs in the clinical field. However, dose-limiting toxicity occurs with doxorubicin therapy and includes myelosuppression and cardiotoxicity.[18] This limitation results from the fact that doxorubicin lacks sufficient selectivity towards tumour cells. For effective cancer chemotherapy, an optimal concentration of anticancer agent must reach the tumour tissues and remain there for the required period of time. To improve its therapeutic activity against tumours, drug delivery systems with long-circulating characteristics have received increasing attention.[19,20] In this work, we attempt to utilize CHGC self-aggregated nanoparticles to increase the aqueous solubility, reduce side effects and enhance the antitumour efficacy of doxorubicin.

Herein, doxorubicin was physically entrapped in the CHGC nanoparticles by a dialysis method. We investigated the physicochemical properties of the doxorubicin-loaded CHGC nanoparticles' (DCNs') drug release behaviour *in vitro*. Furthermore, the in-vivo pharmacokinetics and antitumour activity of DCNs were studied.

Materials and Methods

Materials

CHGC was obtained during a previous study by the author.[17] Doxorubicin hydrochloride was supplied by Beijing Huafeng United Technology Co. Ltd. (Beijing, China). Daunorubicin was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Murine sarcoma 180 cell line (S180) was purchased from the Institute of Biochemistry and Cell Biology (Shanghai, China). Acetonitrile was of HPLC grade (Merck, Darmstadt, Germany). All other chemicals were of analytical grade.

Animals

Sprague–Dawley male rats, 200–230 g, and male ICR strain mice, 6–8 weeks old, 18–22 g, were purchased from the Laboratory Animal Center of Zhejiang University. All care and handling of animals were performed in compliance with the Animal Management Rules of the Ministry of Health of the People's Republic of China (Document No. 55, 2001) and the guidelines for the Care and Use of Laboratory Animals of Zhejiang University. The study protocols were approved by the Institutional Animal Care and Use Committee of Zhejiang University.

Preparation of doxorubicin-loaded nanoparticles

Doxorubicin-loaded nanoparticles were prepared by a dialysis method. First, blank nanoparticles were prepared by probe sonication in aqueous medium as previously reported.[17] Second, different amounts of doxorubicin hydrochloride were stirred with excess triethylamine (molar ratio of triethylamine to doxorubicin hydrochloride = 3 : 1) in 10 ml of dimethyl sulfoxide (DMSO) overnight to obtain doxorubicin base. The doxorubicin base solution was slowly added to 10 ml of the above CHGC nanoparticle suspension. After 6 h stirring, the mixture was placed into a dialysis bag (molecular weight cut-off (MWCO) 14 000) for dialysis against distilled water (1 L) for 24 h at 4°C. The outer solution was exchanged at 3-h intervals. Subsequently, the dialysis solution was filtered through a 0.8-μm membrane and freeze-dried. Based on the different ratio of feed drug to carrier (2 : 100, 8 : 100, 16 : 100, w/w), three kinds of DCN were coded as DCN-2, DCN-8 and DCN-16, respectively.

Characterization of doxorubicin-loaded CHGC nanoparticles

The morphology of the nanoparticles was observed using a transmission electron microscope (TEM) (JEM-1230; Jeol, Japan). The samples were re-suspended in water and placed onto copper grids to dry for TEM analysis. The size and size distribution of the nanoparticles were measured with a Malvern Zetasizer NanoS90 (Malvern Instruments Ltd., Malvern, UK).

To measure the loading content (LC) and encapsulation efficiency (EE) of DCNs, freeze-dried samples were dispersed in aqueous solution and disrupted by the addition of DMSO. The solution was vigorously stirred for 0.5 h followed by sonication for 10 min. The amount of doxorubicin was analysed using a UV spectrophotometer at 479 nm. The LC and EE were calculated with the following equations:

$$\text{LC}\ (\%) = [(\text{amount of doxorubicin in nanoparticles})/(\text{amount of doxorubicin-loaded nanoparticles})] \times 100 \quad (1)$$

$$\text{EE}\ (\%) = [(\text{amount of doxorubicin in nanoparticles})/(\text{amount of feed doxorubicin})] \times 100 \quad (2)$$

In-vitro doxorubicin release study

Doxorubicin release behaviour was studied *in vitro* by a dialysis method in 1/15 M phosphate-buffered saline (PBS) at different pH conditions (pH 5.5, 6.5 and 7.4). Briefly, 1 ml of doxorubicin-loaded nanoparticles was sealed in a dialysis bag (MWCO 14 000) and dialysed against 20 ml of the release medium at 37°C in an air-bath shaker at 100 rev/min. At predetermined time intervals, the entire medium was removed and replaced with the same amount of fresh medium. The amount of the released doxorubicin was determined using a fluorescence spectrophotometer (Jasco FP-6000; Jasco, Tokyo, Japan). Measurements were made at an excitation wavelength (Ex) of 470 nm and an emission wavelength (Em) of 585 nm.

