Special Issue

http://dx.doi.org/10.3786/sincs2011.12

NANO-MICRO LETTERS ARTICLE

www.nmletters.org

(Proceedings of 2011 Shanghai International Nanotechnology Cooperation Symposium, SINCS 2011, Published online 10 January 2012)

In Vitro Evaluation of pH-sensitive Doxorubicin Nanoliposomes Modified with Carboxymethyl Chitosan

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Abstract: The property in vitro of pH-sensitive doxorubicin nanoliposomes modified with carboxymethyl chitosan (CMCS-DOX-NL) was investigated. CMCS-DOX-NL had an encapsulation efficiencies of (88.8±0.84)% and an average particle size of (21.7±10) nm. The drug release in vitro of the CMCT-modified DOX nanoliposomes showed obvious pH sensitivity. Cells viability assay showed that cytotoxicity of CMCS-DOX-NL on HepG-2 cells was less than that of the common DOX nanoliposomes (DOX-NL). The results for the flow cytometry analysis and fluorescence microscopy micrographs suggested that the cells treated with CMCS-DOX-NL exhibited significant increase in cellular association or DOX uptake.

Keywords: doxorubicin nanoliposomes; carboxymethyl chitosan; pH-sensitive; HepG-2 cells

Citation: Shanxiang Yang, Xiuzhen Qian, Yunlong Xu, Xinyi Tao and Chongjun Zhao, "In Vitro Evaluation of pH-sensitive Doxorubicin Nanoliposomes Modified with Carboxymethyl Chitosan", Proceedings of Shanghai International Nanotechnology Cooperation Symposium, 46-50 (2011). http://dx.doi.org/10.3786/sincs2011.12

Introduction

Liposomes are artificial microspheres which are prepared with the direct order lipid bilayer [1]. As drug carriers, liposomes have been extensively investigated as a drug carrier system in attempt to enhance the therapeutic efficacy of anticancer drugs and references shows that liposomes can change the distribution of DOX from the blood to tissue and remarkably reduce cardiotoxicity [2-5]. However compared with normal tissue, the vascular endothelial gap increased in tumor tissue. Preparation of specific pH-sensitive nanoliposomes have been concerned in order to enhance the combination between drugs and cells [6,7]. Liposomes were not three-dimensional stabilized phospholipids. Using the structure of biomaterials changes at different pH values, liposomes decorated with pH-sensitive biomaterials can show pH dependence [8,9]. Carboxymethyl chitosan is a kind of biocompatible, biodegradable and non-toxic polymer. These properties make it have wide biomedical applications in tissue engineering, as excipient for drug delivery. In addition, its hydrophilic surface promotes cell adhesion, proliferation, and differentiation, and evokes a minimal foreign body reaction.

Herein, using the surface-active carboxyl group of liposomes as the best chemical modification site, pH-sensitive liposomes were prepared by modifying with carboxymethyl chitosan [10-12]. The objective of the present study was to investigate the property in vitro of pH-sensitive doxorubicin nanoliposomes modified with carboxymethyl chitosan.

Materials and methods

Materials

Doxorubicin hydrochloride was purchased from

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Hisun Pharmaceutical group (Zhejiang province, China). Soybean phosphatidylcholine was purchased from Lipoid corporation (Germany). Carboxymethyl chitosan was purchased from Chunlu Biotech corporation (Shanghai). Cholestrol, V_E and polycarbonate membrane filters were all purchased from Yuanju biotech (Shanghai). Alcohol and all other chemicalsn were commercial products of analytical reagent grade. BD FACS flow cytometry was a product of Becton Dichinson (USA). Fluorescence microscope was produced by Lecia corporation (Germany).

Preparation of pH-sensitive nanoliposomes

PC, cholesterol and V_E with a mass ratio 4:1:0.1 were dissolved in alcohol in a pear-shaped flask. The alcohol was evaporated to dryness under vacuum with a rotrary evaporator, and the lipid film was then hydrated with PBS buffer solution (pH 4.0) by sonication in the water bath for 20 min and blank nanoliposome suspension was formed. Then the liposomes were successively extruded through polycarbonate membranes with pore size of 220 nm for 3 times to make homogeneous nanoliposomes. The pH value of liposomes suspension was then adjusted to 7.4 with NaOH solution. Doxorubicin hydrochloride was dissolved in the proper amount double-distilled water and heated to 37℃, then added into the above prepared nanoliposome suspension. The flask was incubated in the water bath for 0.5 h, and then 0.5wt% carboxymethyl chitosan solution was added into doxorubicin nanoliposome suspension.

Encapsulation efficiency

Drug-loaded liposomes (1 mL) were putted into dialysis tubing (MW cut off 14000 below) sealed at both ends with clips, and then the tubing was placed into the beaker containing 50 mL of PBS buffer solution as release medium. The release experiment was performed at a constant stirring rate at 37° for 24 h. A volume of 5 mL release solution was taken out at different time from the beaker, followed by an immediate supplement of the same volume of the fresh release medium. Then doxorubicin concentrations were then measured with a UV spectrophotometer at 480 nm. The total doxorubicin liposomes were lysed directly with 10% TritonX-100 (V/V). Encapsulation efficiency (EE) was estimated with the following formula:

$$EE = (M_{total}\text{-}M_{free})/M_{total} \times 100\%$$

Release of doxorubicin from the pH-sensitive nanoliposomes

The release of doxorubicin from the pH-sensitive nanoliposomes was estimated by the 2.3 method. The time points were adapted as the follows: 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 7, 8, 12, 16, 24 h. Each experiment was performed in triplicate.

Particle size and zeta potential

Liposomal size and zeta potential were measured by laser diffraction particle size analyzer.

Cytotoxicity

HepG-2 cells were seeded in a 96 well plate at a density of 3×10^4 cells per well. The culture plates were incubated for 24 h at 37° and 5% CO_2 to reach the exponential phase of growth, and then incubated for 24 h with various drug formulations, including free doxorubicin, common doxorubicin nanoliposomes (DOX-NL), CMCT-DOX-NL. Blank medium was used as control. The cultures were washed with PBS for 3 times and added 20 μl MTT solution (5 μg/mL) for 4 h. After it, 200 μl DMSO were added and measured with Microplate Reader (Synergy Mx, Bitotek, USA) at 490 nm.

Intracellular uptake

Doxorubicin formulations uptake by HepG-2 cells was studied by fluorescence microscopy and flow cytometry methods. 1×10^5 HepG-2 cells were seeded into 24 well plates. It was incubated in a humidified incubator maintained with 5% CO_2 and 37℃. After 12 h the cells was washed with incomplete media and were incubated with various drug formulations at a doxorubicin concentration of 10 μg/mL for fluorescence microscopy. The various drug formulations were used for flow cytometry at 37℃. After 1 h of incubation the cells were washed to remove free doxorubicin and flow cytometry was performed on a BD FACS (Becton Dickinson, USA) with a laser of 480 nm wavelength and fluorescence was detected at 560 nm.

Results and discussion

Encapsulation efficiency, particle size and zeta potential

The results showed that the mean encapsulation efficiencies of doxorubicin-loaded liposomes were 88.8±0.84%. The measured particle size was showed in Fig. 1. The average measurements of particle size were about 21.7±10 nm. The zeta potential values were about −16.5±1.0 mV and the modification of carboxymethyl chitosan didn't change the surface potential of liposomes.

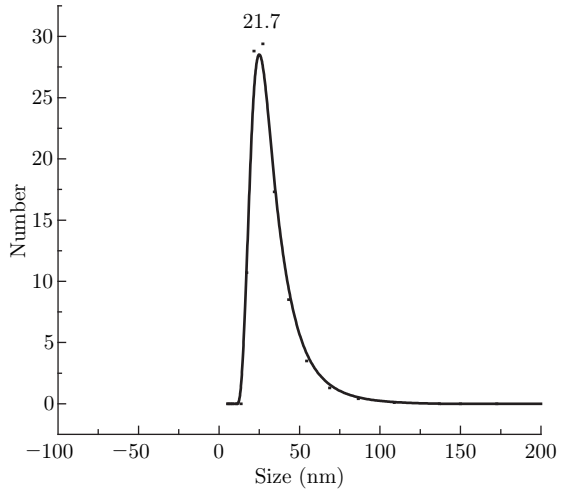


Fig. 1 The size of the pH-sensitive nanoliposomes.

