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Surface charge effect on mucoadhesion of chitosan based nanogels for local anti-colorectal cancer drug delivery

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ABSTRACT

To develop more effective anticancer mucoadhesive drug delivery system for the treatment of colorectal cancer, chitosan based nanogels (NGs) were prepared by electrostatic interaction between chitosan (CS) and carboxymethyl-chitosan (CMCS). By respectively using tripolyphosphate (TPP) and $CaCl_2$ as ionic crosslinker, two well-characterized doxorubicin hydrochloride (DOX) loaded NGs with opposite zeta potential (DOX:CS/CMCS/TPP NGs, -32.6 ± 1.1 mV and DOX:CS/CMCS/Ca^{2+} NGs, $+31.8 \pm 0.9$ mV) were obtained. Compared with DOX:CS/CMCS/TPP NGs, DOX:CS/CMCS/Ca^{2+} NGs were taken up to a greater extent by colorectal cancer cells, resulting in greater reduction in percentage of cell viability. Owing to high binding capability to mucin and inhibited paracellular transport by colon, DOX:CS/CMCS/Ca^{2+} NGs exhibited improved mucoadhesion and limited permeability. This is beneficial to prolong the contact time of formulation onto intestinal mucosa and improved local drug concentration. The results provided evidence DOX:CS/CMCS/Ca^{2+} NGs to be exciting and promising for the treatment of colorectal cancer.

1. Introduction

Colorectal cancer is the fourth most common type cancer and the third leading cause of death in the world [1,2]. Chemotherapy remains to be the necessary approach for treatment of colorectal cancer [3,4]. However, the chemotherapeutic agents for colorectal cancer, such as 5-fluorouracil, oxaliplatin, mitomycin and doxorubicin, often cause severe side effects due to their non-specific toxicity to normal organs and poor curative effect at low dosage [5–7]. Targeted delivery of chemotherapeutic agents to colon and anticancer effect improvement are still challenges for treatment of colorectal cancer.

Mucoadhesive nanocarriers offer strategy by combining targeted drug delivery and enhanced anticancer ability in one entity [8–10]. The targeted therapy effect is achieved through preventing the drug release in stomach and triggering release at the target site [8,11]. The specific surface characteristics of nanocarriers can increase the contact between loaded drug and intestinal mucosa and enhance cellular uptake of formulation [3,12,13]. High local drug concentration is created in lesion, resulting in high mortality of cancer cell, which is beneficial to decrease the required dosage and frequency of administration, reduced toxicity and cost [14,15].

Compared with artificial polymer, nature polysaccharides are more suitable for mucoadhesive nanocarriers owing to its non-toxicity, biodegradability and biocompatibility [16,17]. Chitosan (CS), a nature polycation derived from chitin, has strong mucoadhesive properties and is able to promote cellular uptake into cancer cells and improve anticancer effects [18]. We previously reported the formulation and characterization of mucoadhesive doxorubicin hydrochloride (DOX) loaded nanogels (DOX:CS/CMCS NGs) composed of chitosan and carboxymethyl chitosan (CMCS) [19]. The drug release rate of DOX:CS/CMCS NGs was much lower in simulating gastric acid environment(<18%) than that in simulating intestinal environment, which was in favor of targeted delivering drug to colon [20]. For effective colorectal cancer therapy, targeted delivery of anticancer drug to colon is prerequisite but not the last. The internalization of anticancer drug by colon cancer cell and afterwards cell damage determine the final curative effect of chemotherapy. In this process, surface charge of nanocarriers can significantly affect the efficiency and pathway of cellular uptake by influencing the adhesion of the nanocarriers and their interaction with cells [21–24]. For example, polymeric nanoparticles with higher positive surface charge were phagocytized more efficiently

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by murine macrophage [25], while quantum dots nanoparticles with negative surface charge were easier to be uptaken by human epidermal keratinocytes [26]. It indicates that the efficiency of cellular uptake is not only depended on surface characteristic of nanocarriers but also determined by cell types. However, study about the effects of surface characteristics of CS based NGs in intestinal epithelial cell is lacking. This study focuses on the surface charge effect on the mucoadhesion and anticancer efficiency of DOX:CS/CMCS NGs. Two types of DOX:CS/CMCS NGs with similar composition, shape, size but opposite surface potential were prepared by ionic gelation using different ion crosslinker. Caco-2 cell line was used to assess endocytosis and anticancer activity of formulation. *In vitro* and *ex vivo* mucoadhesion of DOX:CS/CMCS NGs was studied qualitatively and quantitatively.

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 [27]. Fluorescein isothiocyanate (FITC), calcium chloride, acetic acid and sodium tripolyphosphate (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 and characterization of DOX loaded and DOX free nanogels

CS/CMCS/TPP NGs were prepared according to a modified process originally based on our previous work [19,20]. Briefly, 2 mL of TPP (0.25 mg/mL) was added into 3 mL of CS solution (1 mg/mL) under magnetic stirring for 30 min. Subsequently, 4 mL CMCS solution (1 mg/mL) was added into the mixture dropwise under constant stirring for 1 h until nanogels formation.

CS/CMCS/Ca^{2+} NGs were prepared using Ca^{2+} as ion crosslinker as follows. 2 mL of $CaCl_2$ (0.5 mg/mL) was added into 4 mL of CMCS solution (1 mg/mL) under magnetic stirring for 30 min. Then, 3 mL CS solution (1 mg/mL) was added into the mixture dropwise under constant stirring for 1 h until nanogels formation.

DOX loaded CS/CMCS/TPP NGs (DOX:CS/CMCS/TPP NGs) and DOX loaded CS/CMCS/Ca^{2+} NGs (DOX:CS/CMCS/Ca^{2+} NGs) was generated by premixing DOX (1 mg/mL, 1 mL) with TPP or $CaCl_2$ solution in preparation, respectively.

To determine the loading efficiency (*LE*) of DOX in NGs, the obtained DOX:CS/CMCS/TPP NGs and DOX:CS/CMCS/Ca^{2+} 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. 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, Kyoto, Japan). The *LE* were calculated using Eq. (1) [28], respectively:

$$LE\,(\%) = \frac{D_a - D_f}{D_a} * 100\% \quad (1)$$

where D_a and D_f was total dose of DOX added and free DOX in supernatants, respectively.

The size distribution, zeta potential and polydispersity index (PDI) of DOX free and DOX loaded NGs in H_2O, Dulbecco's Hanks Balanced Salt Solution (D-Hanks, pH 7.4) and Dulbecco's modification of Eagle's medium Dulbecco (DMEM, pH 7.4) were respectively measured by photon correlation spectroscopy using nano ZS90 Zetasizer (Malvern Instruments, UK) at a detector angle of 90°, 670 nm, and 25.2 °C. The DOX free and DOX loaded NGs suspensions were stained by phosphotungstic acid and placed onto copper grid and dried at room temperature, then observed by TEM (100 CX II, Japan).

2.3. In vitro cellular uptake assays

In this study, FITC was used as a fluorescent marker to qualitative study cellular uptake of NGs by human colon adenocarcinoma (Caco-2) cell line (purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China, between 25 and 35 passages). CMCS was covalently labeled by FITC through amide bond formation between primary amino group on CMCS and isothiocyanate group on FITC at pH 6.9, and then dialyzed in tri-distilled water for 3 days to remove unreacted FITC refer to previous report [19,20]. FITC labled DOX free and DOX loaded NGs were prepared as described in Section 2.2.

The qualitative study of cellular uptake of nanogels was investigated by the fluorescence microscopy according to reference [29]. Caco-2 cells were cultured in six-well culture plate containing 18-mm coverslips for 24 h to form a confluent monolayer. The culture media was then replaced with D-Hanks (pH 7.4) and preincubated at 37 °C for 30 min. After equilibration, culture media was replaced by D-Hanks (pH 7.4) containing equivalent doses of DOX, DOX:CS/FITC-CMCS/TPP NGs or DOX:CS/FITC-CMCS/Ca^{2+} NGs of 100 μg/mL. The cells treated with CS/FITC-CMCS/TPP NGs, CS/FITC-CMCS/Ca^{2+} NGs were used as negative controls. After 3 h of incubation, the cover slides were washed three times with D-Hanks (pH 7.4) and the nuclei were stained with 4′, 6′-diamidino-2-phenylindolyl hydrochloride (DAPI, Sigma, 1 μg/mL) in D-Hanks as previously described [30]. Cover slides were then washed three times with D-Hanks and observed by fluorescence microscopy (BX41, Olympus, Tokyo, Japan).

The uptake of loading DOX by Caco-2 cell was quantitatively investigated according to literature [31]. Caco-2 cells were cultured to 96-well culture plates and incubated for 24 h to form a confluent monolayer. Then the culture medium was replaced by D-Hanks (pH 7.4) and preincubated at 37 °C for 30 min. After equilibration, culture media was replaced by D-Hanks (0.2 mL pH 7.4) containing equivalent doses of DOX, DOX:CS/CMCS/TPP NGs and DOX:CS/CMCS/Ca^{2+} NGs of 6.25, 12.5, 25, 50 and 100 μg/mL. At time points of 1, 2 and 3 h, The experiment was terminated by washing the cell monolayer three times with ice-cold phosphate-buffered saline (pH 7.4), then 0.2 mL of 0.5% Triton X-100 in 0.2 M NaOH solution was added to lyse the cells. Then the DOX concentrations were determined by flourescence microplate reader at Ex/Em 537/584 nm. Cell uptake efficiency (*UE*, %) was calculated by Eq. (2):

$$UE\,(\%) = \frac{C_u}{C_i} * 100\% \quad (2)$$

where C_u and C_i were the uptake and initial concentrations of DOX, respectively.

2.4. MTT assay

The Caco-2 cells were cultured in DMEM (high glucose) and supplemented with 10% (v/v) fetal bovine serum and penicillin/streptomycine at 37 °C and maintained in a saturated humidity containing 5% CO_2. Briefly, cells were seeded in 96 well plates at 3×10^5 cells/well and allowed to attach for 24 h. The culture media was then replaced with media containing equivalent doses of DOX, DOX:CS/CMCS/TPP NGs and DOX:CS/CMCS/Ca^{2+} NGs of 6.25, 12.5, 25, 50 and 100 μg/mL. The cells treated with CS/CMCS/TPP NGs and CS/CMCS/Ca^{2+} NGs were used as negative controls. At time points of 24, 48 and 72 h, cell viability (%) compared to untreated cells was measured by MTT assay [32].

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