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Chitosan-modified lipid nanovesicles for efficient systemic delivery of L-asparaginase

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ABSTRACT

The goal of this study was to evaluate the enhanced catalytic activity, increased stability, *in vitro* anti-cancer effects on H446 cells and *in vivo* bioavailability of novel enzyme delivery nanovesicles (L-asparaginase containing chitosan modified lipid nanovesicles, ACLNs) when administered intravenously. It was the first time for the chitosan-modified lipid nanovesicles to be fabricated to deliver L-asparaginase (ASP, a therapeutic enzyme) efficiently. It was indicated that ACLNs markedly increased the enzymatic activity, improved the temperature/acid-base/proteolytic stabilities and favorably changed the *in vivo* kinetic characteristics. Moreover, ACLNs exhibited higher anti-lung-cancer activity than free ASP. The possible existence status of ASP in ACLNs and the fluorescence changes of ACLNs reflecting the conformational changes after heat treatment were preliminary explored. ACLNs might be novel promising nanovesicles for effective systemic delivery of therapeutic enzyme ASP.

1. Introduction

Based on functional mechanisms, enzyme therapies can be classified into three main categories: enzyme replacement therapy [1], directed enzyme prodrug therapy [2] and enzyme enhancement therapy such as L-asparaginase (ASP) therapy. ASP has been extensively used in clinics for many years as a first-line therapeutic agent for acute lymphoblastic leukemias and lymphomas [3]. Recently, some new indications of ASP such as nasal type extranodal natural killer/T-cell lymphoma and ovarian cancer, have been confirmed [4] or evaluated in different clinical trial phases [5]. ASP is a tetrameric amidohydrolase able to catalyze the deamination of L-asparagine and glutamine. Since glutamine can provide the substrate (ammonia) of L-asparagine synthetase to synthesis L-asparagine, the decreased glutamine level will certainly contribute to the decreased L-asparagine level. The normal cells can synthesis L-asparagine while cancer cells can't, so lack or reduction of L-asparagine will induce death or growth inhibition of cancer cells [6]. Being a therapeutic enzyme and essentially a protein drug, ASP had intrinsic drawbacks such as low catalytic activity under physiological situation, low stability and short circulating life. A few of delivery systems such as ASP conjugated zinc oxide nanobiocomposites [7], polyethylene glycol-conjugated ASP [5], ASP erythrocytes [8] and ASP chitosan-tripolyphosphate nanoparticles [9] had been developed to meet different needs: increase the anti-cancerous activity against MCF-7 breast cancer or advanced ovarian cancer cells, or prolong the *in vitro* release half-life of ASP.

Recently, lipid nanovesicles have showed attractive potential in the effective delivery of all kinds of drugs such as paclitaxel (a phytodrug for colon adenocarcinoma) [10], pentapeptide mimic 1,4-bis(9-O dihydroquinidinyl)phthalazine/hydroquinidine 1,4-phathalazinediyl diether (a cinchona alkaloid for B-precursor leukemia) [11], doxorubicin (a chemodrug for epidermal carcinoma) [12], recoverin (a protein used to treat retinal diseases) [13] and porphyrin (a multimodal photonic contrast agent) [14]. Furthermore, modification of drug delivery systems by natural polysaccharide chitosan have increased the stability and efficacy as reported in the cases of vancomycin chitosan coated liposomes [15], macrophage-targeting gene chitosan nanoparticles [16] and bovine lactoferrin chitosan-modified solid lipid particles [17]. Moreover, nanovesicles have been employed to improve the activities of the enzymes used in different enzyme therapies. For example, alkaline enzymosomes was formulated to improve the biological properties and hypouricaemic effects of uricase [1] in enzyme replacement therapy, magnetic iron oxide nanoparticles was applied to achieved the enhanced and selective delivery of β-glucosidase to 9L-glioma tumor [2] in directed enzyme prodrug therapy. Here, novel types of nanocarriers, i.e., chitosan-modified lipid nanovesicles, were fabricated to deliver therapeutic enzymes efficiently and obtain

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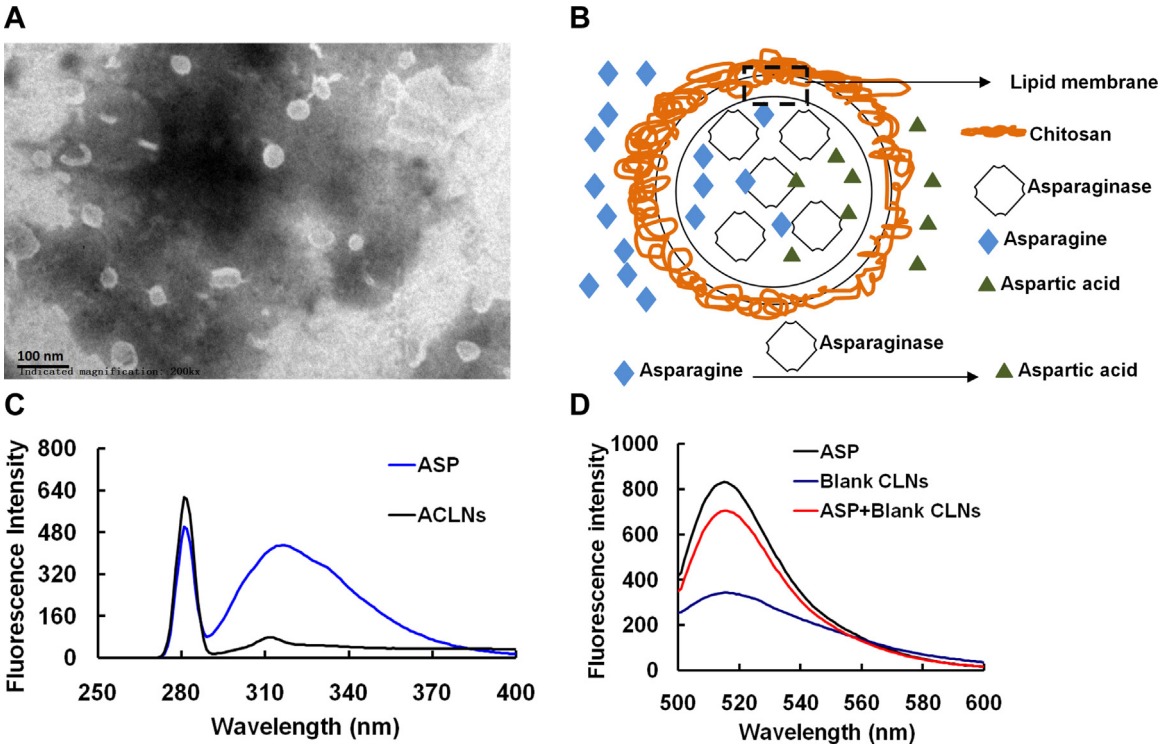

