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Amphiphilic hyaluronic acid derivative with the bioreducible bond: Synthesis and its implication for intracellular drug delivery

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ABSTRACT

Since most of the anticancer drugs exert their action in the intracellular environment, it is imperative to deliver them selectively inside the cancer cells for effective therapy. In an attempt to develop the potential carrier for intracellular drug delivery, we prepared the amphiphilic hyaluronic acid conjugate (HA-SS-DDT) bearing the disulfide bond which could be selectively cleaved at the intracellular environment. Owing to its amphiphilicity, the HA-SS-DDT conjugate formed self-assembled nanoparticles in an aqueous condition. The nanoparticles were stable under the physiological condition (pH 7.4, 37 °C) for at least five days. Doxorubicin (DOX), chosen as the model anticancer drug, was effectively encapsulated into HA-SS-DDT nanoparticles with high efficiency (>70%) by the oil-in-water emulsion method. In the physiological solution (PBS, pH 7.4), DOX was released from HA-SS-DDT nanoparticles in a sustained manner. Interestingly, in the presence of 10 mM glutathione, a peptide abundant in the cytoplasm of the cancer cell, the release rate of DOX remarkably increased, which was due to disintegration of nanoparticles by the cleavage of the disulfide bond. DOX-loaded HA-SS-DDT (HA-SS-DDT-DOX) nanoparticles were efficiently taken up by squamous cell carcinoma (SCC7) cells via receptor-mediated endocytosis, followed by rapid release of DOX in the cytoplasm of the cells. Overall, these results indicate that HA-SS-DDT nanoparticles have promising potential as the carrier for intracellular DOX delivery.

1. Introduction

Self-assembled nanocarriers, composed of polymeric amphiphiles, have shown great potential for the tumor-targeted drug delivery because of their ability to encapsulate a quantity of hydrophobic anticancer drugs and to be preferentially accumulated in solid tumors by the enhanced permeation and retention (EPR) effect [1–3]. Although such nanocarriers can effectively deliver the drugs into the tumor tissue *in vivo*, they do not always guarantee the translocation of encapsulated drugs into the subcellular site of action. To maximize the therapeutic efficacy, it is highly essential to develop nanocarriers that can be selectively internalized by and rapidly release encapsulated drugs in the tumor cells.

Hyaluronic acid (HA) and its derivatives have been widely investigated as the constituents of drug carriers for cancer therapy, since they are selectively taken up by tumor cells over-expressing HA receptors such as CD44 and RHAMM [4–6]. In addition, owing to its excellent biocompatibility and biodegradability, HA has been often used for biomedical applications, including tissue engineering and molecular imaging [7]. Our research group has also developed several HA-based nanocarriers for cancer therapy and imaging [8–12]. The *in vivo* studies using tumor-bearing mice demonstrated that the HA-based nanoparticles could be selectively accumulated in the tumor site by the EPR effect and effectively taken up by the tumor cells through receptor-mediated endocytosis [10].

Since the therapeutic efficacy of the anticancer drug can be enhanced by its selective delivery into the intracellular compartments of tumor cells, the nanocarrier has been prepared by incorporating the cleavable linkage which is sensitive to a specific stimulus. Once the linkage is cleaved in response to the stimulus, these nanocarriers are disintegrated to release the anticancer drug.

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For example, in order to target the acidic microenvironment in tumor tissue, many investigators have prepared nanocarriers with acid-labile linkages such as ester, acetal, and hydrazone groups [13–15]. The pH-sensitive nanoparticles, however, release a significant portion of the drug at the extracellular level (pH < 6.8) in tumor tissue before they reach the acidic intracellular compartments, including endosome and lysosome. In recent years, bioreducible nanocarriers with the disulfide bond have received increasing attention for cancer therapy because there is the concentration gradient of disulfide bond-degrading glutathione (GSH) between subcellular compartments (1–10 mM) and extracellular milieu (2–20 μM) [16–19]. In general, the disulfide bond has been introduced between the hydrophilic and the hydrophobic blocks [20–23]. The resulting block copolymers, forming micellar structure in biological fluids, are stable during circulation in the bloodstream, whereas their three-dimensional structure is disintegrated at the cytoplasm of the cell through the cleavage of the disulfide bond in the presence of GSH, leading to triggered release of drugs.

In this study, we attempted to develop the HA-based nanocarrier which can be specifically taken up by the tumor cells via the receptor-mediated endocytosis and release the anticancer drug at the intracellular compartments (Fig. 1). The amphiphilic HA derivative (HA-SS-DDT), capable of being self-assembled into nano-sized particles in an aqueous condition, was prepared by chemical modification of HA with dodecanethiol (DDT) via the disulfide linkage. Doxorubicin (DOX), chosen as the model anticancer drug, was encapsulated into the nanoparticles by the oil-in-water emulsion method. The *in vitro* drug release behavior and cellular uptake pattern of drug-loaded nanoparticles were also evaluated.

2. Experimental

2.1. Materials

HA (MW = 6.7 × 10^4 g/mol) was purchased from Lifecore Biomedical Inc. (Chaska, MN, USA). *N*-(3-dimethylaminopropyl)-*N*′-ethyl carbodiimide hydrochloride (EDC), 1-hydroxybenzotriazole (HOBt), 2-mercaptoethylamine hydrochloride (2-MEA), 2,2′-dipyridyldisulfide, dithiothreitol (DTT), tetradecylamine (TDA), DDT, GSH, and DOX were obtained from Sigma Aldrich (St. Louis, MO, USA). All other chemicals were of reagent grade and used as received. Water, used in all the experiments, was purified by AquaMax-Ultra system (Younglin Co., Anyang, Korea).

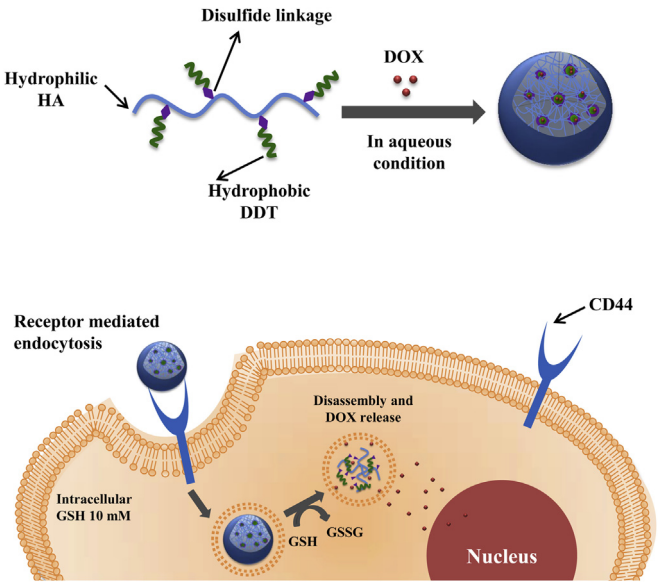

Fig. 1. Schematic illustration for the preparation of DOX-loaded HA-SS-DDT nanoparticles, cellular uptake, and GSH-mediated intracellular release of DOX from the nanoparticles.

2.2. Synthesis and characterization of HA-SS-DDT conjugate

2-(pyridyldithio)-ethylamine hydrochloride (PDA·HCl) was first synthesized via a simple thiol-disulfide exchange reaction [24]. Thereafter, the HA-PDA conjugate was prepared through a carbodiimide-mediated coupling reaction. In brief, HA (100 mg, 0.001 mmol) in 25 ml of distilled water was mixed with PDA·HCl (11.72 mg, 0.053 mmol) in 25 ml of methanol. After the addition of EDC (40.48 mg, 0.211 mmol) and HOBt (28.53 mg, 0.211 mmol) in 2 ml of distilled water/methanol (1v/1v), the mixture was stirred for 24 h at room temperature. The resulting solution was dialyzed against excess amount of distilled water/methanol (1v/1v) for 2 days and distilled water for an additional day using a membrane tube (MWCO = 12,000–14,000; Spectrum®, Rancho Dominquez, CA). Finally, the HA-PDA conjugate was obtained after lyophilization. The degree of substitution (DS) of PDA in the conjugate, defined as the number of PDA groups per 100 sugar residues of HA, was estimated by the integration ratio of the characteristic peaks from PDA and HA in ^{1}H NMR spectra.

The HA-SS-DDT conjugate was synthesized using the thiol-disulfide exchange reaction between the HA-PDA conjugate and DDT. In a typical reaction, HA-PDA (100 mg, 0.001 mmol) was dissolved in distilled water at a concentration of 5 mg/ml. Subsequently, DDT (4.07 mg, 0.020 mmol) in ethanol and 10 μl of glacial acetic acid were slowly added to the HA-PDA solution under stirring. The reaction was allowed to proceed at room temperature, and the progress of the reaction was monitored from the absorbance of released pyridine-2-thione at 343 nm using UV–Visible spectrophotometer (Optizen 3320, Mecasys Inc., Korea). The reaction was stopped when the peak intensity did not increase further. The solution was dialyzed against water/ethanol (1v/1v) for 1 day and distilled water for an additional day using a membrane tube (MWCO = 12,000–14,000), followed by lyophilization to obtain the HA-SS-DDT conjugate. The chemical structure of the conjugate was confirmed using ^{1}H NMR spectral data, recorded on a JEOL JNM-AL300 instrument (300 MHz, Tokyo, Japan). As a reduction-insensitive control, hydrophobic tetradecylamine (TDA) was covalently attached to HA in the presence of EDC and HOBt to obtain an amphiphilic HA-TDA conjugate. The DS of DDT (or TDA) in the conjugate, defined as the number of DDT (or TDA) groups per 100 sugar residues of HA, was also calculated from ^{1}H NMR spectra.

The size distribution of nanoparticles was determined using dynamic light scattering (DLS) (FPAR-1000 fiber-optics particle analyzer, Photal Otsuka Electronics, Kyoto, Japan). Nanoparticle solutions were filtered through a 0.45-μm syringe filter before measurements, and all the measurements were carried out at 25 °C with a scattering angle of 90°. The zeta-potential values of the nanoparticles were measured using the zetasizer (Malvern Instrument Ltd., UK) at 632 nm. For the stability test, the nanoparticles were dispersed in PBS (pH 7.4) at 37 °C and their particle size was measured as a function of time for 5 days.

2.3. Preparation of DOX-loaded HA-SS-DDT (HA-SS-DDT-DOX) nanoparticles

DOX-loaded nanoparticles were prepared by the water-in-oil emulsion method [12]. In brief, DOX·HCl and triethylamine (3 equivalent amount of DOX) in chloroform were slowly added

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