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Hyaluronic acid derivative-coated nanohybrid liposomes for cancer imaging and drug delivery

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ABSTRACT

Nanohybrid liposomes coated with amphiphilic hyaluronic acid–ceramide (HACE) was fabricated for targeted delivery of anticancer drug and *in vivo* cancer imaging. Nanohybrid liposomes including doxorubicin (DOX) and Magnevist, a contrast agent for magnetic resonance (MR) imaging, with 120–130 nm mean diameter and a narrow size distribution were developed. DOX release from the developed formulation was improved at acidic pH (pH 5.5 and 6.8) *versus* physiological pH (pH 7.4). Cytotoxicity induced by the blank plain liposome was reduced by coating the outer surface of the nanohybrid liposome with HACE. Cellular uptake of DOX from the nanohybrid liposome was enhanced by HA and CD44 receptor interaction, *versus* the plain liposome. *In vivo* contrast-enhancing effects revealed that the nanohybrid liposome can be used as a tumor targeting MR imaging probe for cancer diagnosis. In a pharmacokinetic study in rats, *in vivo* clearance of DOX was decreased in the order DOX solution, plain liposome (F2), and nanohybrid liposome (F3), indicating prolonged circulation of the drug in the blood stream and improved therapeutic efficacy of the nanohybrid liposome (F3). Based on these findings, the nanohybrid liposomal system may be a useful candidate for real-time cancer diagnosis and therapy.

1. Introduction

Recently, nano-sized systems have been widely investigated for both diagnostic and therapeutic applications. With the development of *in vivo* imaging techniques, several probes for magnetic resonance (MR), positron emission tomography (PET), computed tomography (CT), single-photon emission computed tomography (SPECT), and fluorescence imaging have been introduced in nano-sized delivery systems [1–3]. The combination of disease diagnosis and drug delivery, the so-called "theranostics," may provide precise drug delivery to a target site and convenience for patients. In the cancer diagnosis and therapy area, tumor-targeting strategies, generally consisting of passive and active targeting, have also been applied to theranostic systems. These techniques may maximize therapeutic efficacy and minimize side effects in the chemotherapy process.

For theranostic systems, various formulations, such as nanoparticles, micelles, liposomes, emulsions, and nanogels, have been used as platforms [4–7]. Among them, liposomes have attracted much interest because of their size control properties, biocompatibility, and loading capacity with hydrophilic and hydrophobic drugs [8]. With these advantages for *in vivo* applications, surface modification has been attempted to further modulate *in vivo* performance. In particular, modification with hydrophilic polymers, such as polyethylene glycol (PEG), poly-N-vinylpyrrolidone, polyvinyl alcohol, can improve the stability and circulation time in the blood stream of the liposome after intravenous administration [9–11]. By coating the outer surface of the liposome with hydrophilic polymers, the stability of a colloidal dispersion can be increased *via* the formation of a protective shell [8,12]. It has been reported that steric protection against interactions with biological components was related to the interaction of liposomes with opsonins in the blood stream [12]. In this regard, the development of polymer-hybridized liposomes has gained attention in terms of altering their *in vivo* stability.

In this investigation, amphiphilic hyaluronic acid derivative (hyaluronic acid–ceramide; HACE)-coated nanohybrid liposomes were prepared for targeted delivery of anticancer drug and MR imaging of cancer. HACE, as an amphiphilic HA oligomer, has been used in tumor-targeted delivery of anticancer drugs and *in vivo* cancer imaging using

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near-infrared fluorescence (NIRF) and MR techniques [1,4,13]. Amphiphilic HACE was embedded into the lipid bilayer of the liposomal formulation and hybrid nanoliposomes were formed. This nanohybrid liposome can exhibit the advantages of both components; the tumor targetability of HACE and the *in vivo* stability of the liposome. Specifically, the hydrophobic residue (CE) may be anchored in the hydrophobic lipid bilayer and the hydrophilic chain (HA) attached to the outer surface of the liposome. It is known that HA can bind to the CD44 receptor overexpressed in many types of cancer cells; it thus can be used as a tumor-targeting moiety [1,13–16]. Both modulation of physicochemical properties (*i.e.*, <200-nm particle size) of nanocarriers and HA–CD44 receptor interaction can provide passive, due mainly to enhanced permeability and retention (EPR) effects, and active tumor targetability in cancer therapy.

In the current study, the physicochemical properties of the hybrid nanoliposomes, including doxorubicin (DOX) and Magnevist, were investigated. Their cytotoxicity and cellular distribution in MDA-MB-231, a CD44 receptor-overexpressing breast cancer cell line, were evaluated. Cancer diagnostic properties, by MR imaging, and *in vivo* pharmacokinetic properties of DOX were also assessed in animal models.

2. Materials and methods

2.1. Materials

Doxorubicin hydrochloride (DOX HCl) was purchased from Boryung Pharmaceutical Co., Ltd. (Seoul, Korea). Egg phosphatidylcholine (Lipoid E100) was obtained from Lipoid AG (Ludwigshafen, Germany). Magnevist (N-methylglucamine salt of the gadolinium complex of diethylenetriamine pentaacetic acid; gadopentetate dimeglumine) was purchased from Bayer HealthCare Pharmaceutical Inc. (Wayne, NJ, USA). Hyaluronic acid oligomer and DS-Y30 (ceramide 3B; mainly N-oleoylphytosphingosine) were from Bioland Co., Ltd. (Cheonan, Korea) and Doosan Biotech Co., Ltd. (Yongin, Korea), respectively. Chloromethylbenzoyl chloride and tetra-n-butylammonium hydroxide (TBA) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Cell culture medium (RPMI 1640, Waymouth), penicillin, streptomycin, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES) buffer solution, and heat-inactivated fetal bovine serum (FBS) were purchased from Gibco Life Technologies, Inc. (Grand Island, NY, USA). All other reagents were of analytical grade.

2.2. Preparation of DOX-loaded liposomal formulations

HACE was synthesized according to a reported method [13]. Briefly, HA (12.21 mmol) and TBA (9.77 mmol) were solubilized in double-distilled water (DDW; 60 ml) and stirred for 30 min. The resulting activated HA–TBA was acquired by freeze-drying. DS-Y30 ceramide (8.59 mmol) and triethylamine (9.45 mmol) in tetrahydrofuran (THF; 25 ml), and 4-chloromethylbenzoyl chloride (8.59 mmol) in THF (10 ml) were mixed to synthesize DS-Y30 linker. DS-Y30-containing linker was obtained from concentration and recrystallization processes after stirring for 6 h at 60 °C. HA–TBA (8.10 mmol) and the DS-Y30-containing linker (0.41 mmol) were solubilized in THF/acetonitrile mixture (4:1, v/v) and stirred for 5 h at 40 °C. After eliminating impurities and organic solvents, HACE was finally obtained.

The nanoliposomal formulations developed were fabricated according to a modified liposome preparation method [17]. The compositions of the formulations are presented in Table S1. Gd-loaded HACE-coated nanohybrid liposome (F1), DOX/Gd-loaded liposome (F2), and DOX/Gd-loaded HACE-coated nanohybrid liposome (F3) were prepared by a thin-film hydration method. For the F1 formulation, egg phosphatidylcholine (80 mg), cholesterol (20 mg) and HACE (20 mg) were dissolved in methanol (20 ml) and the organic solvent was removed by evaporation (Büchi R-200 rotary evaporator, Flawil, Switzerland) at 60 °C for 30 min. Magnevist (2 ml), diluted with 5 mM HEPES buffer (18 ml), was added to a thin film-coated flask and hydrated at 60 °C for 30 min. The resulting preparation was sonicated with a probe-type sonicator (Vibra-Cell VC 750 ultrasonic processor, Sonics & Materials, CT, USA) for 15 min, and the mixture was passed through a 0.2-μm filter with an extruder (Northern Lipids, Inc., Canada) three times. For F2, egg phosphatidylcholine (80 mg) and cholesterol (20 mg) were solubilized in methanol (20 ml) and the organic solvent was removed by evaporation at 60 °C for 30 min. Ammonium sulfate solution (250 mM, 10 ml) was added to a thin-film-coated flask and hydrated at 60 °C for 30 min. The resulting preparation was sonicated with a probe-type sonicator for 15 min, and the mixture was passed through a 0.2-μm filter with an extruder three times. The liposomal dispersion was then blended with DOX HCl (2 mg/ml) dissolved in 5 mM HEPES buffer (8 ml) and Magnevist (2 ml) mixture. For F3, HACE (20 mg) was added to the composition of F2 while other preparation methods were identical to that of F2. After preparing each formulation, it was incubated at 60 °C for 2 h. To remove unloaded drug and Magnevist, the formulations were in a dialysis bag (molecular weight cut-off: 6–8 kDa) against DDW for 2 days. For storage, 0.6 g of sucrose was added to the dialyzed dispersion and it was lyophilized.

2.3. Characterization of DOX-loaded liposomal formulations

The synthesis of HACE was characterized by ^{1}H-NMR as reported previously [13]. Characteristics of the nanostructured formulations, such as particle size, polydispersity index, and zeta potential, were investigated with a light-scattering spectrophotometer (ELS-Z; Otsuka Electronics, Tokyo, Japan). Mean diameters of the F2 and F3 formulations were monitored in PBS (pH 7.4) and 50% (v/v) FBS (in PBS, pH 7.4) after incubation for 1 or 24 h.

The encapsulation efficiency (EE) and DOX HCl content of each formulation were determined by high-performance liquid chromatography (HPLC) system after dissolving in DW and diluting with 100× the volume of the mobile phase. The drug was assayed using a Waters HPLC system (Waters Co., Milford, MA) combined with a separation module (Waters e2695), a fluorescence detector (Waters 2475), and a reverse-phase C-18 column (Xbridge, RP-18, 250 × 4.6 mm, 5 μm; Waters Co.). Fluorescence wavelengths for the detection of DOX were 480 nm (excitation) and 560 nm (emission). The flow rate was 1.0 ml/min and the injection volume for drug assay was 20 μl. The mobile phase consisted of 10 mM potassium phosphate buffer (pH 2.5) and acetonitrile including 0.1% (w/w) triethylamine (71:29, v/v). The lower limit of quantification (LLOQ) was 25 ng/ml and precision and accuracy were within the acceptable range. Gd (major element of Magnevist) contents of each formulation were determined using an inductive coupled plasma-atomic emission spectrometer (ICP–AES; Optima 4300 DV; PerkinElmer Inc., Wellesley, MA).

The morphology of each nanostructure was observed by transmission electron microscopy (TEM). Nanostructured formulations were stained with 2% uranyl acetate and destained with DDW. They were then placed on copper grids with films, dried for 10 min, and observed by TEM (JEM 1010; JEOL, Tokyo, Japan).

2.4. In vitro drug release

DOX release from F2 and F3 was assessed *in vitro*. This was performed in phosphate-buffered saline (PBS, pH 5.5, 6.8, and 7.4, adjusted with phosphoric acid). The release pattern was monitored for 7 days at 37 °C with 50 rpm rotation. The formulation (equivalent to 50 μg DOX HCl) was added to a dialysis bag (molecular weight cut-off: 12–14 kDa) and was immersed in 20 ml of PBS (pH 5.5, 6.8, and 7.4). At each time point (1, 3, 6, 9, 12, 24, 48, 72, 96, 120, 144, and 168 h), a 0.2-ml aliquot was collected and an equal volume of fresh medium was replenished. To quantify DOX release, its concentration in the samples was determined by the HPLC method described above.