Reduction-Sensitive Hyaluronic Acid Nanoparticles for Targeted Intracellular Delivery of Doxorubicin

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ABSTRACT

Reduction-sensitive, amphiphilic HA conjugates (HA-SS-DDTs) were synthesized using the facile thiol-exchange reaction, and evaluated their potential as the carrier for intracellular delivery of the hydrophobic anticancer drug, doxorubicin (DOX). The HA-SS-DDT conjugates formed self-assembled nanoparticles in aqueous conditions, and DOX was successfully encapsulated into the nanoparticles by an oil-in-water emulsion method. The release rate of DOX from the DOX-loaded HA-SS-DDT (HA-SS-DDT-DOX) nanoparticles was accelerated in the presence of 10 mM glutathione, mimicking the intracellular condition. The HA-SS-DDT-DOX nanoparticles were efficiently taken up by SCC7 cancer cells that over-express the HA receptor (CD44). These results suggest that the HA-SS-DDT nanoparticles have the promising potential as the carrier of DOX for cancer therapy.

Keywords: hyaluronic acid nanoparticle, reduction-sensitive, drug delivery, doxorubicin

1 INTRODUCTION

Self-assembled nanocarriers, made from polymeric amphiphiles, have shown great potential for the tumor-targeted drug delivery owing to their ability to encapsulate a quantity of hydrophobic anticancer drugs, prolong *in vivo* circulation, and exhibit preferential accumulation at solid tumors by the enhanced permeation and retention (EPR) effect [1-2]. To facilitate the release of the encapsulated drugs upon reaching the tumor site, stimuli-sensitive linkages have been incorporated into the nanocarriers.

In recent years, polymeric nanocarriers with reducible disulfide bonds have been extensively studied due to the existence of glutathione (GSH) concentration gradient between subcellular compartments (1-10 mM) and extracellular milieu (2-20 μM) [3]. These drug-loaded nanocarriers with disulfide functionality are stable while circulation in the blood stream and disintegrate their structure through cleavage of disulfide bond after interaction with GSH, resulting in triggered release of the drugs within the intracellular environment. To date, most of the reduction-sensitive nanocarriers have been made using poly(ethylene glycol) as the hydrophilic constituents and synthetic polyesters or polypeptides as the hydrophobic constituents [4,5]. However, to maximize the therapeutic efficacy, it is highly essential to develop nanocarriers that should selectively internalize through the specific mechanism and precisely release the drugs inside of the tumor cells.

Recently, hyaluronic acid (HA) has been widely investigated as the targeting constituents of drug carriers for cancer therapy owing to the over-expression of HA-binding receptors by various tumor cells [7-10]. We also have developed several HA-based nanocarriers and demonstrated that they could selectively accumulate in the tumor site through EPR effect and effectively taken up by the cancer cells through receptor-mediated endocytosis [7-10]. In this regard, preparation of reduction-sensitive HA-based nanocarriers has enormous potential for tumor-targeted drug delivery because the effective targeting and selective release of drugs within the intracellular compartments will improve the therapeutic effects.

Herein, reduction-sensitive HA-SS-DDT conjugates were synthesized from pyridyldisulfide-bearing HA (HA-PDA) conjugates using the facile thiol-exchange reaction. The amphiphilic HA-SS-DDT conjugates that could form self-assembled nanoparticles (Figure 1) were evaluated as the carrier for intracellular delivery of doxorubicin (DOX).