Pharmacokinetics in rat plasma

Twelve Sprague–Dawley male rats were randomly divided into two groups of six rats each. Doxorubicin or DCN-16 were intravenously administered via a tail vein as a single dose of 2 mg doxorubicin per kg. Blood was collected by heparinized tubes at appropriate time intervals. After centrifugation at 12 000 rev/min for 3 min, plasma samples were frozen at −20°C until they were analysed. The extraction of doxorubicin from plasma was carried out as described in the previous report.[21] Daunorubicin solution was used as an internal standard. Plasma (100 μl) was spiked with 20 μl of daunorubicin (5 μg/ml) and vortex mixed. Borate buffer solution (300 μl) was added, and extraction was performed by adding 2.0 ml of chloroform–methanol (4 : 1, v/v) before stirring for 15 min. After centrifugation, the lower organic layer was transferred to a glass tube and evaporated to dryness at 40°C under a stream of nitrogen. The residue was dissolved in 100 μl of the HPLC mobile phase, and 20 μl of this solution were analysed using an HPLC system. The doxorubicin concentration was evaluated using an Agilent 1200 HPLC system equipped with a fluorescence detector (Ex = 470 nm, Em = 580 nm) and a 5 μm ODS C18 column (250 × 4.6 mm). A guard column (Diamonisl C18; 4 mm × 8 mm) was installed ahead of the analytical column. The mobile phase was water–acetonitrile (47.3 : 52.7, v/v) containing 20 mM phosphoric acid and 10 mM sodium dodecyl sulfate. The column temperature was set at 25°C. The flow rate was 1.5 ml/min. The recovery of doxorubicin from plasma was determined to be 91.7%. Pharmacokinetic parameters were determined using DAS (Drug and Statistics) VER 2.0 software (www.drugchina.net).

In-vivo evaluation of antitumour activity

Mice, in groups of ten, were injected subcutaneously with S180 tumour cells (2×10^6 cells/0.2 ml) into the right axillary tissue.[22,23] Four days after inoculation, the drugs were injected via a tail vein. Mice were allocated to four treatment groups as follows: (1) normal saline (control group); (2) CHGC nanoparticles; (3) free doxorubicin; and (4) DCN-16. Drugs were injected once every two days for eight consecutive days. The equivalent dose of doxorubicin administered was 2 mg/kg. The CHGC nanoparticles were injected at a dose of 20 mg/kg (equivalent to the amount of CHGC in DCN-16). The first day that mice received treatment was set as day 0. Tumour dimensions were measured daily with a caliper from day 0 to day 8. The tumour volume (V) was calculated by the following equation:

$$V = \pi/6 \times a \times b \times c \quad (3)$$

where a, b and c are length, width and height, respectively. On day 8, the mice were sacrificed and the tumours were excised and weighted. The inhibition rate of tumour growth (IR) was calculated as follows:

$$IR\ (\%) = [(A - B)/A] \times 100 \quad (4)$$

where A is mean tumour weight of the control mice, and B is that of the treated mice.

Statistical analysis

Results were expressed as mean ± standard deviation. The Kruskal–Wallis nonparametric analysis of variance was used with Nemenyi's post-hoc procedure to analyse differences between more than two groups. The non-parametric Mann–Whitney test was evaluated to determine whether differences between two groups existed. $P < 0.05$ denoted significance in all cases.

