European Journal of Pharmaceutics and Biopharmaceutics 87 (2014) 197–207

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

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

Research paper

Transport mechanism of doxorubicin loaded chitosan based nanogels across intestinal epithelium

Chao Feng [a], Guohui Sun [a], Zhiguo Wang [b], Xiaojie Cheng [a], Hyunjin Park [c], Dongsu Cha [c], Ming Kong [a,*,1], Xiguang Chen [a,*,1]

[a] College of Marine Life Science, Ocean University of China, Qingdao, China
[b] Department of Plastic Surgery, The Affiliated Hospital of Medical College Qingdao University, Qingdao, China
[c] Graduate School Biotechnology, Korea University, Seoul, South Korea

ARTICLE INFO

Article history:
Received 4 October 2013
Accepted in revised form 28 November 2013
Available online 4 December 2013

Keywords:
Carboxymethyl chitosan
Nanogels
Transport pathway
Doxorubicin hydrochloride
Drug permeability
Oral delivery

ABSTRACT

Chitosan/carboxymethyl chitosan nanogels (CS/CMCS-NGs) could enhance the oral bioavailability of doxorubicin hydrochloride (DOX). To identify the mechanisms that support this recent observation, different transport pathways of CS/CMCS-NGs through the small intestine were studied in this work. Transcellular mechanisms were investigated in the presence of different inhibitors of protein-mediated endocytosis. A reduction of 52.32 ± 18% of drug transport was found when clathrin-mediated endocytosis was inhibited, which demonstrated that clathrin-mediated endocytosis played an important role in the transcellular transport of DOX:CS/CMCS-NGs. The paracellular transport results showed that CMCS in NGs could produce a transient and reversible enhancement of paracellular permeability by depriving Ca^{2+} from adherens junctions, whose efficacy as an absorption enhancer was about 1.7–3.3 folds higher than CS in NGs in GI tract. Finally, *in vivo* experiment showed that the transport capacity of DOX:CS/CMCS-NGs was significantly inhibited by extra added Ca^{2+}, which confirmed that the higher capacity to binding Ca^{2+} of CS/CMCS-NGs was beneficial for transport of DOX.

1. Introduction

In the current regimen of chemotherapy, the anticancer drugs are administered through i.v. injection or infusion. Although it is effective for cancer therapy, the side effect due to the direct delivery of high concentration anticancer-drug to bloodstream and the inconveniences during chemotherapy compromise its clinical treatment efficacy [1,2]. Oral administration of anticancer drugs is a viable alternative to intravenous administration, since it can maintain an optimum blood drug concentration and improves convenience and compliance of patients [3,4]. Nevertheless, most anticancer drugs especially those with excellent anticancer effects such as doxorubicin hydrochloride (DOX) and Taxanes (paclitaxel and docetaxel) are not orally bioavailable owing to inadequate transport throughout the intestinal epithelium [5].

To improve oral bioavailability of anticancer drugs, Chitosan based nanogels (CS/CMCS-NGs) consisting chitosan (CS) and carboxymethyl chitosan (CMCS) were successfully developed in our recent study [6]. The rational design of these nanocarriers was to retain the promising behavior of CS as an absorption enhancer [7,8], yet expand the range of absorption enhancement from limited duodenal segment to the entire small intestine. However, the behavior of CS/CMCS-NGs toward the intestinal barrier and different cellular and molecular interactions with intestine epithelium remain to be clarified.

Caco-2 cell model has been widely used to study the intestinal permeability of drugs. After differentiation, Caco-2 cells can form a monolayer of polarized cells which present tight junctions and active transporters (P-glycoprotein) [5]. Several studies using Caco-2 cells model or animal models have shown the uptake of CS-based NGs by Payer's patches [9] and the epithelium [10] as well. The endocytosis of CS-based NGs by Caco-2 cells was saturable, energy- and temperature-dependent, which indicated that this process might be an active transport [10]. However, the endocytosis approach of CS-based NGs especially for CS/CMCS-NGs is poorly characterized [22]. On the other hand, CS-based NGs have ability to open tight junctions (TJs) between intestinal epithelium and facilitate paracellular transport of drugs [11–14]. This capacity depends on the positive charge of CS under weak acidic condition, which is infeasible in intestinal segments other than duodenum [15,16]. But for CS/CMCS-NGs, the introduction of CMCS could maintain continues and efficient absorption enhancement of DOX throughout the

* Corresponding authors. College of Marine Life Science, Ocean University of China, Yushan Road, Qingdao 266003, Shandong Province, China. Tel./fax: +86 0532 82032586.
E-mail addresses: kongming@ouc.edu.cn (M. Kong), xgchen@ouc.edu.cn (X. Chen).
[1] These authors contributed equally to this manuscript as co-corresponding authors.