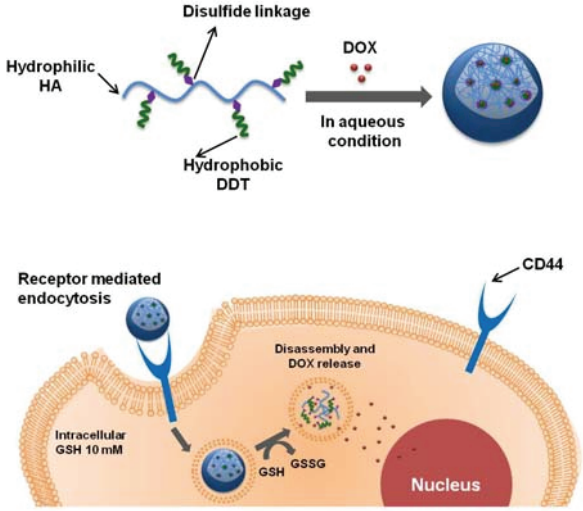

Figure 1. Schematic illustration for the preparation of HA-SS-DDT-DOX nanoparticles, cellular uptake, and GSH-mediated intracellular release of DOX from the nanoparticles.

2 MATERIALS AND METHODS

2.1 Materials

Sodium hyaluronate (Mw = 6.7 × 10^4 Da) was purchased from Lifecore Biomedical (MN, USA). All other chemicals were obtained from commercial sources and used as received. 2-(pyridyldithio)-ethylamine hydrochloride (PDA.HCl) was synthesized using simple thiol-exchange reaction.

2.2 Synthesis of HA-SS-DDT Conjugate

First, HA-PDA conjugate was synthesized through carbodiimide-mediated coupling reaction. In brief, HA (100 mg) and PDA.HCl (11.72 mg) were dissolved in distilled water/methanol (1v/1v). After the addition of 1-ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride (40.48 mg) and 1-hydroxybenzotriazole (28.53 mg), the mixture was stirred for 24 h at room temperature. The solution was dialyzed against water/methanol (1v/1v) for 2 days, followed by lyophilization. The degree of substitution (DS) of PDA in the conjugate, defined as the number of pyridyldisulfide groups per 100 sugar residue, was determined by measuring the absorbance of pyridine-2-thione at 343 nm after addition of DTT using the UV-Visible spectrophotometer (Optizen 3320 UV, Korea). Second, HA-SS-DDT conjugates were synthesized using thiol-exchange reaction between HA-PDA conjugate and dodecanethiol (DDT). In biref, DDT (4.07 mg) dissolved in ethanol were added to a solution of HA-PDA_6 (100 mg) dissolved in distilled water containing 10 µl of glacial acetic acid. After completion of the reaction, the solution was dialyzed and lypholized to obtain HA-SS-DDT_6 conjugate.

2.3 Characterizations

The chemical structure of the conjugate was confirmed using ^{1}H-NMR, recorded on a JEOL JNM-AL300 (300 MHz, Tokyo, Japan) instrument. The size and distribution of the nanoparticles were measured by dynamic light scattering method using FPAR-1000 fiber optics particle analyzer (Photal Otsuka Electronics, Tokyo, Japan). The morphology of the nanoparticles was observed using TEM (JEOL JEM-2100F) operated at an accelerating voltage of 200 kV.

2.4 Drug loading and release

DOX loaded HA-SS-DDT (HA-SS-DDT-DOX) nanoparticles were prepared by water-in-oil emulsion method [10]. The loading efficiency (LE) and the loading content (LC) of DOX in the nanoparticles were determined using UV-Visible spectrophotometer by measuring the absorbance at 480 nm. The release profiles of DOX from the nanoparticles were investigated at two different conditions, in the presence or absence of GSH (10 mM).

2.5 *In vitro* cytotoxicty and cellular uptake

For cytotoxicity, SCC7 (squamous carcinoma) cells were seeded at a density of 1 × 10^4 cells/well in 96-well flat-bottomed plates and incubated with the samples for 24 h. The cells were then washed with PBS to remove any remaining drug and fresh culture medium was added. 20 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/ml in PBS) were added to each well. The cells were incubated for an additional 4 h at 37 °C. Finally, the medium was removed and the formazan crystals were dissolved in DMSO. The absorbance at 570 nm was measured using a microplate reader (BioTek, Seoul, Korea).