Results

Characterization of doxorubicin-loaded CHGC sef-aggreggated nanoparticles

The incorporation of doxorubicin into the CHGC self-aggregated nanoparticles occurred simultaneously during dialysis. The physicochemical properties of DCNs are listed in Table 1. Formulations using three different doxorubicin-to-CHGC nanoparticle weight ratios were prepared. As the weight ratio of feed doxorubicin to CHGC nanoparticles increased from 2/100 to 16/100, the doxorubicin loading content increased from 1.73% to 9.36%. The doxorubicin entrapment efficiency was 63.8% and 75.1% with respect to DCN-16 and DCN-8 ($P > 0.05$). The difference was significant ($P < 0.05$) for comparison of the entrapment

Table 1 Physicochemical properties of doxorubicin-loaded CHGC nanoparticles

Sample	Doxorubicin/carrier[a]	Size (nm)	PI	LC (%)	EE (%)
DCN-2	2/100	237 ± 25.6	0.115 ± 0.032	1.73 ± 0.12	88.0 ± 6.03
DCN-8	8/100	283 ± 18.6	0.137 ± 0.067	5.67 ± 0.32	75.1 ± 4.25
DCN-16	16/100	336 ± 21.2	0.129 ± 0.081	9.36 ± 0.49	63.8 ± 3.34

[a]The ratio of doxorubicin to carrier, based on feed amount (mg/mg). PI, polydispersity index; LC, loading content; EE, encapsulation efficiency. The results represent the mean ± SD, $n = 3$.

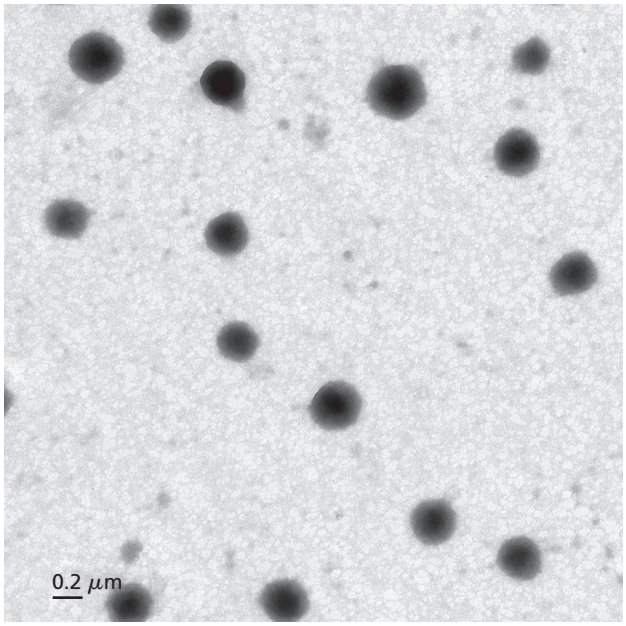

Figure 2 Transmission electron microscopy image of DCN-16.

efficiency of DCN-2 (88.0%) with the others. Based on the results of dynamic light scattering (DLS) experiments, the average diameter of drug-loaded nanoparticles increased as the loading content increased. The mean size of DCN-2, DCN-8 and DCN-16 was 237, 283 and 336 nm, respectively. The size difference between DCN-16 and DCN-2 was statistically significant ($P < 0.01$). This suggests that doxorubicin molecules were entrapped into the hydrophobic CHGC inner cores and the entrapped doxorubicin molecules increased the size of these nanoparticles. As shown in Figure 2, DCN-16 was roughly spherical in shape, determined by TEM. The mean diameter of DCN-16 was about 260 nm, which was smaller than the size (336 nm) determined by DLS. The reason for this is mainly due to TEM depicting the size in the dried state of the samples, whereas DLS measurement used a self-aggregate solution in aqueous medium.

In-vitro doxorubicin release study

In-vitro drug release behaviour was studied at different pHs to simulate the different physiological surroundings *in vivo*. The doxorubicin release profiles are summarized in Figure 3. It is noteworthy that doxorubicin release from DCNs was much faster in PBS at pH 5.5 (Figure 3a) than at pH 6.5 (Figure 3b) or 7.4 (Figure 3c) under the same conditions. For instance, DCN-16 released nearly 51% of the initial drug content in PBS at pH 5.5 after 72 h, which was more than at pH 7.4 and at pH 6.5 ($P < 0.05$). A similar trend was found for DCN-2 and DCN-8. This result was ascribed to the pH-dependent solubility of doxorubicin.

Pharmacokinetics in rat plasma

Figure 4 shows the drug concentration in rat plasma after intravenous administration of free doxorubicin and DCN-16. As expected, free doxorubicin showed rapid clearance from

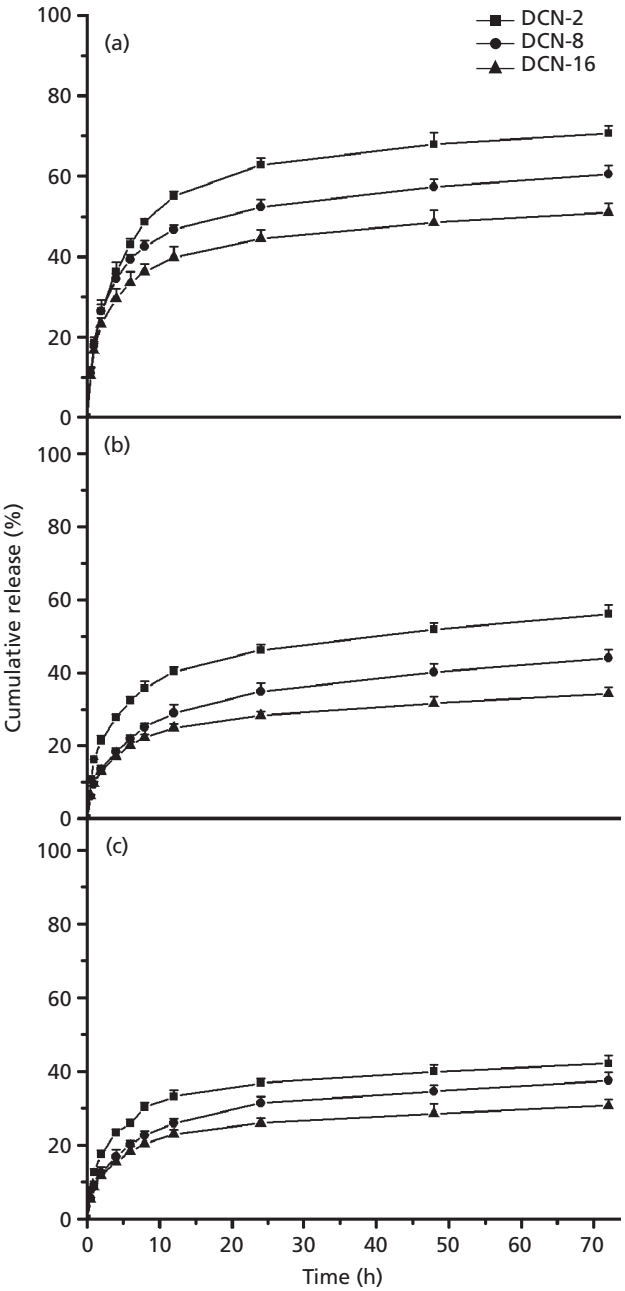

Figure 3 Release profiles of doxorubicin from doxorubicin-loaded CHGC nanoparticles. Release studies were carried out at 37°C in PBS at different pH values: (a) pH 5.5, (b) pH 6.5, (c) pH 7.4.

the plasma. Encapsulation of doxorubicin in the nanoparticles produced a significant change in drug pharmacokinetic parameters. After intravenous administration, DCN-16 exhibited a remarkably delayed blood clearance. Selected pharmacokinetic parameters are listed in Table 2. In comparison with free doxorubicin, DCN-16 had an increased mean residence time (MRT) ($P < 0.01$) and decreased clearance (CL) ($P < 0.01$). The area under the plasma concentration–time curve (AUC) of DCN-16 was 6.61 times higher than that of free doxorubicin ($P < 0.01$).

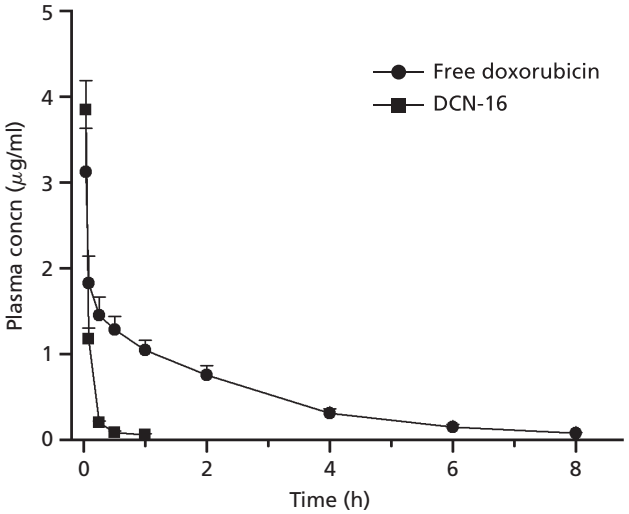

Figure 4 Plasma concentration–time curves of free doxorubicin and DCN-16 in rats. Plasma concentration was measured following the intravenous administration of a single dose of 2 mg doxorubicin per kg to rats, as free doxorubicin or DCN-16. Data are expressed as mean ± SD, $n = 6$.

