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CD44-specific nanoparticles for redox-triggered reactive oxygen species production and doxorubicin release

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ABSTRACT

CD44-specific and redox-responsive nanoparticles were prepared by coating a bioreducible chitosan-based nanoparticles with hyaluronic acid for intracellular glutathione-triggered reactive oxygen species (ROS) production and doxorubicin (DOX) release. Chitosan (CS) was conjugated with a copper chelator, D-penicillamine (D-pen), to obtain a CS-SS-D-pen conjugate through the formation of a disulfide bond. D-pen release from the conjugate was triggered by intracellular glutathione (GSH) via reducing biologically reversible disulfide bonds. Self-assembled CS-SS-D-pen nanoparticles were prepared through ionotropic gelation with tripolyphosphate and subsequently coated with hyaluronic acid (HA). The HA-coated CS-SS-D-pen NPs were reduced by GSH to release free D-pen and trigger ROS production via a series of reactions involving Cu(II)-catalyzed D-pen oxidation and H_2O_2 generation. DOX was loaded into the HA-coated CS-SS-D-pen NPs by a method involving the complexation of DOX with Cu(II) ions. The Cu(II)–DOX complex-loaded NPs exhibited redox-responsive release properties which accelerated DOX release at a higher glutathione level (10 mM). Confocal fluorescence microscopy demonstrated that the Cu(II)–DOX-loaded NPs effectively delivered DOX to human colon adenocarcinoma cells (HT-29) by active targeting via HA–CD44 interactions. Intracellular ROS generated from the HA-coated CS-SS-D-pen NPs sensitized cancer cells to DOX-induced cytotoxicity. *In vitro* cytotoxicity assays revealed that Cu(II)–DOX-loaded NPs sensitized cells to DOX-induced cytotoxicity in CD44-overexpressing HT-29 cells compared to CD44 low-expressing HCT-15 cells.

Statement of Significance

In this manuscript, we develop a CD44-targetable loaded with nanoparticles Cu(II)–DOX complex. The nanoparticles exhibited redox-responsive properties, which triggered reactive oxygen species (ROS) production and accelerated DOX release. The Cu(II)–DOX-loaded nanoparticle sensitized cells to DOX-induced cytotoxicity in CD44-overexpressing HT-29 cells.
To our knowledge, this is the first report showing the combination of CD44-targeting and redox-responsive property for triggering ROS production and subsequent drug release. We believe our findings would appeal to the readership of *Acta Biomaterialia* because the study bring new and interesting ideals in the development of specific and stimuli-responsive nanoparticles as drug carrier for cancer therapy.

1. Introduction

Several studies showed that reactive oxygen species (ROS) mediate both caspase-dependent and mitochondrial-dependent apoptosis of cancer cells [1]. Intracellular increases in ROS induce early cytosolic acidification and are critical for activation of cytosolic caspases. Several copper complexes were recently proposed to generate ROS and free radicals through a copper-mediated redox

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cycling in cells, which was linked to the sensitivity or resistance of cancer cells to some chemotherapeutic agents [2]. Doxorubicin (DOX) is an anthracycline chemotherapeutic medicine that is used to treat a broad range of cancers. Transition metals such as iron and copper were reported to enhance the DNA damage activity of anthracyclines upon metal complexation. Several studies suggested that redox-active copper complexes sensitize cancer cells to DOX-induced apoptosis through modulating biological oxidative reactions [3,4]. A combination chemotherapeutic strategy using drug carriers to co-deliver a copper and DOX may improve its therapeutic efficiencies.

Stimulus-responsive nanoparticles (NPs) can release drugs in response to changes in the external pH, temperature, ultrasound, and magnetic field to reduce multiple-drug resistance and enhance therapeutic efficiencies [5–9]. Chitosan (CS) has become one of the most attractive materials for use in developing NPs-based gene and drug delivery systems [10–12]. Hyaluronic acid (HA) is a negatively charged glycosaminoglycan composed of *N*-acetyl-D-glucosamine and glucuronic acid. It shows high-affinity binding to CD44, an adhesion/homing molecule involved in cancer cell invasion and metastasis [13]. CD44 is overexpressed in some types of colon cancers and is the major cell-surface receptor for HA. Moreover, HA is susceptible to hyaluronidases (HAdases) which are involved in malignant transformation and cancer progression by degrading this polymer into small molecules. The specific affinity to CD44 and its enzymatic degradability make HA a potential material for targeted cancer therapies [14–19].

