International Journal of Pharmaceutics 517 (2017) 25–34

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Fabrication of self-assembled chitosan-dispersed LDL nanoparticles for drug delivery with a one-step green method

Jing Tian[a], Shasha Xu[a], Hongbing Deng[b], Xinxing Song[c], Xiujuan Li[c], Jiajia Chen[b], Feng Cao[c,*], Bin Li[a,*]

[a] College of Food Science and Technology and the MOE Key Laboratory of Environment Correlative Dietology, Huazhong Agricultural University, Wuhan 430070, China
[b] Hubei International Scientific and Technological Cooperation Base of Sustainable Resource and Energy, Hubei Key Lab of Biomass Resource Chemistry and Environmental Biotechnology, School of Resource and Environmental Science, Wuhan University, Wuhan 430079, China
[c] Department of Cardiology, Cardiology Department of Chinese PLA General Hospital, Beijing, 100853, China

ARTICLE INFO

Article history:
Received 30 August 2016
Received in revised form 23 October 2016
Accepted 10 November 2016
Available online 11 November 2016

Keywords:
Low-density lipoprotein
Chitosan
Self-assembly
Nanocarrier

ABSTRACT

Self-assembled nanoparticles (NPs) composed of chitosan (CS) and low density lipoprotein (LDL) of hen eggs were prepared by a one-step green synthesis of mixing CS solution and LDL suspension. The formulated CS-LDL NPs were then applied to encapsulate doxorubicin hydrochloride (DOX) with the encapsulation efficiency of 51.7%. The average particle size and ζ-potential of DOX-loaded CS-LDL NPs (CS-LDL-DOX NPs) were 179 nm and +48.3 mV, respectively. The encapsulated DOX showed less cytotoxicity than free DOX after 24-h incubation with gastric cancer SGC7901 cells, which may be due to extended release. Cellular uptake of CS-LDL-DOX NPs was significant higher than that of free DOX due to the endocytosis of tumor cells. Thus CS-LDL-DOX NPs showed a potential in reducing cytotoxicity of DOX by extended release behavior and preferential uptake compared to free DOX. In addition, flow cytometry and terminal-deoxynucleotidyl-transferase-mediated dUTP nick-end labeling assay demonstrated that CS-LDL-DOX NPs induced the apoptosis of cancer cells. Autophagy was involved in effects caused by CS-LDL-DOX NPs through blocking AKT/mTOR signaling, which was demonstrated by the analyses of the expression of LC3, p62, AKT, *p*-AKT, mTOR and *p*-mTOR.

1. Introduction

Nanoparticles (NPs)-based drug delivery systems have advantages in the extension of drug release, protection of premature degradation against rapid metabolic activation, enhancement of absorption into a targeted tissue to minimize side-effects and maximize therapeutic efficacy, reversion of multidrug resistance in tumor cells, restoration of sensitivity of tumor cells to anticancer drugs and provision of an affinity to tumor cell membrane (Choi et al., 2012; Li et al., 2010; Ould-Ouali et al., 2005; Yamamoto et al., 2005). Thus nanocarriers have received considerable interest in the development of drug delivery systems (Althuri et al., 2013; Barreto et al., 2011; Brannon-Peppas and Blanchette, 2012; Panyam and Labhasetwar, 2003).

Chitosan (CS) is a natural cationic polysaccharide, which is composed of randomly distributed β-(1–4)-linked D-glucosamine (de-acetylated unit) and *N*-acetyl-D-glucosamine (acetylated unit) (Wang and Chen, 2014). CS has been widely used as a biopolymer-based drug delivery system due to its advantages in biocompatibility, sustained release of encapsulated therapeutics, cell adhesion and tumor inhibition effects *per se* (Hasegawa et al., 2001; Tan et al., 2009).

