International Journal of Pharmaceutics 471 (2014) 118–126

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

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

Pharmaceutical nanotechnology

Enhanced cellular uptake and anti-proliferating effect of chitosan hydrochlorides modified genistein loaded NLC on human lens epithelial cells

Wenji Zhang [a], Jinlu Liu [b], Qi Zhang [c], Xuedong Li [b], Shihui Yu [c], Xinggang Yang [c], Jun Kong [b,**], Weisan Pan [c,*]

[a] School of Traditional Chinese Medicine, Shenyang Pharmaceutical University, 103 Wenhua Road, Shenyang 110016, China
[b] Department of ophthalmology, Affiliated Fourth Hospital of China Medical University, Shenyang 110005, China
[c] Department of Pharmaceutics, School of Pharmacy, Shenyang Pharmaceutical University, 103 Wenhua Road, Shenyang 110016, China

ARTICLE INFO

Article history:
Received 12 March 2014
Received in revised form 20 April 2014
Accepted 18 May 2014
Available online 22 May 2014

Keywords:
Cellular uptake
Chitosan hydrochloride
NLC
Genistein
Apoptosis

ABSTRACT

This study was attempted to increase the cellular uptake of developed genistein loaded nanostructured lipid carriers (NLC) into human lens epithelial (HLE) cells by chitosan hydrochlorides coatings when applied in post lens capsule (PCO) treatment, and to provide further understanding of the uptake and anti-proliferation mechanisms inside. NLCs were produced using melt-emulsification method and were subsequently coated with chitosan hydrochlorides by adsorption. The uptake of various particle sizes were evaluated and visualized by confocal laser scanning microscopy (CLSM), showing a size-dependent manner. The uptake of NLC was proved to be endocytosed in an energy dependent and clathrin-mediated endocytosis to HLE cells by the decrease in uptake at lower temperature, when pre-saturated by blank NLC and in the presence of NaN_3 and sucrose. CH coating improved the uptake percentage of NLC irrespective of the particle size, without influencing the uptake mechanism. Cell apoptosis was tested using PI and Annexin V-FITC/PI staining, followed by flow cytometer analysis. Higher anti-proliferation effect was observed for CH-NLC in inhibiting the growth of HLE cells by causing more apoptosis. Results above indicate that GEN-NLC surface modified by chitosan hydrochlorides could enhance the transcellular performance and anti-proliferating effect as PCO therapy.

1. Introduction

Posterior capsular opacification (PCO) remains the most frequent complication following extracapsular cataract operation and could lead to a secondary blurred vision (Spalton, 1999). Clinically, PCO is caused by the proliferation, migration and myofibroblast transformation of epithelial cells retained in the capsular bag after cataract surgery. Therefore, the inhibition of the epithelial cells growth could effectively reduce the occurrence of PCO and improve the patient compliance after cataract surgery.

Nanostructured lipid carrier (NLC) has been widely accepted as a unique drug carrier due to its characteristic sustained drug release behavior, high drug loading capacity, and good biocompatible property (Souto et al., 2004). Recently, researchers have also found that NLCs are equipped with some additional merits in biological properties like the ease of cell uptake (Su et al., 2011), and the targeting effect by absorption (Maurizio et al., 2005) or ligand-mediated binding to the surfaces of the particles (Zhao et al., 2011; Han et al., 2013).

The author has previously developed optimized genistein loaded NLC using hot melt emulsification technique combined with ultra-sonication, and used them in preliminary cellular uptake research of human lens epithelial (HLE) cells for inhibition effect evaluation (Zhang et al., 2013). The lipid incorporated formulation is helpful in improving the transcellular transportation due to the prolonged drug release and the enhanced lipid affinity with plasma membrane. However, the cell viability still could not decrease to the clinical effective inhibition percent, with less drug uptake efficiency and late efficacy onset.

The cellular uptake enhancement effect of chitosan has been reported by many researchers (Tian et al., 2012; Luo et al., 2011; Tahara et al., 2009). Chitosan is often selected as the excellent

* Corresponding author. Tel.: +86 024 23986313; fax: +86 024 23986313/23953241.
** Corresponding author. Tel.: +86 024 62355002.
E-mail address: pppwwwsss@163.com (W. Pan).

http://dx.doi.org/10.1016/j.ijpharm.2014.05.030
0378-5173/

candidates for surface modification of colloidal drug delivery system for excellent bioadhesiveness and biocompatibility (Luo et al., 2011; Li et al., 2009). However, chitosan is not water-soluble, which limits their use in pharmaceutics. Currently, many chitosan derivatives are developed in methods of reduction in molecular weights, modification of other groups like carboxymethyl groups, and transformed into chitosan hydrochlorides, to improve the solubility for a wider application. Among them, chitosan hydrochlorides (CH) could be produced in a simple process, which retains its unique biological adhesiveness and favorable biocompatibility.

Genistein (GEN) is the inhitor of tyrosine kinase and can effectively inhibit the proliferation, migration and transformation to myofibroblasts of lens epithelial cell by depressing the function of tyrosine kinase in the whole process of PCO formation (Walker et al., 2007). The purpose of this study was to surface modify the previously developed GEN-NLC with self-prepared chitosan hydrochlorides (CH), and investigate the uptake enhancement of CH-GEN-NLC in HLE cells. Physicochemical properties of CHC-GEN-NLC, such as particle size and zeta potential, were investigated to determine cellular uptake. Several uptake pathways were examined to both quantify and elucidate the mechanism and interactions between non- and CH-NLC and HLE cells. The effect of NLC on cell apoptosis was also evaluated.

