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Chitosan nanoparticles as a dual drug/siRNA delivery system for treatment of colorectal cancer

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ABSTRACT

Nanoparticles are widely used to deliver anticancer drugs and inhibit tumor growth without systemic toxicity. Recently, chitosan has received much attention as a functional biopolymer for encapsulation of small interfering RNA (siRNA). Because of cationic nature, chitosan efficiently encapsulate siRNA and forming nanoparticles. Moreover, biocompatible and biodegradable properties represent chitosan as potential candidate for in vivo siRNA delivery. In the present study we designed carboxymethyl dextran (CMD) chitosan nanoparticles (ChNPs) to encapsulate snail siRNA and anticancer drug doxorubicin (DOX). The effect of ChNPs-drug/siRNA on cell growth and Epithelial mesenchymal transition (EMT) gene expression of HCT-116 cell lines were investigated. Furthermore the efficacy of dual agent nanoparticle to induce apoptosis and inhibit migration of colorectal cancer cells were assessed using Annexin-V and wound healing assays, respectively. The results demonstrated that treatment with dual agent nanoparticles led to significant changes of EMT genes (i.e down regulation of MMP-9 and Vimentin and up regulation of E-cadherin), apoptosis cell death and migration inhibition in HCT-116 cells. In conclusion, ChNPs encapsulating DOX and snail siRNA can be considered as an effective anti-cancer drugs delivery system for the treatment of colorectal cancer.

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1. Introduction

Colorectal cancer (CRC) is one of the most common malignancy and fourth leading cause of cancer related death after lung, liver and stomach cancer [1]. Several approaches have been employed for the treatment of cancers including tumor removal, radiotherapy, and chemotherapy. Chemotherapy is the most effective treatment for tumors [2]. However, off-target delivery of cytotoxic drugs to tumor sites that lead to severe side effects is the major cause of failure of chemotherapy [3].

Nano vehicle delivery systems are being implemented to solve several limitations of conventional drug delivery systems such as nonspecific delivery, poor bio distribution water solubility, poor oral bioavailability, low therapeutic indices, high toxicity, and so on. Recently tumor biologist and chemists have focused on polymeric and biodegradable nanoparticles such as chitosan [4].

Chitosan, the linear and partially acetylated (1–4)-2-amino-2-deoxy-D-glucan, as the second most abundant natural polymer is obtained from chitin [5]. Chitosan nanoparticles are widely employed as drug carrier. Slow/controlled release of chitosan nanoparticles improves drug solubility and stability, enhanced efficacy, and reduced toxicity of nanoparticles [6]. Moreover, chitosan directly affects tumor cells and interfere with cell metabolism, inhibits cell growth, or induces cell apoptosis [7].

Carboxymethyl dextran (CMD), an anionic derivative of dextran, provides potential for chemical conjugations due to high density of

Abbreviations: siRNA, small interfering RNA; CMD, carboxymethyl dextran; DOX, doxorubicin; ChNPs, nanoparticles; EMT, Epithelial mesenchymal transition; CRC, colorectal cancer; MMPs, matrix metalloproteinases; FTIR, Fourier transform infrared spectroscopy; FBS, fetal bovine serum; PI, propidium iodide; ALL, acute lymphoblastic leukemia; AML, acute myeloblastic leukemia.

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carboxymethyl groups [8]. Structurally modified CMD had shown to be cleared more slowly from circulation than the unmodified form and to exhibit low-fouling activity. Furthermore, the carboxymethyl groups allow for covalent fixation of the polymer to the particles' surface which has a favorable impact on the nanoparticles' colloidal stability [9]. Coating nanoparticles with polymers allows for colloidal stabilization through steric repulsion, which is less susceptible to the effects of high ionic strength of the biological medium [10].

Discovery of RNA interference (RNAi)-based gene silencing has drawn much attention to cancer therapy due to its unique properties to silence a broad range of genetic targets. RNAi is a process, whereby the introducing of 21–23 bp small interfering RNA (siRNA) in cells results in the degradation of homologous mRNA and specific protein knock-down [11]. However, the delivery of siRNA into the body has raised several issues, including rapid degradation, low intracellular uptake, and limited stability in the blood stream. To overcome these limitations, it is of interest to identify a suitable delivery vehicle that increases the stability of siRNA in vivo and intracellular uptake [12].

Doxorubicin (DOX) is widely used in chemotherapy due to its effectiveness in wide range of cancers. The off-target effects often complicates cancer treatment by limiting dosage and diminishing the quality of patients' life during and after DOX treatment [13,14]. Therefore, targeted and controlled release of DOX using nanoparticles [15] has drawn attention [16].