Drug release in vitro

The releases of DOX from pH-sensitive nanoliposomes in PBS buffer medium from pH 5.0 to pH 7.4 were investigated. The percentage of release of DOX from the pH-sensitive nanoliposomes has been presented in Fig. 2. The release rate of DOX in the media with different pH values was different. The release showed pH-dependent. The release rate of DOX at neutral or alkalescent pH was slow and sustained. However in the weak acidic environment surrounding the tumor releases were much faster. It was found that the pH value of dissolution medium affect the drug release rate of DOX. The drug release rate reduced with the increasing of the pH value of dissolution medium. The faster drug release rate in lower pH medium could be based in two factors: the one is the structure of the liposomes modified with carboxymethyl chitosan. The loose structure caused by the stronger protonation of amino groups of carboxymethyl chitosan in lower pH surrounding the tumor; the other is the higher solubility of DOX in lower pH [11,13].

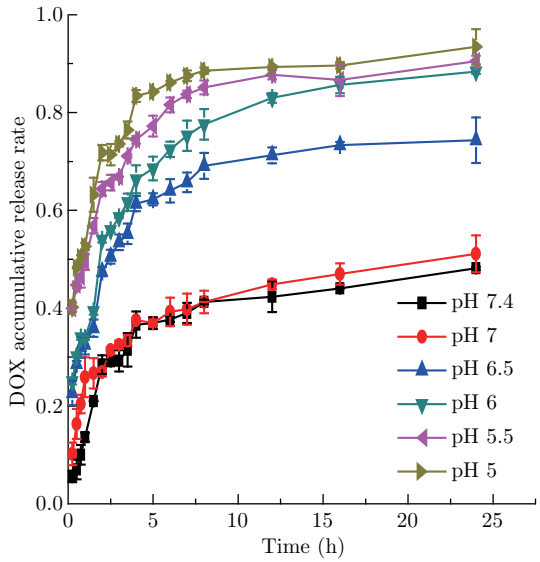


Fig. 2 Cumulative release profile of DOX at different pH value.

In vitro toxicity studies

The toxicity of the liposomes modified with carboxymethyl chitosan was checked using the MTT methods. As shown in Fig. 3, it was suggested that CMCT-DOX-NL enhanced the cellular uptake efficiency. It was previously reported that carboxymethyl chitosan had no significant cytotoxicity and as a biomaterial it had many biological use. So the enhancement is most probably due to the mechanism which caused by the nanoliposomes modified by carboxymethyl chitosan.

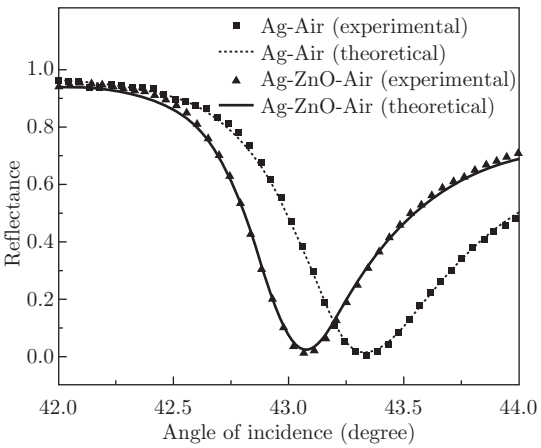
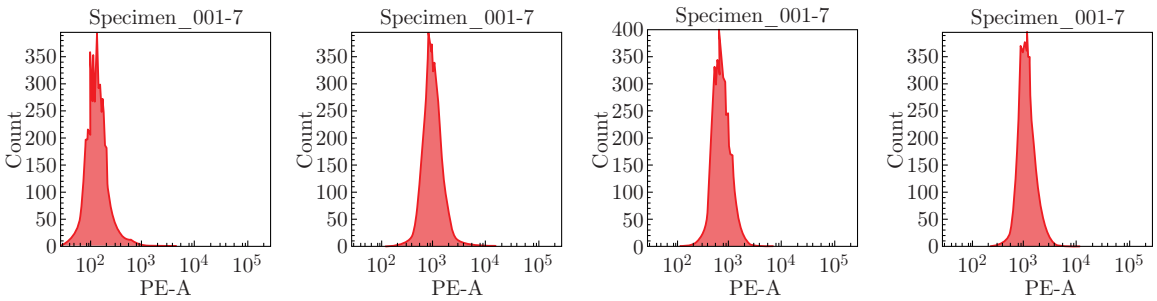


Fig. 3 Cell viability after exposure to Pure-DOX, DOX-NL, CMCT-DOX-NL at various drug concentrations at 37℃ for 24 h.

Intracellular uptake of pH-sensitive nanoliposomes

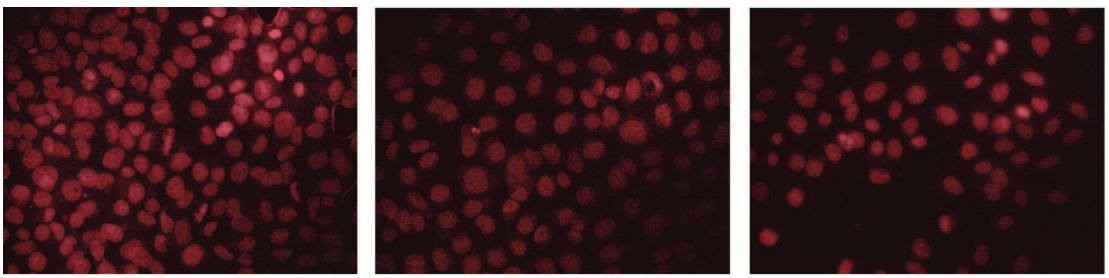
Flow cytometry was employed to study the behavior of various drug formulations with HepG-2 cells. There was an increase in the fluorescence intensities from DOX-NL to CMCT-DOX-NL in Fig. 4. The untreated cells showed low level fluorescence intensity. However the pure doxorubicin solution showed the highest uptake after 1 h of incubation among all formulations. From the Fig. 4 the mean values of fluorescence intensities for HepG-2 were 149, 745, 982, and 1178 respectively. To study the uptake/internalization of CMCT-DOX-NL, fluorescence microscopy was performed on HepG-2 cells. From the fluorescence images, Pure DOX showed more intense and bright fluorescence than the other two among the three drug formulations: Free DOX, DOX-NL, and CMCT-DOX-NL. However CMCT-DOX-NL showed more intense and brighter than the DOX-NL. The different uptake and cytotoxicity could be contributed to three factors:

the one was the release amount of doxorubicin from various drug formulations were different and free doxorubicin was higher. The second was the potential values between the DOX-NL and CMCT-DOX-NL which may incubate with HepG-2 cells for the potential effect. The above results in 3.1 showed that carboxymethyl chitosan didn't change the surface potential of nanoliposomes. The third was for the combination of doxorubicin formulation with HepG-2 cells which may be via various pathways, including diffusion, membrane fusion and endocytosis. Different ways may result in different consequent with cells. The mechanism of membrane fusion is related to materials; however the materials were identical in this study. Previously mentioned, the release amount in the cell culture media with FCS didn't differ significantly, so doxorubicin amount through such way should be equal. As the low pH value of cells interstitial, the release rate of CMCT-DOX-NL was faster compared to DOX-NL (Fig. 2). Incubated with the cells, CMCT-DOX-NL released more free drug than DOX-NL by diffusion. This may be the main reason of the fluorescence intensity differed between DOX-NL and CMCT-DOX-NL.



(a) Untreated (b) Pure DOX (c) DOX-NL (d) CMCT-DOX-NL

Fig. 4 Flow cytometric measurement of doxorubicin uptake into HepG-2 cells.



(a) Pure DOX (b) DOX-NL (c) CMCT-DOX-NL

Fig. 5 Fluorescence microscopy micrographs of HepG-2cells treated with Pure-DOX, DOX-NL, CMCT-DOX-NL.

Conclusions

In this study, a pH-sensitive nanoliposomes modified with carboxymethyl chitosan were prepared by film-sonicate-pH gradient. Release behaviors of CMCT-DOX-NL showed obvious pH dependence and sustained release pattern. The release amount of doxorubicin from CMCT-DOX-NL increased with the decreasing pH value. In vitro cytotoxicity test using MTT method, flow cytometry and fluorescence microscopy showed that CMCT-DOX-NL enhanced the cellular uptake. Due to high specific intracellular uptake, this liposomes modified with carboxymethyl chitosan may further be explored for its applications in delivery of many other anti-cancer drugs. In addition, in vivo testing of pH-sensitive nanoliposomes is in progress.

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