2. Materials and methods

2.1. Reagents and cell lines

Genistein (GEN) was purchased from Huike Botanical Development Co., Ltd. (Xian, China). Compritol 888 ATO and Gelucire 44/14 were both kindly gifted by Gattefosse (Paris, France); Miglyol 812 N was obtained from Sasol (Witten, Germany); Solutol® HS15 was sampled from BASF (Ludwigshafen, Germany). Egg phosphatidylcholine (EPC) was from Taiwei Pharmaceutical Co., Ltd. (Shanghai, China). Chitosan (deacetylation degree 97% and molecular weight 540 kDa) was purchased from Haidebei Biochemical Corp. (Shandong, China). The fluorescent dye Rhodamine B (RhB) and propidium iodide (PI) were supplied by Sigma–Aldrich Co. (St. Louis, MO, USA); Annexin/PI kit was obtained from TCI Development Co., Ltd. (Tokyo, Japan). Filipin and cytochalasin D were also purchased from Sigma–Aldrich (St. Louis, MO, USA). Purified water was used after deionization and filtration in a Millipore® system home supplied.

Human lens epithelial (HLE) cell line was provided by China Medical University; Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), trypsin were all obtained from Gibco technology (Shanghai, China); Cell Counting Kit-8 (CCK-8) was acquired from Dojindo Molecular Technology Inc. (Shanghai, China); trypan blue and penicillin–streptomycin for cell culture were both the products of Beyotime Technology (Jiangsu, China). TrypLETM Express was obtained from Invitrogen (Carlasbad, CA). All other reagents and solvents used were of analytical grade.

2.2. Preparation of GEN-NLC and cationic GEN-NLC

2.2.1. Preparation of chitosan hydrochlorides.

Chitosan hydrochlorides were produced as Signini with minor modifications (Signini and Campana, 1999). Briefly, 1 g of commercial chitosan products was dissolved in 100 ml of dilute hydrochloric acid (1% by weight). The dissolution was assured by stirring the initial suspension in an airtight container for 10 h with moderate speed at 40 °C. The final solution was subsequently filtered through Millipore membrane (5.0 μm) and dialyzed against deionized water. Chitosan hydrochlorides were finally obtained after freeze-drying (FD-1; Boyikang, Beijing, China). The final product was white powders soluble in pure water.

2.2.2. Preparation of NLC and CH-NLC.

GEN-NLC was produced using melt emulsification technique combined with ultra-sonication as described in our previous study (Zhang et al., 2013). Briefly, designed amounts of GEN, Compritol 888 ATO, Miglyol 812 N, and Gelucire 44/14 were mixed and heated under moderate stirring at 85 °C to bring a transparent and uniform oil phase. Meanwhile, Solutol® HS 15 and EPC were dissolved in 20 ml purified water before heated up to 85 °C and dropped into the oil phase, with stirring for 10 min at 600 rpm. After the coarse emulsion was formed, it was homogenized (JY-92-II; Xinzhi, Ningbo, China) for various time durations. The obtained nanoemulsion was rapidly solidified in ice bath (0–4 °C) to form GEN-NLC.

The NLC labeled with RhB was prepared by the same method as described above. RhB was added to the oil phase after the oil phase was uniformly melted. The finally obtained NLC was dialyzed against distilled water for 24 h using a dialysis sack (MW cutoff: 1000) with regular water changing to remove unencapsulated RhB. Samples were carefully protected from light throughout the experimental procedure. All formulations were made isotonic with an appropriate amount of glycerin.

CH-NLC with different CH concentrations was prepared as follows. Firstly, various amounts of CH powders were dissolved in 0.9% NaCl to form solutions. Then the above mentioned GEN-NLC or RhB-NLC was slowly added to an appropriate portion of CH solutions, followed by magnetic agitation at 25 °C for 10 min, and the volume was adjusted by adding 0.9% NaCl in order to keep the drug concentration (0.1%).

2.3. Physicochemical properties of nanoparticles

2.3.1. Mean particle size and zeta potential

The mean particle size (PS) and zeta potential (ZP) were assessed by photon correlation spectroscopy (PCS) using a Zetasizer Nano (Malvern Instruments, Worcestershire, UK) at 25 °C. The PS and PI values were obtained at an angle of 90° in respect to the incident beam in 10 mm disposable polystyrene cells. The zeta potential was measured in disposable plain folded capillary zeta cells by determining the electrophoretic mobility on the particle surface using the same instrument. Each measurement was made in triplicate.

2.3.2. Encapsulation efficiency (EE)

GEN-NLC or CH-GEN-NLC was separated from unentrapped drug using a Sephadex G-50 column (2.5 cm × 1.0 cm). Briefly, Sephadex G-50 soaked in 100 °C distilled water for a few hours was loaded into a 2.5 ml syringe and then centrifugated at 2000 rpm for 2 min to obtain a dehydrated column. Subsequently, 0.2 ml of NLC suspensions was added onto the column and centrifugated (2000 rpm, 2 min). Afterwards, the column was washed four times with 0.2 ml of distilled water each time. After centrifugation, the eluent was collected and destroyed using a mixed solvent of dichloromethane and methanol (1:4, v/v). The amount of encapsulated GEN was determined by HPLC with conditions as follows: a Diamasil® C18 column (200 mm × 4.6 mm, 5 μm, Dikma, China) was used. The mobile phase was the mixture of methanol-0.05% phosphoric acid aqueous solution (60/40, v/v). The flow rate was 1.0 ml/min. The wavelength was set at 260 nm. Additional 0.2 ml of GEN-NLC or CH-GEN-NLC suspension was destroyed with methanol and dichloromethane before adding onto the column and assessed by HPLC in the same procedure. EE was calculated according to the following equations: