Journal of Controlled Release 222 (2016) 78–85

Contents lists available at ScienceDirect

Journal of Controlled Release

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

Reactive oxygen species responsive drug releasing nanoparticle based on chondroitin sulfate–anthocyanin nanocomplex for efficient tumor therapy

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ARTICLE INFO

Article history:
Received 6 September 2015
Accepted 8 December 2015
Available online 11 December 2015

Keywords:
Reactive oxygen species
Chondroitin sulfate
Anthocyanin
Doxorubicin
Tumor therapy

ABSTRACT

To develop a reactive oxygen species (ROS) sensitive drug carrier, a chondroitin sulfate (CS)–anthocyanin (ATC) based nanocomplex was developed. Doxorubicin hydrochloride (DOX) was loaded in the CS–ATC nanocomplex (CS–ATC–DOX) *via* intermolecular stacking interaction. The nanocomplex was fabricated by a simple mixing method in the aqueous phase. The morphology and size of CS–ATC–DOX were determined by ATC content. In the group with 1.5 mg/ml of ATC loaded CS–ATC–DOX (CS–ATC2–DOX), the drug content and loading efficiency were 8.5% and 99.1%, respectively. The ROS sensitive drug release of CS–ATC2–DOX was confirmed under *in vitro* physiological conditions. The results demonstrated that 1.67 times higher DOX release occurred in CS–ATC2–DOX for 48 h compared to CS–DOX (ATC absent sample). Drug release and nanocomplex destruction were induced by ROS mediated ATC degradation. We determined that 66.7% of ROS was scavenged by CS–ATC2–DOX. Additionally, an HCT-116 tumor bearing animal model was used to confirm ROS sensitive therapeutic effects of CS–ATC2–DOX. The results indicate that DOX was released from the intravenously injected CS–ATC2–DOX in the tumor tissue. Thus, nuclei shrinkage and dead cells were observed in H&E staining and TUNEL assay, respectively. These data suggest that the tumor growth was effectively inhibited. This study means that CS–ATC2–DOX has potential in improving tumor therapy.

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1. Introduction

During the past several years, disease sensitive nanomedicine has been highlighted because of its unique drug releasing behaviors at targeted disease sites. Many disease sensitive drug delivery mechanisms have been reported in the field, which include pH [1], temperature [2], magnetic field [3] and reactive oxygen species (ROS) responsive systems [4]. The results from these studies suggest that improved therapeutic effects against various diseases were achieved by these advanced nanomedicine technologies.

Tumor regions have unique environmental characteristics that include enrichment of reactive oxygen species (ROS) [5] and low pH [6]. Both pH sensitive polymeric drug carriers and ROS sensitive drug carriers have been designated as promising candidates for efficient tumor therapy [7]. However, safety concerns and low sensitivity of the previously developed pH and ROS sensitive drug carriers still remain as limitations such as low ROS sensitivity, low drug release and toxicity of molecule.

Previously, we developed a chondroitin sulfate (CS)–anthocyanin (ATC) based nanocomplex as an efficient ROS scavenging material [8]. Each CS and ATC nanocomplex was approved by FDA for clinical use. The CS is naturally occurring glycosaminoglycans (GAGs) present in the extracellular matrix (ECM) of cartilage and an anionic polyelectrolyte consisting of repeated disaccharide units of β-1,4-linked D-glucuronic acid and β-1,3-linked *N*-acetyl galactosamine (GalNAc). The ATC is a low molecular weight natural compound and has different pharmacological properties, such as anti-aging [9], anti-inflammatory [10], antibacterial [11], anti-tumorigenic [12], and anti-carcinogenic [9] effects. The CS–ATC nanocomplex reacted with ROS and then was decomposed into water soluble compounds. In addition, the CS–ATC had high ROS scavenging capacity (redox potential of ATC; 0.23–0.75) so that ultra-sensitive nanocomplex destruction was shown. Additionally, during the preparation process, π–π stacking interactions were formed in the core of the CS–ATC nanocomplex.

Based on this, we developed a doxorubicin hydrochloride (DOX) loaded CS–ATC nanocomplex for efficient tumor therapy (Scheme 1). The DOX was loaded in the core of the CS–ATC nanocomplex *via* π–π stacking interaction. We assumed that the loaded DOX could be released *via* CS–ATC nanocomplex destruction at the ROS enriched tumor region.

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http://dx.doi.org/10.1016/j.jconrel.2015.12.009
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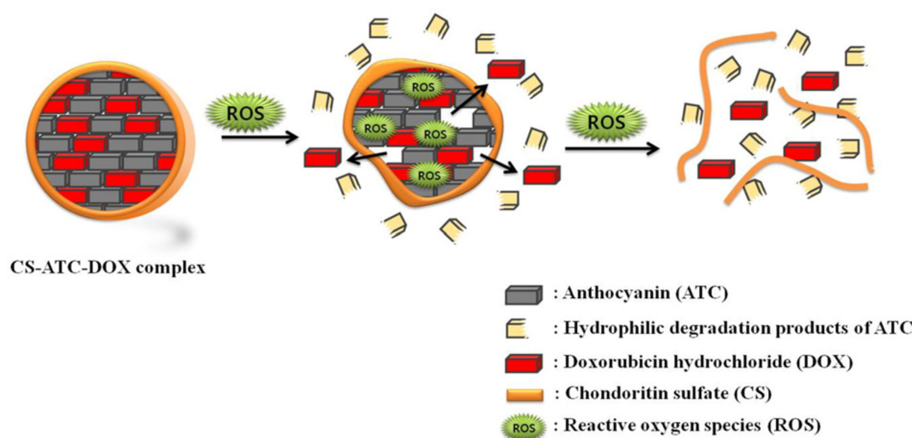

Scheme 1. Schematic illustration of ROS sensitive CS–ATC–DOX nanocomplexes. ROS acts as a triggering molecule for drug release of the CS–ATC–DOX nanocomplex. The ATC scavenged ROS is immediately degraded to water soluble substances. DOX release occurs by nanocomplex destruction.

In this study, the ROS sensitive drug releasing behavior of CS–ATC nanocomplex was confirmed under various conditions. Additionally, tumor inhibition effects of the DOX loaded CS–ATC nanocomplex were confirmed by *in vitro* and *in vivo* experiments.

2. Materials and methods

2.1. Materials

CS was purchased from Carl Roth (Schoemperlenstra, Karlsruhe, Germany). ATC (extracted from black soy bean) was provided by the rural development administration of Korea. DOX was purchased from ILDONG Pharm. Co., Ltd. (Ansung-si, Gyeonggi-do, South Korea). 30% hydrogen peroxide solution was purchased from Junsei Chemical (Tokyo, Japan). 2-diphenyl-1-picrylhydrazyl (DPPH) and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Preparation of DOX loaded CS–ATC nanocomplex

Preferentially ATC was dissolved in a water/ethanol solution (1:1, *v*/*v*), and the ethanol was evaporated at 40 °C. To remove the remaining insoluble impurities in the solution, ATC in the water was filtered using a syringe filter. And then 1 mg of DOX and predetermined amounts of ATC (0.67 and 1.5 mg of ATC for CS–ATC1–DOX and CS–ATC2–DOX, respectively) were dissolved in 1 ml of distilled water (Table 1). 10 mg of CS was added into the DOX–ATC solution. The mixed solution was stirred for 5 min. To prepare the homogeneous DOX loaded CS–ATC nanocomplex, the solution was stored at 4 °C for 12 h. The color of the solution then changed to dark-purple. For the control experiments, CS–DOX was prepared by the same method without ATC. All of these samples were used within 24 h of sample preparation for experiments.

2.3. Characterization of DOX loaded CS–ATC nanocomplex

The ATC content of the CS–ATC nanocomplex was determined by the absorbance of unloaded ATC. The unloaded ATC was extracted by centrifugation in an amicon tube (MwCO: 3500, Millipore®) at 3000 rpm for 10 min. The absorbance of the unloaded ATC was detected at 523 nm. The ATC content was calculated by following equation:

$$\text{ATC content }(\%) = \frac{\text{weight of loaded ATC}}{\text{total weight of CS} - \text{ATC nanocomplex}} \times 100$$

Also, using of the same manner, the DOX content and the loading efficiency of the CS–DOX, CS–ATC1–DOX and CS–ATC2–DOX nanocomplexes were determined by the UV/visible spectrophotometer at 490 nm. Finally, the DOX content and DOX loading efficiency were calculated by following equations:

$$\text{DOX content }(\%) = \frac{A_1}{A_0} \times 100$$

$$\text{DOX loading efficiency }(\%) = \frac{B_0 - B_1}{B_0} \times 100$$

where A_0 is the total weight of CS–DOX, CS–ATC1–DOX or CS–ATC2–DOX nanocomplex, and A_1 is weight of loaded DOX. Also, B_0 is the feed DOX, and B_1 is the unloaded DOX. The DOX loaded CS–ATC nanocomplex, hydrodynamic volumes were measured by dynamic light scattering (DLS) system (Nanosizer, Malvern Instruments) at 25 °C. Additionally, the morphology of the nanocomplex was observed by field emission scanning electron microscopy (FE-SEM, S4800, Hitachi). To prepare the FE-SEM samples, 10 μl of nanocomplex solution was deposited on a cover glass and completely dried for 24 h at room temperature. All of the FE-SEM images were randomly obtained.

Table 1
Composition of the CS–ATC–DOX nanocomplexes.

Sample name	CS (mg)	ATC (mg)	DOX (mg)	CS contents (%)	ATC contents (%)	DOX contents (%)	Loading efficiency (%)
CS–DOX	10	–	1	91.1	–	8.9	97.3
CS–ATC1–DOX	10	0.67	1	85.8	5.7	8.5	99.1
CS–ATC2–DOX	10	1.5	1	80.0	12.0	8.0	99.5

2.4. In vitro ROS sensitive drug release test

To confirm the ROS sensitive drug release behaviors of the CS–ATC nanocomplex, 1 ml of the nanocomplex solution was dialyzed against 10 ml of H_2O_2 solutions (0, 10, 50 and 100 mM of H_2O_2 containing 0.1 M phosphate buffered saline solution, pH 7.4) using a cellulose acetate membrane (Spectra, MWCO: 3500) in a shaking water bath (50 rpm, 37 °C). At the designated time intervals, the solution was exchanged for fresh H_2O_2 solution. The released DOX was quantified using UV/visible spectrophotometer at 490 nm.

2.5. Drug release and complex destruction

A relationship between drug release and ATC degradation was investigated for confirmation of the drug releasing mechanism. DOX fluorescence images and its intensity with various concentrated H_2O_2 solutions

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