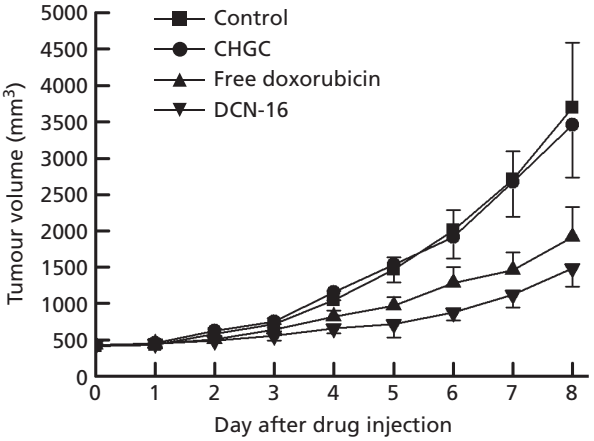

Figure 5 Growth inhibition of S180-bearing mice by intravenous administration of DCN-16 or free doxorubicin. Tumour size was measured for each mouse daily following the intravenous injection of a single dose of 2 mg doxorubicin per kg, as free doxorubicin or DCN-16; tumour volume in control mice and mice injected with CHGC was also monitored. Data are expressed as mean ± SD, $n = 10$.

Table 2 Pharmacokinetic parameters of doxorubicin and DCN-16

Parameters	Free doxorubicin	DCN-16
$AUC_{0\rightarrow\infty}$ (mg h/l)	0.666 ± 0.103	4.403 ± 0.712*
MRT (h)	0.125 ± 0.016	2.477 ± 0.297*
CL (l/h/kg)	3.005 ± 0.467	0.454 ± 0.083*

The pharmacokinetic parameters were determined after intravenous administration of the same dose of aqueous solution of free doxorubicin and DCN-16, respectively. *$P < 0.01$, compared with free doxorubicin.

Table 3 Antitumour activity of DCN-16 and free doxorubicin in S180 tumour-bearing mice

Group	Tumour weight (g)	IR (%)
Control	1.259 ± 0.242	NA
CHGC	1.241 ± 0.139	1.43
Free doxorubicin	0.611 ± 0.088*	51.5
DCN-16	0.452 ± 0.093**	64.1

IR, inhibition rate of tumour growth calculated using Equation 4; NA, not applicable. Tumour weight is expressed as mean ± SD, $n = 10$. *$P < 0.05$, **$P < 0.01$, compared with control group.

In-vivo evaluation of antitumour activity

To determine whether DCN-16 could suppress S180 tumour growth in-vivo, an S180-bearing model was established by subcutaneous injection of S180 tumour cells into the right axillary tissue of each mouse. Figure 5 shows tumour growth in terms of mean tumour size (mm^3), and Table 3 summarizes the antitumour efficacy shown in the four groups. At eight days post-injection, the difference between the CHGC group and control group was not significant. With regard to the change in the tumour volume, it was evident that treatment with either DCN-16 or free doxorubicin effectively suppressed the tumour growth. Tumour volumes of the mice treated with DCN-16 ($P < 0.01$) or free doxorubicin ($P < 0.05$) were significantly smaller than those of untreated mice. Moreover, the tumour volumes of the mice treated with DCN-16 were smaller than those treated with free doxorubicin. Based on the tumour weight, the difference was highly significant ($P < 0.01$) for comparison of the DCN-16 group with the control group (Table 3). In addition, the difference between the free doxorubicin group and control group was significant ($P < 0.05$). This was consistent with the results of volume inhibition.

Discussion

The doxorubicin-loaded CHGC nanoparticles (DCNs) formed self-assembled aggregates in aqueous medium and were observed by TEM to be almost spherical in shape. The mean diameters of these nanoparticles determined by DLS were in the range of 237–336 nm as the doxorubicin-loading content increased from 1.73% to 9.36%. In-vitro study indicated that doxorubicin release from DCNs was much faster in PBS at pH 5.5 than at pH 6.5 and 7.4, and the release rate was dependent on the loading content of doxorubicin in the nanoparticles. The finding of pH-dependent release behaviour of doxorubicin from the CHGC self-aggregated nanoparticles *in vitro* is of particular interest in achieving tumour-targeted doxorubicin delivery. Accelerated drug release in weak acidic solution is considered to be an advantage in an antitumour drug delivery system. Since doxorubicin-loaded nanopartcles were relatively stable in the blood circulation at pH 7.4, doxorubicin can be released at the tumour tissue where the local pH value is reported to be lower than that of normal tissue.[24,25] This character of drug release can reduce the toxicity of the antitumour drug to