To determine cellular uptake and intracellular release of DOX from the nanoparticles, SCC7 cells were incubated with the nanoparticles for 1 or 3 h. For nuclear staining, the cells were incubated with 4,6-diamino-2-phenylindole (DAPI) for 10 min at room temperature, followed by washing with PBS (pH 7.4). The intracellular distribution of DOX from the nanoparticles was observed using an IX81-ZDC focus drift compensating microscope (Olympus, Tokyo, Japan).

3 RESULTS AND DISCUSSION

The synthetic strategy for the preparation of HA-SS-DDT conjugate is illustrated in Figure 2.

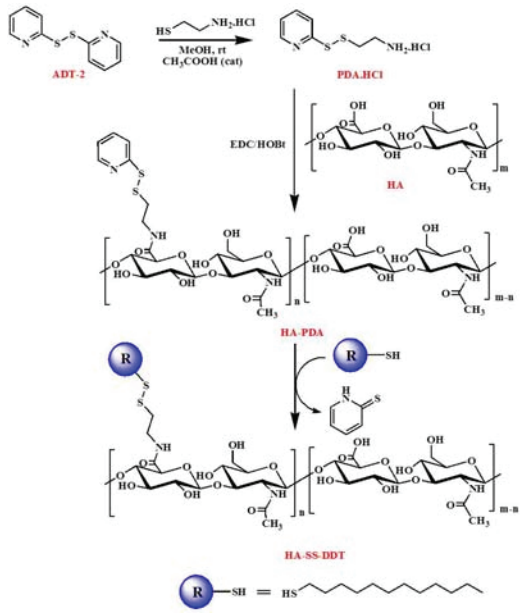

Figure 2. Reaction scheme for the sythesis of HA-SS-DDT conjugaes.

First, thiol-reactive HA-PDA conjugates were synthesized by chemically conjugation of PDA to the

carboxylic acid of HA backbone using carbodiimide chemistry. The successful formation of HA-PDA conjugate was confirmed using ^{1}H-NMR spectrum, which showed the characteristic aromatic peaks at 8.42, 7.87 and 7.28 ppm (corresponding to the pyridyl ring of PDA) and methyl peak at 2.01 ppm (from N-acetyl glucosamine) of HA. The DS of PDA moieties in the conjugates was quantitatively determined using absorption spectroscopy, which indicates increasing the molar feed ratio of PDA to HA increased the DS of the HA-PDA conjugate. It has been widely recognized that pyridyl disulfide group exhibits highly efficient, quantitative, facile and selective thiol-disulfide exchange reaction with sulfhydryl groups under very mild reaction conditions. By taking the advantage of thiol-disulfide exchange reaction, HA-SS-DDT conjugates were readily prepared by reacting HA-PDA and DTT under ambient conditions. The ^{1}H-NMR spectrum of HA-SS-DDT conjugate showed the absence of aromatic peaks and existence of new characteristics aliphatic peaks at 1.28 ppm (-CH_2-) and 0.89 ppm (-CH_3) of DDT, indicating formation of HA-SS-DDT conjugates.

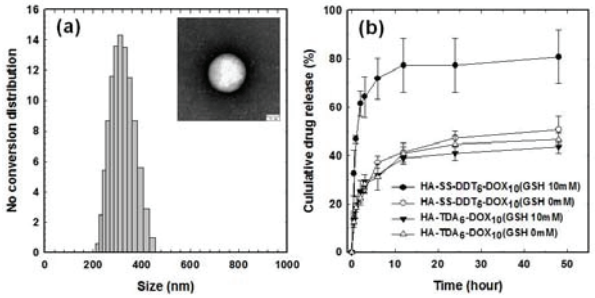

Figure 3. Size distributions of (a) HA-SS-DDT_6, where the inset is representative TEM image, and the scale bars indicates 70 nm; and (b) *In vitro* release pattern of DOX from HA-SS-DDT_6 and HA-TDA_6 in the presence and absence of GSH (10 mM). The error bars in the graph represent standard deviation (n = 3).