Low-density lipoprotein (LDL) from hen eggs are the main constituent of yolk. They are spherical particles (17–60 nm in diameter with a mean value of about 35 nm) with a lipid core at a liquid state (triglycerides and cholesterol esters) surrounded by a monofilm of phospholipid and protein (Anton, 2007). LDL has been found being greatly required by malignant cells (Firestone, 1994). Therefore LDL has been extensively used as drug delivery vehicles by chemical-linking or association via deposition or mixture in various ways for malignant cells expressing LDL receptors (Firestone, 1994). Due to the resemblance of natural lipoprotein structures, LDL-like NPs have been considered highly biocompatible, low immunogenicity and safe (Ng et al., 2011). In previous studies LDL-like NPs have

* Corresponding authors.
E-mail addresses: hbdeng@whu.edu.cn (H. Deng), wind8828@gmail.com (F. Cao), libinfood@mail.hzau.edu.cn (B. Li).

http://dx.doi.org/10.1016/j.ijpharm.2016.11.030
0378-5173/

been developed from LDL isolated from human plasma, which have limitation in large quantity production and the possible introduction of pathogens (Harisa and Alanazi, 2014; Zhang et al., 2014; Zhu et al., 2014). LDL of hen egg origin provide an alternate safe and favorable template for the development of LDL-like NPs due to its convenient, massive and economic production.

Doxorubicin hydrochloride (DOX) is one of frontline chemotherapeutic pharmaceutics against a variety of cancers and one of ideal candidates for the study of a drug delivery system due to its cytotoxicity-caused systemic side-effects and auto-fluorescent characteristics (Soares et al., 2016). Potential carriers have been developed to load DOX by chemical conjugation or physical entrapment via manipulation of experimental conditions (i.e. gelation or pH adjustment) or the addition of organic chemicals (Li et al., 2008; Qi et al., 2010; Tan et al., 2009; Yu et al., 2006; Zhu et al., 2013), which may introduce unhealthy reagents. In addition these processes were not friendly with pH-sensitive or chemical-sensitive drugs, which may result in lowered encapsulation efficiency or efficacy of drugs.

The purpose of the study is to develop a simple, green and low-cost process that produces NPs having advantages of CS and LDL mentioned above and having potentials in reducing the systemic side-effects of DOX by extending DOX release and enhancing accumulation of DOX in cancer cells. It is hypothesized that electrostatic forces of opposing charges carried by CS and phospholipids on the surface of LDL could be harnessed to formulate NPs via self-assembly technique. Moreover because of the slowed penetration of buffer into biopolymer shell and the diffusion of substances through biopolymer matrix (Liang et al., 2015; Tu et al., 2015), CS-based NPs are expected to have a pattern of extended release behavior of encapsulated therapeutics, which may result in reduced cytotoxicity of DOX-loaded NPs compared to free DOX. Because LDL tends to aggregate in aqueous solution at around pI 6.5 (He et al., 2015), in presented study CS acidulous aqueous solution was applied to disperse agglomerated LDL aqueous solution under stirring without further process or chemicals applied forming electrostatic-force-held NPs. The structure of NPs was characterized with dynamic light scattering (DLS), transmission electron microscopy (TEM) and atomic force microscopy (AFM). The cytotoxic effects of DOX-loaded LDL-CS NPs on gastric cancer cells SGC7901 were evaluated; The underlying mechanisms were investigated including the involvement of apoptosis and autophagy. To date LDL from hen eggs easily and fast dispersed with CS forming stable NPs to encapsulate anticancer pharmaceutics DOX via self-assembly technique in a one-step green method has not yet been reported.

2. Materials and methods

2.1. Materials

Low-density lipoprotein (LDL) was extracted and purified according to the previous study (Anton et al., 2003). LDL with a purity of more than 97% were obtained by lyophilization. Chitosan (CS, M_W 680 kDa, 87.5% deacetylated) was purchased from Zhejiang Yuhuan Co. (Zhejiang, China) and purified according to the previous study (Gan and Wang, 2007). The chemical reagents including sodium hydroxide, hydrochloric acid and glacial acetic acid were purchased from Sinopharm Chemical Regent Co., Ltd (Shanghai, China). Doxorubicin hydrochloride (DOX) was obtained from Shanxi Pude Pharmaceutical Co. Ltd (Shanxi, China). Human gastric cancer SGC 7901 cell lines were obtained from the State Key Laboratory of Cancer Biology at Fourth Military Medical University in China. RPMI-1640 cell culture medium and fetal bovine serum were purchased from Hyclone (Thermo Fisher Scientific Inc., USA). All other reagents were of analytical grade unless stated otherwise. All aqueous solutions were prepared using purified water with a resistance of 18.2 MΩ cm.

2.2. Preparation of nanoparticles (NPs) formed by CS and LDL

CS solution was prepared by dissolving purified CS powder in 0.5% (w/w) of glacial acetic acid solution with a concentration of 0.5% (w/w) and stirred overnight. LDL was suspended in ultra-pure water and the resultant slurry was stirred at room temperature for 1 h. Then CS solution was added into the slurry of LDL with the weight ratio of 1/6 under continuous stirring for 2 h, and afterwards CS-LDL NPs were formed.

2.3. Characterizations

The particle size and polydispersity of samples were determined with Malvern Nano-ZS (Malvern Instruments, UK). Each sample was measured for three times. Samples for transmission electron microscopy (TEM) analysis were obtained by placing a drop of colloid dispersion containing NPs onto a copper grid. The drop was dried under ambient condition on the copper grid prior to the examination with TEM. Energy-dispersive X-ray (EDX) spectroscopy analysis was performed with a JEOL2000 FX-II transmission electron microscope (JEOL Ltd., Japan). NPs solution was dropped on conducting resin and dried at room temperature. Afterwards the morphology of NPs surface was observed with field emission scanning electron microscopy (FE-SEM, JSM-6700F, JEOL, Japan). NPs solution was dried on a freshly cleaved mica surface in a desiccator at room temperature two days before atomic force microscopy (AFM) analyses. Images were acquired in tapping mode on a digital instruments nanoscope IV (Aglient, USA). The composition of NPs was examined with X-ray photoelectron spectroscopy (XPS) using an axis ultra DLD apparatus (Kratos, UK). ζ-potential measurements were carried out on a Nano-ZS Zetasizer with a 633 nm He-Ne laser (Malvern Instrument, UK). ζ-potential was calculated by Malvern software package using Henry's equation. The measures were conducted at a constant temperature of 25 °C and the model was Smoluchowski, F(ka) = 1.5.

2.4. Encapsulation of DOX

DOX was dissolved in ultra-pure water with a concentration of 100 μg/mL. Then LDL was dissolved into as-prepared DOX solution to reach the concentration of 0.5 mg/mL. CS was dissolved in 0.5% of glacial acetic acid solution with a concentration of 0.5% (w/w) and then was added into the slurry of LDL solution containing DOX. The mixture was maintained stirring for 2 h at room temperature. Afterwards the obtained mixture was centrifuged (MWCO 10 k) at 3500 rpm for 20 min (He et al., 2015; Tu et al., 2015). NPs encapsulated with DOX was obtained by removing supernatant and labeled as CS-LDL-DOX NPs. The fabrication process of CS-LDL-DOX NPs was illustrated using schematic diagram in Fig. S1. The amount of DOX in the supernatant was determined with a UV–vis spectrophotometer (UV-1100, MAPADA) at 480 nm (Qi et al., 2010; Tu et al., 2015). The encapsulation efficiency (EE) was calculated as follows:

$$EE = (W_0 - W_1)/W_0 \times 100\%$$

Where W_0 (mg) was the weight of DOX in initial solution, and W_1 (mg) was the weight of free DOX in solution.

2.5. Cell culture maintenance

Human gastric cancer cell lines SGC7901 were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum and