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Low-density lipoprotein-coupled N-succinyl chitosan nanoparticles co-delivering siRNA and doxorubicin for hepatocyte-targeted therapy

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ABSTRACT

Developing safe and effective carriers of small interference RNA (siRNA) is a significant demand for the systemic delivery of siRNA. In this study, low-density lipoprotein (LDL) was isolated from human plasma and loaded with cholesterol-conjugated siRNA to silence the multidrug resistant gene of tumors. Chol-siRNA/LDL-coupled N-succinyl chitosan nanoparticles loaded with doxorubicin (Dox-siRNA/LDL-SCS-NPs) were then prepared and characterised. The Dox-siRNA/LDL-SCS-NPs had average particle size of 206.4 ± 9.2 nm, entrapment efficiency of 71.06% ± 1.42%, and drug-loading amount of 12.35% ± 0.87%. *In vitro* antitumor activity revealed that cell growth was significantly inhibited. The accumulation of Dox by fluorescence microscopy and flow cytometry showed that LDL-coupled nanoparticles were more easily taken up than Dox-SCS-NPs. Results of confocal microscopy and reverse transcription-PCR revealed the highly efficient uptake of siRNA and the decrease in *mdr1* mRNA expression. LDL-coupled nanoparticles protected siRNA from macrophage phagocytosis by dynamic observation using live cell station. *In vivo* tumor-targeting suggested that Cy7-labelled Dox-LDL-SCS-NPs were markedly accumulated in an analyzed *in situ* liver tumor model. Results indicated that LDL-SCS-NPs were effective tumor-targeting vectors and that the preparation form may provide a new strategy for co-delivering siRNA and anti-tumor drugs.

1. Introduction

Multidrug resistance (MDR) hinders the improvement of anti-tumor effects. Mechanisms that explain MDR include the increase in drug efflux pumps on the cell membrane and the increase in antiapoptotic pathways [1]; MDR can be reversed from these processes. P-glycoprotein (P-gp) is a member of ATP-binding cassette transporter family (ABC) and an important MDR protein; the inhibition of P-gp expression restores the intracellular drug levels to required concentrations [2,3].

RNA interference (RNAi) has been widely applied in biology and medicine because of specific and effective gene silencing. RNAi is mediated by small interfering RNAs (siRNAs) that can be incorporated into the RNA-induced silencing complex (RISC) and degrade target mRNA to inhibit the expression of target protein [4]. Although siRNA interference is valid, transport and transfection of siRNA is hindered because of large molecular weight, strong negative charge, and instability under nuclease and serum proteins [5]. Exogenous molecules internalized into the cells through endocytosis will be transported through endosomes and lysosomes, followed by enzymatic degradation. The processes increase the difficulty for siRNAs to initiate the silencing activities in the cytoplasm.

Developing effective siRNA vehicles is important to overcome the disadvantages. Several siRNA delivery systems based on lipid, polymer and protein have been developed [6–8]. Negatively

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charged siRNAs easily combine with the cationic polymers, such as polyethyleneimine (PEI), poly(amino amide) (PAMAM), and chitosan via electrostatic interactions [9–11]; cationic polymers are thus alternative and attractive. However, problems have occurred in applying these vehicles, such as nonbiodegradability, systemic toxicity, interactions with serum proteins, and macrophage phagocytosis [12–14].

Low-density lipoprotein (LDL) is advantageous because of endogenous degradability, nontoxicity, compatibility with cell membranes, and escape from reticuloendothelial system (RES) [15,16]. LDL comprises of a lipidic core surrounded by a monolayer of phospholipids, in which cholesterol and apolipoprotein B-100 (ApoB-100) are embedded [17]. The uptake of LDL is mediated by LDL receptor (LDLr), an integral plasma membrane glycoprotein abundantly expressed in tumors that recognize ApoB-100 [18,19]. The active Lys residues of ApoB can be properly modified to retain the binding capability to LDLr [20]. Although LDL is a useful vehicle of lipophilic drugs, its application is largely limited because of low entrapment efficiency and failure to obtain therapeutically effective LDL drug complexes. These problems can be solved with the use of LDL as a targeting ligand to conjugate the nanoparticles.

In this study, LDL was isolated from human plasma and incorporated with cholesterol-conjugated *mdr1* siRNA. N-succinyl chitosan (SCS) was synthesized and used to prepare SCS nanoparticles loaded with Dox, followed by coupling with chol-siRNA/LDL to give polymeric nanoparticles (Scheme 1). The *in vitro* antitumor activity, intracellular uptake, and silencing effects of siRNA were investigated. Macrophage phagocytosis and tumor-targeting effects were evaluated.

2. Materials and methods

2.1. Materials and cell lines

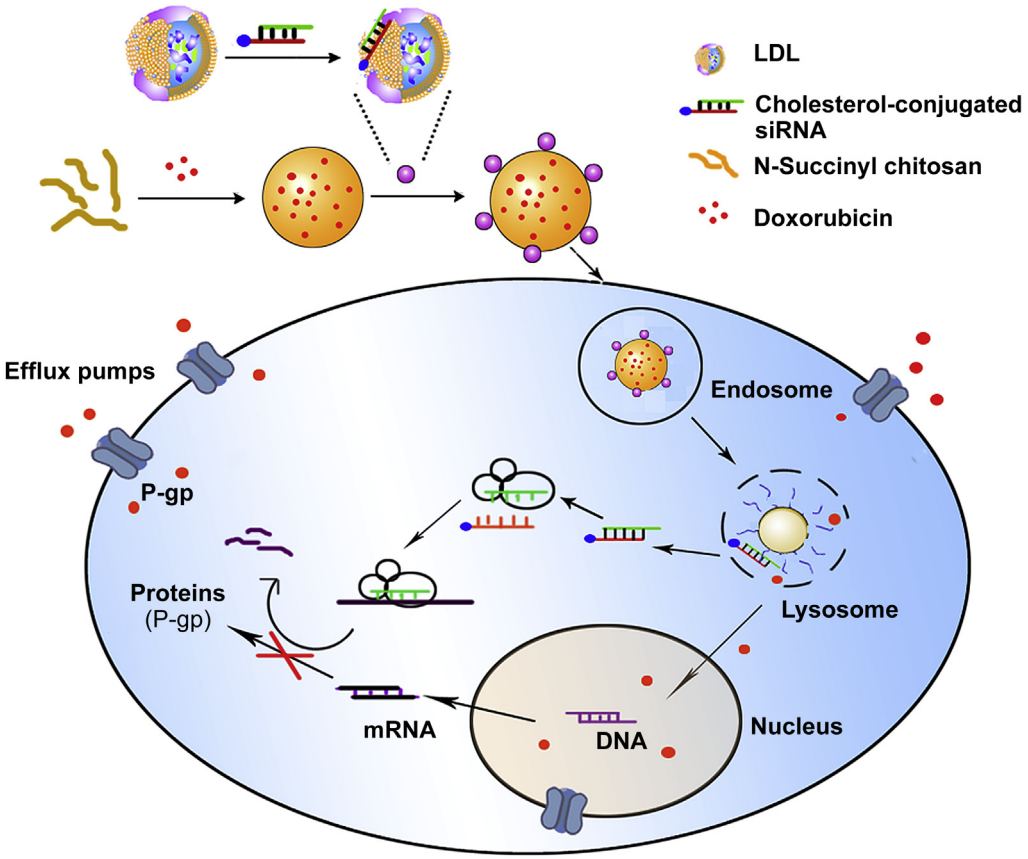
Virus inactivation human plasma was provided by the First Affiliated Hospital of Soochow University, Suzhou, China. Cholesterol-conjugated siRNAs (chol-siRNAs) have respective sense and antisense patterns given as 5′-GGAAAAGAAACCAACUGUCdTdT-(cholesterol)3′ and 5′-GACAGUUGGUUUCUUUUCCdTdT-3′; targeting *mdr1* mRNA was chemically synthesized at Shanghai Genepharma Co., Ltd. (Shanghai, China). Chitosan with molecular weight (MW) of 8–10 kDa and deacetylation degree (DD) of 93.1% was obtained from Xingcheng Biochemical Co., Ltd. (Nantong, China). Succinic anhydride, 1-(3-dimethylaminopropyl)-3-ethyl carbon carbodiimide hydrochloride (EDC·HCl) and N-hydroxysuccinimide (NHS) were purchased from Aladdin-Reagent Co., Ltd (Shanghai, China). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was from Sigma–Aldrich (St. Louis, Missouri, USA). Fetal bovine serum (FBS), RPMI-1640, and DMEM medium were purchased from Hyclone (Thermo-Fisher Biochemical Products Co., Ltd., Beijing, China). Heptamethine cyanine fluorescence dyes (Cy7) were provided by Baomanbio Co., Ltd. (Shanghai, China). All other chemicals were of analytical grade and used without further purification.

Female nude mice (age, 4–6 w) were provided by the Experimental Animal Centre of Soochow University (Suzhou, China). All animals were kept in an environment that complied with the guidelines of the National Institutes of Health for the care and use of laboratory animals. All animal procedures were performed following the protocols approved by the Institutional Animal Care.

Human hepatoblastoma cell line HepG2 and human hepatocyte L-02 from Jiangsu Province Key Laboratory of Biotechnology and Immunology (Suzhou, China) were maintained in DMEM and RPMI-1640 media supplemented with 10% (v/v) fetal bovine serum, respectively. Human Adriamycin-resistant hepatoblastoma cell line HepG2/ADM was purchased from Aiyan Biotechnical Co., Ltd. (Shanghai, China) and maintained in DMEM medium with 10% fetal bovine serum and 0.1 nmol mL^{-1} Dox.

2.2. Isolation of LDL

LDL was isolated from human plasma by sequential density gradient ultracentrifugation [21] and stored at 4 °C until further use within 2 w. Protein concentration was determined by BCA method. The particle distribution of LDL was measured by Zetasizer nanoparticle analyzer (HPPS-5001; Malvern Instruments, Worcestershire,



Scheme 1. LDL-coupled SCS nanoparticles co-delivering siRNA and Dox reversing multidrug resistance.