CS/HA complex NPs have been used as an attractive nanocarrier to deliver genes and drugs [20,21]. In this study, we synthesized a bioreducible conjugate from CS and a copper chelator, D-penicillamine (D-pen), via the formation of biologically reversible disulfide bonds. The CS and D-pen conjugate (CS-SS-D-pen) can release D-pen through a glutathione (GSH)-mediated disulfide-thiol exchange reaction. HA-coated CS-SS-D-pen NPs (HA/CS-SS-D-pen NPs) were prepared for cancer cell-specific targeting and drug delivery because HA can target CD44 and is easily depolymerized by reactive oxygen species (ROS) [22]. DOX and Cu(II) ions were incorporated in the NPs in the form of a Cu(II)–DOX complex to increase drug loading and improve the stability of DOX-loaded NPs. Intracellular GSH reduced disulfide bonds of CS-SS-D-pen to release the copper chelator (D-pen). The released free D-pen initialized ROS production via a series of reactions that involve Cu(II)-catalyzed D-pen oxidation and subsequently removed Cu(II) ions from the Cu(II)–DOX complex to release DOX. ROS production due to the redox reaction and hyaluronidases overexpressed in colon cancer cells resulted in the depolymerization of HA, therefore further increased DOX release from the NPs. We also demonstrated NP-directed cellular localization of DOX to CD44-overexpressed human colon adenocarcinoma (HT-29) cells and sensitization of the cells to DOX-induced apoptosis due to induction of ROS production by Cu(II)–DOX loaded HA/CS-SS-D-pen NPs. Scheme 1 illustrates the GSH-reducible and CD44-targetable NPs for active loading of DOX (formation of the Cu(II)–DOX complex), and GSH-triggered intracellular ROS production and subsequent DOX release.

2. Materials and methods

2.1. Materials

Water soluble chitosan was purchased from Nicechem Co., Ltd (Shanghai, China). HA (Mw 15–30 kDa), DOX, D-pen, L-glutathione reduced (GSH), sodium tripolyphosphate (TPP), dithiothreitol (DTT), 3-(2-pyridyldithio)propionic acid *N*-hydroxysuccinimide ester (PDP-NHS), and copper(II) D-gluconate were obtained from Sigma-Aldrich (St. Louis, MO). 5,5-Dithiobis(2-nitrobenzoic acid) (DTNB) was purchased from Tokyo Chemical Industry (Tokyo, Japan). All other chemicals used were of reagent grade.

2.2. Synthesis of the CS-SS-D-pen conjugates

The CS-SS-D-pen conjugate was synthesized according to procedures described in the literature with some modifications [23]. Briefly, CS (1 mg/mL) was reacted with PDP-NHS for 2 h at 35 °C. Subsequently, excess PDP-NHS was separated from the CS using dialysis tubing with a MWCO of 8000–10,000. The CS-PDP conjugate was mixed with D-pen (1.50 mmol) in borate buffer (50 mM) by stirring. After 12 h of reaction at 35 °C, the CS-SS-D-pen conjugate was purified by the above-mentioned dialysis treatment. A schematic diagram of the synthesis of the CS-SS-D-pen conjugate is shown in Fig. 1A.

2.3. Characterization of CS-SS-D-pen conjugate

A Bruker Avance 500 MHz nuclear magnetic resonance (NMR) spectrometer was used to record the ^{1}H NMR spectra of CS-SS-D-pen conjugates. The degree of disulfides formed in the CS-SS-D-pen conjugates was determined by reducing the disulfide bonds with a strong reductant, DTT. After dialysis, free thiol groups formed due to the reduction of disulfide bonds were quantified by Ellman's reagent. Ellman's reagent was prepared by dissolving 4 mg of 5,5-dithiobis(2-nitrobenzoic acid) (TNBS, Sigma-Aldrich) in 0.1 M sodium phosphate buffer (10 mL, pH 8.0). An amount of 0.5 mg of the CS-SS-D-pen conjugate was added to a tube containing 250 mL of 0.5 M phosphate buffer (pH 8.0) and 5 mL of Ellman's reagent. After reacting for 3 h, the mixture was centrifuged, and the supernatant was spectrometrically analyzed at a wavelength of 412 nm with an enzyme-linked immunosorbent assay (ELISA) reader. Disulfide contents in the CS-SS-D-pen conjugates were determined by calculating the increased thiol contents from a cysteine standard curve.

2.4. Bioreduction of disulfide bonds

Disulfide bonds of CS-SS-D-pen conjugates were reduced in PBS buffer (pH 7.4) containing 10 mM GSH to simulate the bioreduction of disulfide bonds in the cytoplasm [38]. The reduction ratio was determined by measuring the increased thiol contents with Ellman's reagent according to the above-described procedure.

2.5. Preparation and characterization of HA/CS-SS-D-pen NPs

HA-coated NPs (HA/CS-SS-D-pen NPs) were prepared according to the polyelectrolyte complexation method of Almalik et al. [21]. Brifely, aqueous CS-SS-D-pen (1.5 mg/mL) was mixed with TPP (1.0 mg/mL) in a 1:9 TPP to CS-SS-D-pen mass ratio. After centrifugating the nanoparticles using glycerol as stabilizer, the nanoparticles were redispersed in 100 mM acetate buffer (pH 5.0). The colloidal dispersion was added to an equal volume of HA (1.5 mg/mL) dissolved in deionized water (pH 6.0) with magnetic stirring at 100–500 rpm for 10 min to obtain HA/CS-SS-D-pen NPs. A Malvern 3000HS Zetasizer was used to measure the particle size and zeta potential. The morphology of each colloidal suspension was characterized by transmission electron microscopy (TEM). A drop of the colloidal suspension was placed onto a carbon-coated copper grid. Two minutes after deposition, surface water on the grid was removed with filter paper. Samples were observed by a Hitachi H-600 TEM (Nissei Sangyo, Japan).