Epithelial mesenchymal transition (EMT) genes are a functional family of genes involve in tumor progression and migration. For instance, matrix metalloproteinases (MMPs), degrade extracellular matrix components and play a critical role in tissue remodeling during development in pathological process, including inflammation, tissue repair, tumor invasion, and metastasis [17]. Over expression of MMP-1, -2, -3, -7, -9, -13, and MT1-MMP have been demonstrated in human colorectal cancers [18]. Recently, vimentin, a major constituent of the intermediate filament family of proteins, has been recognized as a marker for EMT. Vimentin is ubiquitously expressed in normal mesenchymal cells and is known to maintain cellular integrity and provide resistance against stress. Vimentin's overexpression in cancers correlates with accelerated tumor growth, invasion and poor prognosis [19,20]. Adhesion protein E-cadherin plays a central role in the process of epithelial morphogenesis. Expression of this protein is down regulated during the acquisition of metastatic potential at late stages of epithelial tumor progression [21]. Moreover inhibition of snail in epithelial cancer cell lines, not expressing E-cadherin protein, restores the expression of the E-cadherin gene.

The aim of the present study was to design chitosan/CMD nanoparticles to effectively encapsulate anti-cancer drugs, DOX and snail siRNA. We furthur investigated physicochemical, growth inhibitory, pro-apoptotic and anti-migratory properties of dual delivery of DOX-snail siRNA CMD-Chitosan nanoparticles in colorectal cancer cells.

2. Methods and materials

2.1. Preparation of nanoparticles

Chitosan nanoparticles were prepared as previously described [22]. Briefly, 2 g of chitosan with average molecular weight of 400 kDa along with 12.7% acetylation degree (primex, Karmoy, Norway), was dissolved in 10 ml of CH_3COOH (acetic acid) 6% V/V. Subsequently, 10 ml of $NaNO_3$ (sodium nitrate, 1 mg/ml) was added to chitosan solution and kept at room temperature for 2 h with slow rotational motion. pH was adjusted to 9 by NaOH (sodium hydroxide) 4 M. Precipitated chitosan was filtered and washed three times with acetone and dissolved in 40 ml of CH_3COOH (0.1 N). Prepared stock was dialyzed using dialysis bag (cut-off 12 kDa), dried in freeze dryer (Alpha 2–4 LD plus; Christ, Osterode am Harz, Germany) and kept at 4 °C for further use [23].

2.2. Preparations of pharmaceutical groups

Briefly, 10 mg of ChNP was dissolved in 1 ml of sterile distilled water, with pH 5.5 (adjusted by acetic acid 1 N) and stirred for 3 h at the room temperature. Subsequently, 100 μl of CMD (1 mg/ml) and 5 μl (100 nM) of snail siRNA (Santa Cruz Biotech, USA) was poured on the 10 μl, 5 μl, 2.5 μl and 1.25 μl of doxorubicin (50 mg/25 ml, Pfizer, USA) and vortexed for 15 s (1500 rpm). In the next step, prepared stocks of CMD, DOX and siRNA were poured gently on the 1 ml of chitosan solution. Solution was mixed and incubated at room temperature for 30 s and 30 min respectively. SiRNA-CMD-ChNP and CMD-ChNP were prepared accordingly.

2.3. Scanning electron microscopy

The surface morphology of the freshly prepared chitosan siRNA-CMD-ChNPs was examined using a scanning electron microscope (SEM XL 30; Philips, Eindhoven, The Netherlands). SiRNA-CMD-ChNPs were dried on an aluminum disk at room temperature. The coated samples were examined using SEM.

2.4. Size and zeta potential measurement

Particle size and zeta potential of the siRNA-CMD-ChNPs complexes were determined by photon correlation spectroscopy and laser Doppler anemonetry, respectively (Nano-ZS; Malvern Instruments, Malvern, UK). The size measurement was performed with a wavelength 633 nm at 25 °C with an angle detection of 90 °C. Scattering angle was recorded for 180 s for each measurement. Each sample was measured at least 3 times.

2.5. Fourier transform infrared spectroscopy (FTIR)

Fourier transform infrared (FTIR) spectra were analyzed using a Nicolet FTIR spectrometer (Magna IR 550; Madison, WI) at 4 cm resolution. The sample was mixed and pressed on a plate to investigate the chemical reactions between the drug and siRNA-CMD-ChNPs.

2.6. In vitro release study of DOX and siRNA

4 ml of CMD-ChNPs was dispersed in a freshly prepared phosphate-buffered saline (PBS; pH = 7.4) as a release medium in a dialysis membrane sac (mw cut-off 12 kDa; Sigma Aldrich). The nanoparticles containing SAC dialysis in 50 ml PBS in the same condition as slowly released to the media. The beaker was placed in a shaking incubator at 37 °C under mild agitation (90–100 rpm). For each sample, 2 ml of the release medium was soaked at prearranged time intervals and substituted by the same medium under the same circumstance.

Utilizing UV–vis spectrometry, quantity of released DOX in the medium was assessed by UV absorbance values (λ = 480 nm) and compared to the primary total DOX which was resuspended in 50 ml of PBS. Medium which was collected from unloaded NPs was used as negative control. Also, the release of snail siRNA calculated at optical densities of 260 nm using the same protocol for DOX release calculation.

$$\text{Released snail siRNA}\,(\%):$$
$$[(\text{OD of snail siRNA in PBS}/\text{initial total content of snail siRNA})]$$

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