0939-6411/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.ejpb.2013.11.007

entire small intestine [6]. The mechanism of this phenomenon is still not clear yet.

This study was to investigate the mechanism regarding the transport of CS/CMCS-NGs throughout the intestinal barrier, particularly focused on the cellular and molecular mechanisms. Two possible routes have been studied by using well defined Caco-2 cell model. As for the transcellular pathway, different endocytosis mechanisms, including macropinocytosis, clathrin-, caveolae- and clathrin-independent mediated endocytosis have been studied in the presence of different inhibitors of protein-mediated endocytosis [17]. On the other hand, the transport of nanogels by paracellular pathways focused on the mechanism that CS/CMCS-NGs enhance intestinal absorption of anticancer drugs throughout the entire small intestine. Finally, the transport mechanism of this nanocarrier was evaluated *ex vivo* and *in vivo* using animal models and confirmed the findings of *in vitro* studies.

2. Materials and methods

2.1. Materials

CS (molecular weight, MW: 10 kDa, degree of deacetylation, DD: 89%) was obtained from Biotech Co. (Mokpo, Korea). CMCS (MW: 12 kDa, DD: 81%, Degree of substitution, DS: 92%) was synthesized and characterized by the method described by Chen [18]. Cy3-SE (Cy3-N-hydroxy-succinimide ester), fluorescein isothiocyanate (FITC), acetic acid and sodium triphosphate (TPP) were purchased from Sigma (St. Louis, USA). DOX was supplied by Zhejiang Hai zheng Co. Ltd. (China). All other reagents and solvents were of analytical grade.

2.2. Preparation of Cy3-CS and FITC-CMCS

Cy3-labeled chitosan (Cy3-CS) and FITC-labeled carboxymethyl chitosan (FITC-CMCS) were synthesized according to the methods described in the literature [19,20].

The synthesis of Cy3-CS was based on the reaction between the free amines on the chitosan and N-hydroxy-succinimide on Cy3–SE. A solution of Cy3–SE in DMSO (1 mg/mL) was prepared and added gradually to soluble chitosan (1 mg/mL, pH 6.6) with continuous stirring for 12 h in the dark.

The synthesis of FITC-CMCS was based on the reaction between the amine groups of O-CMCS and the isothiocyanate group of FITC. Briefly, CMCS (30 mg) was dissolved in water (15 mL) in a weakly acidic condition with 1 M HCl, and the pH was adjusted to 6.9 with 1 M NaOH. FITC (2.1 mg) was added, and the mixture was stirred at room temperature for 24 h.

After reaction, Cy3-CS and FITC-CMCS were dialyzed in tri-distilled water for 3 days to remove unreacted Cy3 or FITC. The products were precipitated with ethanol and then freeze-dried.

2.3. Preparation and characterization of DOX:CS/CMCS-NGs

DOX-loaded CS/CMCS-NGs (DOX:CS/CMCS-NGs) were prepared according to a modified process originally based on our previous work [6]. Briefly, DOX aqueous solution (1 mg/mL, 1 mL) was premixed with CMCS (1 mg/mL, 4 mL) under magnetic stirring for 30 min. Subsequently, CS solution (1 mg/mL, 3 mL, pH 6.6) and TPP (0.25 mg/mL, 2 mL) were blended with the mixture under constant stirring for 1 h, and nanogels formed.

The size distribution and zeta potential of the prepared DOX:CS/CMCS-NGs at pH 1.2, 2.5 (HCl), pH 6.6, 7.0, 7.4 (phosphate-buffered saline, PBS) were measured with a Zetasizer ZEN 3600 Nano Series apparatus (ZEN, UK).

The morphology of the prepared NGs was examined by scanning electron microscopy (SEM, JSM-6010LA, JEOL Ltd., Japan) and transmission electron microscope (TEM, JEM-1200EX, JEOL Ltd., Japan) at pH values 1.2, 6.6, 7.0 and 7.4, simulating environments of the gastric acid, duodenum, ileum or intercellular spaces of enterocytes, respectively.

The obtained DOX:CS/CMCS-NGs were washed with deionized water 3 times to remove the DOX onto the surface of the particles, collected via ultracentrifugation at 12,000 rpm for 30 min, and then freeze-dried for 48 h. To determine the loading efficiency (*LE*) and loading content (*LC*) of DOX in nanogels, the free DOX in supernatants was filtered through a membrane filter (0.45 μm), whose concentration was examined spectrophotometrically at 481 nm (UV-1100, Shimadzu Ltd., Japan). The *LE* and *LC* were calculated using Eqs. (1) and (2), respectively:

$$LE\ (\%) = \left(Does_{added} - Does_{free}\right)/Does_{added} * 100\ \% \quad (1)$$

$$LC\ (\%) = \left(Does_{added} - Does_{free}\right)/W_{nanogels} * 100\ \% \quad (2)$$

where $Does_{added}$ is total amount of DOX added, $Does_{free}$ is free DOX in solution, and $W_{nanogels}$ is weight of nanogels

2.4. Cytotoxicity studies

The human colon adenocarcinoma (Caco-2) cell line obtained by the American Type Culture Collection (Manassas, USA, between 25 and 35 passages) was utilized to evaluate the cytotoxicity of blank-NGs (CS/CMCS-NGs). The cells were cultured in DMEM (high glucose) and supplemented with 10% (v/v) fetal bovine serum and penicillin/streptomycin at 37 °C and maintained in a saturated humidity containing 5% CO_2. Briefly, cells (3×10^4 cells/mL) were seeded in 96-well culture plates. Various concentrations (0–1000 μg/mL) of CS/CMCS-NGs in cell culture medium were incubated for 24, 48, or 72 h followed by MTT assay. Meanwhile, the cytotoxicity of CS/CMCS-NGs (200 μg/mL) at different pH values (6.6, 7.0 and 7.4) was also tested after 24 h of incubation time with Caco-2 cells.

2.5. Transcellular transport

2.5.1. Passive or active transport

Biofluorescence NGs (Cy3-CS/FITC-CMCS-NGs) were prepared using Cy3-CS and FITC-CMCS as per the procedure described in Section 2.3 and used for fluorescence microscope or CLSM study. After 21 days of differentiation, the Caco-2 cells were incubated with Cy3-CS/FITC-CMCS-NGs (200 μg/mL, dissolved in Hank's Balanced Salt Solution, HBSS, pH 7.0) for 4 h in the dark at 4 °C or 37 °C. And then, the slides were washed in HBSS and nuclei were stained with 4′,6′-diamidino-2-phenylindolyl hydrochloride (DAPI, Sigma, 1 μg/mL) in HBSS [21]. Slides were then washed three times in HBSS and observed by fluorescence microscopy (Nikon Eclipse Ti-S, Nikon Ltd., Japan) and confocal laser scanning microscopy (CLSM, LSM510, Zeiss Ltd., Germany), respectively.

2.5.2. Characterization of DOX:CS/CMCS-NGs endocytosis

In order to identify the approach of transcellular transport used by DOX:CS/CMCS-NGs, transport experiments were performed in the presence of specific inhibitor for different types of endocytosis. Caco-2 cell were cultured on the tissue-culture-treated polycarbonate filters (diameter 24.5 mm, growth area 4.7 cm^2) in Costar Transwell 6 wells/plates (Corning Costar Corp., Corning, NY) 24–30 days after seeding (Transepithelial electrical resistance [TEER] values in the range of 600–800 $\Omega\,cm^2$) to form Caco-2 cell monolayer. Firstly, the cell monolayer was incubated with the medium containing the specific inhibitor for 1 h at 37 °C. Then, the transport experiment was performed in the presence of