Fig. 1. Preparation and characteristics of ACLNs. Transmission electron photomicrographs (bar: 100 nm) (A) and schematic diagram (B) of ACLNs. Fluorescence changes of ASP induced by entrapment in ACLNs (C) or by interaction between ASP and CLNs membrane (D).

increased antitumor effects in enzyme enhancement therapy. Since L-asparaginase (ASP) was a clinically used enzyme which usage was restricted by its instability and low catalytic activity *in vivo*, it was chosen as a model of therapeutic enzyme in this study.

In the experiments outlined below, L-asparaginase-containing chitosan-modified lipid nanovesicles (ACLNs) were prepared to deliver the therapeutic enzymes efficiently for the first time. Their catalytic activity, physicochemical and enzymatic stabilities, anti-lung-cancer activity, *in vivo* kinetic property and bioavailability were investigated. Our study indicated that ACLNs markedly increased the enzymatic activity, improved the stability, and favorably changed the *in vivo* kinetic characteristics. Moreover, ACLNs had higher anti-lung-cancer activity than that of free ASP. The possible existence status of ASP in ACLNs and the fluorescence changes of ACLNs reflecting the conformational changes after heat treatment were preliminary explored. ACLNs might be promising nanovesicles for effective systemic delivery of therapeutic enzyme ASP.

2. Materials and methods

2.1. Materials

L-asparaginase from *Escherichia coli*. (ASP, activity of 225 units/mg powder at 37 °C, purity >96.0%) was obtained from ProSpec-Tany TechnoGene Ltd. (Rehovot, Israel). L-asparagine, Cholesterol and fluorescein isothiocyanate (FITC) were obtained from Sigma (St. Louis, MO, USA). Soybean phospholipid (Lipoid S 100) was obtained from Lipoid GmbH (Ludwigshafen, Germany). Chitosan hydrochloride (deacetylation 80.0%-90.0%, viscosity 10–120 mpa.s, average molecular weight ~50 kDa) was obtained from Golden-Shell Pharmaceutical Co., Ltd. (Yuhuan, China). Coomassie blue G-250 was obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). HPLC-grade water was purified by a Milli-Q system equipped with cellulose nitrate membrane filters (0.2 mm, Whatman, Maidstone, UK). All other reagents used in the study were of analytical grade. All male Sprague Dawley rats (200 g ± 20 g) were supplied by the Laboratory Animal Center at Chongqing Medical University (Chongqing, China). All animals were acclimatized at a temperature of 25 ± 3 °C and a relative humidity of 70 ± 5% under natural light/dark conditions for 7 d before dosing.

2.2. Fabrication and characterization of ACLNs

2.2.1. Preparation of ACLNs

Firstly, ASP lipid nanovesicles were prepared by a modified reverse-phase evaporation method [18]. Briefly, soybean phospholipid and cholesterol at a molar ratio of 1:1 were dissolved in 30 mL of chloroform. After chloroform was removed by rotary evaporation, lipid films were obtained and redissolved in 30 mL of diethyl ether. Then, they were further added to 10 mL of Tris–HCl buffer (50 mmol/L, pH 7.3) containing ASP. The mixture was sonicated in a bath sonicator until a homogeneous opalescent dispersion was obtained. Next, diethyl ether was removed from the dispersion by a rotary evaporator under reduced pressure to form an aqueous suspension (i.e., ASP lipid nanovesicles). Secondly, chitosan modified nanovesicles were prepared by an electrostatic deposition method [15]. Chitosan hydrochloride (200 mg) was dissolved in 100 mL of double distilled water to form chitosan solution (0.2%, weight/volume), which was further dropwisely added to the ASP lipid nanovesicle suspension under magnetic stirring for 1 h at 25 °C and then incubated at 4 °C for another 12 h.

2.2.2. Characterization of ACLNs

The size and zeta potential of ACLNs were determined at 25 °C by dynamic light scattering (Zeta-Sizer Nano-ZS90, Malvern, UK). The morphology of ACLNs was observed by the transmission electron microscopy (JEM-1400Plus, JEOL, Japan). The samples were prepared by diluting ACLNs with 19 times volume of Tris–HCl buffer.