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Preparation of HIFU-triggered tumor-targeted hyaluronic acid micelles for controlled drug release and enhanced cellular uptake

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ABSTRACT

In this study, a novel type of high intensity focused ultrasound (HIFU)-triggered active tumor-targeting polymeric micelle was prepared and investigated for controlled drug release and enhanced cellular uptake. Amphiphilic hyaluronic acid (HA) conjugates were synthesized to form docetaxel loaded micelles in aqueous conditions with high encapsulation efficiencies of over 80%. The micelle sizes were limited to less than 150 nm, and they varied slightly according to the encapsulated drug amount. Modifying the micellar surface modification with polyethylene glycol diamine successfully inhibited premature drug leakage at a certain level, and it can be expected to prolong the circulation time of the particles in blood. In addition, high-intensity focused ultrasound was introduced to control the release of docetaxel from micelles, to which the release behavior of a drug can be tuned. The in-vitro cell cytotoxicity of docetaxel-loaded micelles was verified against CT-26 and MDA-MB-231 cells. The IC50 values of drug-loaded micelles to CT-26 and MDA-MB-231 cells were 1230.2 and 870.9 ng/mL, respectively. However, when exposed to HIFU, the values decreased significantly, to 181.9 and 114.3 ng/mL, suggesting that HIFU can enhance cell cytotoxicity by triggering the release of a drug from the micelles. Furthermore, cellular uptake tests were conducted via the quantitative analysis of intracellular drug concentration within CT-26 (CD44 negative), MDA-MB-231 (CD44 positive), and MDA-MB-231 (CD44 blocked), and then imaged with coumarin-6 loaded micelles. The results verified that intracellular drug delivery can be enhanced efficiently via the CD44 receptor-mediated endocytosis of HA micelles. Moreover, HIFU enhanced the cellular uptake behavior by altering the permeability of the cell membrane. It was also able to aid with the extravasation of micelles into the interior of tumors, which will be explained in further research. Therefore, the present study demonstrates that the micelles prepared in this study can emerge as promising nanocarriers of chemotherapeutic agents for controlled drug release and tumor targeting in cancer treatment.

1. Introduction

In recent decades, it has been demonstrated that nanoparticles have great merit for application in drug delivery and for overcoming the limitations of conventional cancer chemotherapies [1–5]. Nano-sized amphiphilic copolymer micelles represent a type of nanoparticle that is rather promising, due to their high loading capacity of water-insoluble drugs, such as docetaxel (DTX) [5–7]. In previous studies, most micelles were prepared on nanoscale and hydrophilic surfaces, which prolonged their circulation time in the blood stream, reduced the side effects of chemotherapeutic agents, and enhanced their accumulation in tumor sites via the enhanced permeation and retention effect (EPR) [8–10]. However, during the blood circulation process, nanoparticles can be readily recognized and eliminated by the reticuloendothelial system (RES), due to the adsorption to plasma proteins as opsonins, which leads to undesired side effects on RES organs and a reduction in the therapeutic effect [11,12]. Typically, to limit phagocytosis by RES cells and extend the systematic circulation time, nanoparticles are modified by polyethylene glycol (PEG) to inhibit the absorption of plasma proteins [12–14]. However, before practical applications can be implemented, several intractable problems remain that need to be addressed [15–17]. First, a controlled release of chemotherapeutic agents is of great significance for the maintenance of effective local drug concentration in the tumor site and reduction in side effects

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due to premature drug leakage [16]. More importantly, although passive targeting strategies have emerged as more efficient drug delivery methods than conventional therapy via free therapeutic agents, specific drug delivery to an actual tumor site has not been successfully achieved thus far due to the lack of selective cellular recognition and uptake strategies in the passive tumor targeting process [17].

Drug-bearing micelles are typically far from meeting these requirements, due to premature drug leakage and inadequate drug release concentrations in the tumor region [16,18]. An optical carrier is expected to retain the drug in a compact manner during the circulation process and release it in a sustained profile in response to a specific stimulus in the tumor site [19]. Thus far, temperature, magnetic field, light, pH, enzymes, and ultrasound have been used as the stimulus to trigger the drug release from the micelles [16,18–21]. In particular, in addition to its use as a stimulus for drug release, ultrasound has been reported to induce certain biological effects, providing it with advantages over other methods [22,23]. Among these methods, high-intensity focused ultrasound (HIFU) can be considered the most promising candidate, as it can effectively penetrate deep into the interior of the body and specifically focus on the target. The polymer chains of micelles can be disassembled by sheer force by the acoustic streaming and cavitation effect induced by HIFU [24,25]. Thus, the release of embedded drugs can be triggered, allowing for a relatively effective drug concentration in the local region of the tumor. In addition, various beneficial bio-effects can be created through ultrasound exposure, such as altering the permeability of cell membranes, enhancing the extravasation of therapeutic agents, and increasing local interstitial transport. These effects can be attributed to one or a combination of the following four mechanisms: cavitation, radiation pressure, acoustic streaming, and ultrasound-induced heating [26,27]. Therefore, HIFU-mediated drug delivery can noninvasively and effectively enhance the site-specific intracellular delivery of therapeutic agents to targeted tumors.