As the hydrophilic HA is grafted with liphophilic lengthy alkyl chain molecule through disulfide bond, the amphiphilic HA-SS-DDT conjugate can form self-assembled nanoparticles in aqueous conditions. The particle sizes of HA-SS-DDT were in the range of 291-333 nm, depending on the amount of hydrophobic constituent. This indicates that increasing the density of liphophilic moieties in the conjugates allowed the formation of compact inner cores. The conjugate with DS of 6 was selected for the further experiment. As shown in Figure 3a, the HA-SS-DDT_6 nanoparticles showed unimodal size distribution with spherical morphology. Further, the nanoparticles exhibited negative surface charge (ζ = -23.8 mV) due the presence of polyanionic HA shell. The stability of the nanoparticles in PBS (pH = 7.4) was investigated using DLS method. The mean hydrodynamic particle size of the nanoparticles did not show any significant change for at least week. This indicates that the nanoparticles could preserve fairly good stability under aqueous conditions.

DOX, a cytotoxic anthracyline antibiotic, has been widely used for the treatment of several solid malignant tumors. The mechanism of action of DOX is known to occur through binding to DNA and inhibition of nucleic acid synthesis. Therefore, it is highly indispensible to deliver DOX inside the cell to exert its therapeutic effect. However, the lack of selectivity of conventional DOX formulations induce severe side effects such as hypersensitivity and cardiotoxicity. There has been considerable research efforts focused on the development of safe and more effective carrier system for DOX.

In order to investigate the potential of HA-SS-DDT nanoparticles as drug carrier for intracellular delivery, DOX was loaded into the nanoparticles by oil-in-water emulsion method. The DOX encapsulation ability of HA-SS-DDT_6 conjugates was evaluated by varying the feed ratio 10 and 20 wt.%. The characteristics of DOX-loaded nanoparticles are summarized in Table 1. The hydrodynamic size of DOX-loaded nanoparticles decreased when the DOX loading content increased. Moreover, the size of all the DOX-loaded nanoparticles were smaller than the bare nanoparticles without drugs, indicating that more compact nanoparticles were formed by the hydrophobic interactions between DOX and the hydrophobic segments.

Sample	Feed amount of DOX	LC	LE	Size
HA-SS-DDT_6-DOX_{10}	10	7.5	75.77	290±5
HA-SS-DDT_6-DOX_{20}	20	14.6	73.49	264±3
HA-TDA_6-DOX_{10}	10	7.9	78.95	233±3

Table 1: Physicochemical characteristics of DOX-loaded nanoparticles.

To mimic the physiological and intracellular GSH condition, the *in vitro* drug release experiments were carried out in the absence and presence of PBS solution containing 10 mM GSH, respectively. HA-SS-DDT_6-DOX_{10} and DOX-loaded reduction-insensitive tetradecylamine conjugated HA (HA-TDA_6-DOX_{10}) were selected as representative samples for the drug release experiments. The DOX release profiles from the nanoparticles are depicted in Figure 3b. In the absence of GSH, only 41% of DOX were released from the nanoparticles for 12 h, indicating sustained release. On the other hand, at 10 mM GSH (mimicking intracellular environment), the release of DOX from the nanoparticles was much faster, 77% of DOX were released after 12 h. This accelerated release of DOX might be due to the disassociation of nanoparticles resulting from the cleavage of disulfide bonds by GSH. The HA-TDA_6-DOX_{10} nanoparticles without any disulfide bonds showed about 38-40% of DOX release both in the presence and absence of 10 mM GSH, after 12 h. Taken together, these results clearly

suggest that the cleavage of the disulfide by GSH is responsible for the accelerated drug release of DOX from HA-SS-DDT_6-DOX_{10} nanoparticles.