normal tissue and improve its antitumour effect, consistent with results reported by our group and other groups.[26–28] As the drug loading content of these nanoparticles increased, the release rate of doxorubicin was much slower under the same conditions. Comparison of the release profiles of DCN-2, DCN-8 and DCN-16 in PBS (pH 7.4) is depicted in Figure 3c. The total amount of doxorubicin released from DCN-16 after 72 h was 31%, which is markedly lower than 38% from DCN-8 and 42% from DCN-2 ($P < 0.05$). This may be the result of doxorubicin release from the drug-loaded nanoparticles by the process of diffusion. In addition, doxorubicin appeared to be released in a biphasic way, which was characterized by an initial rapid release period followed by a step of slower release. The burst effect was observed during the first 8 h. After this initial effect, doxorubicin was released in a continuous way for up to 72 h.

Encapsulation in the nanoparticles significantly changed doxorubicin's pharmacokinetic parameters in rats. DCN-16 exhibited delayed blood clearance, increased MRT, decreased clearance and increased AUC compared with free doxorubicin. The increase in the AUC of DCN-16 compared with that of free doxorubicin might be due to the sustained release of doxorubicin from the nanoparticles *in vivo* and the decrease in time-dependent excretion from the body. The prolonged circulation in the blood compartment strongly suggests that DCN-16 is capable of avoiding uptake by the reticuloendothelial system (RES).[29] As reported previously, three kinds of self-assembled glycol chitosan nanoparticles were detected in the blood for three days.[30] Thus, the hydrophilic group's glycol chitosans on the surface of drug-loaded nanoparticles might offer steric hindrance to plasma opsonin. Therefore, this result indicated that the long circulation time in plasma of DCN-16 could contribute to enhanced uptake of doxorubicin at target sites (e.g. tumours).

In the S180 tumour model in mice, DCN-16 was found to suppress tumour growth. The prolonged circulation time of doxorubicin was responsible for the enhanced tumour inhibition rate of doxorubicin-loaded nanoparticles. For DCN-16, the enhanced permeability and retention (EPR) effect in solid tumours also contributed to the enhanced antitumour activity.[6,7] As reported previously, long circulation in the blood could be one of the most significant factors determining tumour targeting efficiency.[31] In recent years, considerable attention has been paid to the modification of the carrier surface with polyethylene glycol (PEG) and poly (2-ethyl-2-oxazoline), which can prolong the half-life of the carrier during circulation in blood by reducing opsonization and thus minimizing the carrier's clearance by the RES, which is mainly composed of the macrophages in liver and spleen.[32–34] This long-circulating characteristic of the carrier endows an EPR effect more valuable in cancer passive targeting.[7] Many studies show that the nanoparticulate polymeric carriers or liposomes, when their average size is less than 100 nm, have high potential for prolonged circulation in the blood and accumulation in the tumour.[35,36] However, DCN-16, with higher particle size (336 nm), exhibited long-term circulation in the bloodstream and showed stronger activity than free doxorubicin. A similar result was reported by Park *et al*.[30] Recent studies suggest that the deformability of nanoparticles should be considered to understand their RES uptake phenomena, because the real particle size can be changed against the deformability of particles in the bloodstream.[37] Thus, the in-vivo fate of blank and drug-loaded nanoparticles requires further investigation. It was also observed that the body weight of DCN-16-treated mice increased gradually, while that of mice treated with free doxorubicin decreased after treatment (data not shown). This indicated that free doxorubicin was delivered not only to tumour cells but also to other normal cells and produced side effects, whereas DCN-16 can reduce the unwanted side effects.

Conclusions

In this study, doxorubicin was successfully entrapped into the CHGC self-aggregated nanoparticles by a dialysis method. The mean diameter of three kinds of DCNs measured by DLS ranged from 237 to 336 nm. Doxorubicin release from DCNs was much faster in PBS at lower pHs, and was dependent on the loading content of doxorubicin in these nanoparticles. In the pharmacokinetics study, DCN-16 exhibited a prolonged circulation time and showed a greater AUC value than free doxorubicin in rat plasma ($P < 0.01$). By means of comparing the inhibition rate of S180 tumour growth, DCN-16 was therapeutically more active than free doxorubicin. Therefore, the CHGC nanoparticles are a promising carrier for the anticancer drug doxorbubicin.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflict of interest to disclose.

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