In recent years, specific active tumor targeting strategies had been investigated by modifying the nanoparticles with tumor-targeting moieties—including antibodies, proteins, and ligands to overcome the inherent limitation of passive tumor targeting [28–30]. Various previous studies have demonstrated that site-specific targeted nanoparticles conjugated to targeting moieties accumulate specifically on the tumor site in-vivo [13,16,17,28–30]. Incorporated with tumor-targeting moieties, nanoparticles can be recognized specifically by corresponding receptor overexpressing tumor cells, and thus, be taken up into cells via receptor-mediated endocytosis and release the drug inside the cells after circulation in the blood system [13,17,27–29]. It is believed that the internalization of nanoparticles into tumor cells could enhance the therapeutic effect of chemotherapy in cancer treatment [17,34].

Hyaluronic acid (HA) is a naturally occurring, biodegradable, biocompatible, and nonimmunogenic linear polysaccharide that is mainly distributed in the extracellular matrix (ECM), making it an ideal material for biomedical applications, such as tissue engineering and drug delivery [35,36]. In particular, HA acts as a native tumor-targeting moiety that can bind specifically to CD44 receptors, which are overexpressed abundantly on the surface of various types of tumor cells, such as those in breast, colon, and ovarian cancers [37]. Thus, its tumor-targeting ability induces the utilization of HA as a tumor-targeting ligand in modifying nonspecific nanocarriers, which highly enhances drug delivery [38]. Various amphiphilic HA copolymer-based micelles have been produced for active tumor imaging and delivery of therapeutic agents, such as docetaxel, paclitaxel, doxorubicin, siRNA, and antibodies, displaying highly specific accumulation at the tumor site and enhancing the therapeutic effect [13,17,31–33].

Herein, we propose a novel HA derivative-based micelle for active tumor-targeted intracellular controlled drug release (Fig. 1). A copolymer HA conjugated with hexadecylamine was synthesized for the embedment of DTX; we evaluated the loading ability and encapsulation efficiency of DTX. The in-vitro DTX release pattern was investigated and HIFU was used as the stimulus to carry out the controlled release of the drug from the micelles [39]. In particular, the intracellular uptake efficiency of DTX via the HA-CD44 interaction was verified on CT-26 (CD44 receptor negative) and MDA-MB-231 (CD44 receptor positive) cell lines [38]. The cellular distribution of the micelles was also investigated via fluorescent coumarin-6, and an enhanced cellular uptake effect was investigated under HIFU exposure [22].

2. Materials and methods

2.1. Materials

Sodium hyaluronate (MW = 4.8 kDa) was purchased from Bioland Co. Ltd. (Cheonan, Korea). Hexadecylamine, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), poly (ethylene glycol) bis (amine) (PEG diamine, MW = 3.0 kDa), pyrene, coumarin-6, and 4′, 6-diamidino-2-phenylindole (DAPI) were obtained from Sigma-Aldrich Co. (St. Louis, MO). Docetaxel Anhydrous (DTX) was supplied by Samyang Biopharmaceuticals Co. (Daejeon, Korea). Cell culture medium (DMEM, RPMI-1640), penicillin, streptomycin, and fetal bovine serum (FBS) were obtained from Gibco BRL (Gaithersburg, MD). All other chemicals were analytical grade and used in the form that they were received, without further purification.

2.2. Synthesis and characterization of HA-hexadecylamine (HA-C_{16}) conjugates

The synthesis of the hexadecyl derivative of hyaluronic acid and a similar octadecyl derivative has been reported in several previous studies with high molecular weight HA [33,40]. In this paper, a low molecular weight HA hexadecyl derivative was synthesized through an EDC-NHS reaction with a slightly modified process, shown in Fig. 2(a).

Briefly, 1 mM HA was dissolved in 10 mL anhydrous DMF; then, 3 mM each EDC and NHS were added to the reaction solution and stirred for 1 h to activate the carboxylic groups in the HA structure. Then, 3 mM hexadecylamine dissolved in 10 mL DMF was added to the reaction solution and stirred for another 4 h under heating and nitrogen conditions. The resultant solution was cooled to room temperature and dialyzed against water/ethanol (1 v/1 v) for two days and distilled water for two days with a dialysis bag (MWCO 3500). Then, the resultant solution was filtered to remove the large residue and lyophilized to obtain a uniform powder, using a 10 μm filter. The content of the hexadecyl chains in the HA conjugate was determined by proton nuclear magnetic resonance (^{1}H NMR) spectrophotometry (400 MHz, AVANCE III HD 400; Bruker, Billerica, MA). HA and HA-C_{16} were dissolved in D_2O and DMSO-d_6, respectively. The existence of the peptide bond was determined with a Fourier transform infrared (FT-IR) spectrometer (Spectrum 400; PerkinElmer Co.).

Critical micelle concentration (CMC) was determined by fluorescence measurement, using a standard pyrene procedure as follows. Pyrene was prepared in acetone in a tube, and the acetone was evaporated for 2 h under vacuum. Different concentrations of HA-C_{16} solution, ranging from 0.1 to 1000 μg/mL, were added to the previous pyrene to achieve a final concentration at of 6×10^{-7} M. Then, pyrene fluorescence spectra were measured with a