The cytotoxic effects of bare and DOX-loaded nanoparticles against SCC7 cancer cells were evaluated using MTT colorimetic assay. Owing to their excellent biocompatibility of HA, bare HA-SS-DDT_6 and HA-TDA_6 nanoparticles did not show any toxicity to the SCC7 cells at the concentrations range tested (Figure 4a). On the other hand, both HA-SS-DDT_6-DOX_{10} and HA-TDA_6-DOX_{10} nanoparticles showed dose-dependent cytotoxicity (Figure 4b), indicating that the nanoparticles were efficiently taken up by the cells. In particular, HA-SS-DDT_6-DOX_{10} exhibited slightly higher toxicity compared to the HA-TDA_6-DOX_{10} nanoparticles. As both nanoparticles possess the hydrophilic HA surface, the increase in cytotoxicity of HA-SS-DDT_6-DOX_{10} could be attributed to the rapid release of DOX from the nanoparticles by the cleavage of the disulfide bonds at the intracellular environment.

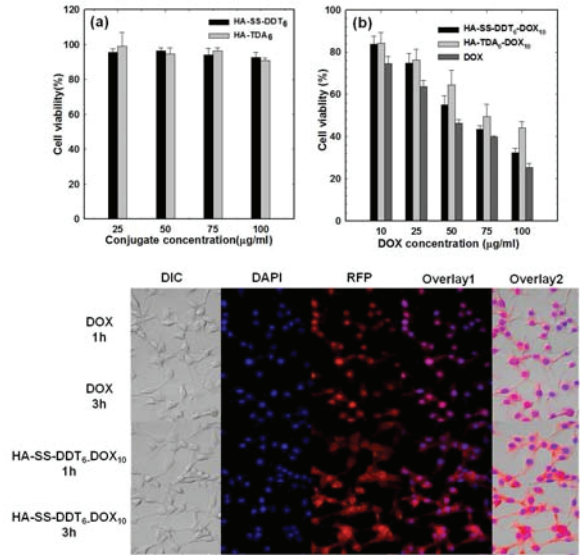

Figure 4. *In vitro* cytotoxicity of (a) bare and (b) DOX-loaded nanoparticles. (c) Fluorescence microscopic images of SCC7 cells treated with free DOX and HA-SS-DDT_6-DOX_{10} after 1 h and 3h incubation.

The cellular uptake and intracellular release of DOX from the HA-SS-DDT_6-DOX_{10} nanoparticles were investigated by incubating the nanoparticles with SCC7 cancer cells, and monitoring the uptake using the fluorescence microscope at different time intervals. As shown in Figure 4c, HA-SS-DDT_6-DOX_{10} nanoparticles exhibited considerable DOX fluorescence signals at the cytoplasm of the cells just 1h after incubation, indicating fast internalization of nanoparticles through receptor-mediated endocytosis and rapid release of DOX inside the cells. This result is consistent with previous studies, which also demonstrated efficient and fast uptake of HA-based nanoparticles by CD44-overexpressing cancer cells [8]. On the other hand, after 1 h incubation, free DOX showed nuclear accumulation, which could be attributed to simple diffusion. Interestingly, the fluorescence signals at the cytosol for the cells treated with HA-SS-DDT_6-DOX_{10} nanoparticles increased after 3 h of incubation, and some of the DOX transported into the cell nucleus. These results suggest that HA-SS-DDT_6-DOX_{10} nanoparticles could internalize the cells through CD44-mediated endocytosis, and efficiently release the DOX at the intracellular environment to exert their therapeutic effect.

4 CONCLUSION

In summary, we have synthesized reduction-sensitive HA-SS-DDT conjugates using HA-PDA conjugates, via facile thiol-exchange reaction. The conjugates formed self-assembled nanoparticles in aqueous conditions.. The HA-SS-DDT_6-DOX_{10} nanoparticles showed accelerated release of DOX in the presence of GSH, due the disintegration of the nanoparticular structure resulting from the cleavage of disulfide bonds. While bare nanoparticles exhibited negligible cytotoxicity, HA-SS-DDT_6-DOX_{10} nanoparticles showed dose-dependent cytotoxicity to SCCC7 cancer cells, due to enhanced cellular uptake and effective DOX release in the intracellular environment. Overall, these results suggest that HA-SS-DDT nanoparticles might have promising potential as the carrier for targeted intracellular delivery of DOX.

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