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Facile preparation of biocompatible nanostructured lipid carrier with ultra-small size as a tumor-penetration delivery system

Xin Zhao[a,1], Dongyang Tang[b,1], Tie Yang[c], Cheng Wang[d,*]

[a] Department of Pharmacy, Xinxiang Central Hospital, Xinxiang, Henan 453000, PR China
[b] Department of Experimental Center, Henan Institute of Science and Technology, Xinxiang, Henan 453003, PR China
[c] Nanjing Research Center, Jiangsu Chiatai Tianqing Pharmaceutical Co. Ltd, Nanjing, Jiangsu 210042, PR China
[d] College of Pharmaceutical Sciences, Zhejiang University, 866 Yuhangtang Road, Hangzhou, Zhejiang 310058, PR China

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ABSTRACT

Insufficient tumor penetration is one of the major obstacles for satisfactory cancer therapy. As a result, the ability to push the lower limits of size for nanoparticle platforms that have traditionally existed in larger forms is highly desirable. In our study, a facile solvent diffusion method was applied to prepare an ultra-small nanostructured lipid carrier (usNLC) which was capable of encapsulating hydrophobic molecules. Our results demonstrate that the as-prepared usNLC is composed of homogeneous particles with size around 25 nm. In addition to its preferable colloidal stability, negligible hemolysis as well as strong tumor homing property, the as-prepared usNLC shows preferable tumor penetration capacity both *in vitro* and *in vivo*. The paclitaxel (PTX) loaded usNLC shows comparable *in vitro* cytotoxicity on HepG2 cells and multicellular tumor spheroids to Taxol with the best *in vivo* anti-tumor efficacy, which all indicate its potential to be a promising candidate for cancer therapy.

1. Introduction

Cancer remains to be one of the most lethal diseases around the world, whereas current cancer treatments are still far from perfection. The most widely adopted cancer chemotherapy usually subject to some evident obstacles, *i.e.*, insolubility and undesired side effect of the anti-cancer drugs [1]. As a result, drug delivery vehicles are widely employed with the aim to increase drug accumulation to the tumor tissue [2]. The first and foremost requirement of drug delivery vehicles intended to be administered *in vivo*, by any mode and for any biomedical application, is that they should be safe [3]. Although synthetic materials gain increasing interests owing to their diverse origins and versatile functions which can be easily tailored to meet various demands [4]. Nevertheless, their safety profiles are still to be questioned compared with their physiological originated counterparts which severely limit their clinical applications [5].

Solid lipid nanoparticles (SLNs) are a class of particulate drug carriers typically constructed by physiological lipids (including: fatty acids, steroids, waxes, mono-, di-, or triglyceride mixtures) [6], which have been employed as a preferable drug delivery system in the past two decades due to their various merits, including good tolerability, brain targeting capacity, facile surface modifications and possibility for large scale production [7]. Nanostructured lipid carriers (NLCs) are the second generation of SLNs which basically composed of solid lipid matrix with a certain content of liquid lipid [8]. Due to the introduction of liquid lipid, the resulted NLC matrix have great imperfections in the crystal lattice and offer enough space to accommodate drug molecules [9], thus rendering the NLCs with improved drug loading capacity. Moreover, liquid lipid can also increase the flexibility of the as-prepared NLC which give rise to the possibility to push the lower limits of its size [10].

The *in vivo* performance of nanoparticles, such as circulation time and biodistribution, are closely related to their size [11]. It is generally recognized that nanocarriers with size under 200 nm can preferably take advantage of the permeability of tumor vasculature to target tumor tissue *via* the “enhanced permeability and retention” (EPR) effect [12]. However, due to the existing of an interconnected network of collagen fibers at the tumor extracellular matrix (ECM), large nanoparticles are usually being blocked and trapped in perivascular regions without further penetration [13]. As a result, ultra-small particles with size under 100 nm or lower range, are reported to be ideally suited to the design of tumor targeting vehicles as it allows for not only broad range of blood pharmacokinetics, but also superior tumor accumulation and permeation capability [14,15].

* Corresponding author.
E-mail address: 11519016@zju.edu.cn (C. Wang).
[1] These authors contributed equally to this work.

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In addition to size, surface modification also plays a significant role in dominating the *in vivo* fate of nanoparticles. It has been well recognized that surface modification of nanoparticles with polyethylene glycol (PEG) can greatly decrease their capture by reticuloendothelial system and increase the circulation cycle which are favorable for their passive targeting to the tumor tissue [16,17]. On the other hand, by further introducing tumor-targeting moieties [18–20], such as folic acid (FA) [18], the tumor-homing profile of the nanoparticles can be further enhanced due to the selective recognition of modified targeting moieties with the over-expressed receptors on the surface of cancer cells.

Due to the disappointing fact that the size most of the reported NLCs were over 100 nm [21–23]. Here, by fine-tuning the component ratios, temperature, and concentrations, we demonstrate a facile approach to prepare an ultra-small NLC (usNLC) with surface modification of stealthy polymer (PEG) and targeting moiety (FA) with a final size around 25 nm. The ability to engineer an ultra-small version of the NLC has the potential to significantly boost the utility of this already promising platform for a variety of applications, including brain-targeted delivery and deep tumor penetration [24].

2. Materials and methods

2.1. Materials

Glycerin monostearate (GM), oleic acid (OA) and paclitaxel (PTX) were purchased from Sigma-Aldrich (St Louis, MO, USA). Folic acid (FA), Hoechst 33,342, 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindotricarbocyanine iodide (DiR), 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI), methylthiazolete-trazolium (MTT) and coumarin 6 (C6) were offered by Thermo Fisher Scientific (Massachusetts, USA). Phosphatidylcholine (PC) was obtained from Lipoid GmbH (Ludwigshafen, German). Stearic acid-polyethylene glycol-folate (SA-PEG-FA) was purchased from Nanocs Inc. (New York, USA). Other Chemicals involved in the study were analytically pure obtained from Shanghai Chemical Reagent Co. Ltd., (Shanghai, China) and used without further pretreatment.

2.2. Cell culture

Human liver carcinoma cell line (HepG2) and mouse fibrosis cell line (NIH3T3) were cultured in folate free DMEM medium (Sigma-Aldrich, USA) containing 10% fetal bovine serum (FBS), 100 U/mL of penicillin and 100 μg/mL of streptomycin (Gibco, USA) at 37 °C using a humidified 5% CO_2 incubator (311, Thermo scientific, USA).

2.3. Multicellular tumor spheroids (MCTS) model

To establish the HepG2 MCTS, HepG2 and NIH 3T3 cells were detached from monolayers, pipetted into single cell suspensions (1.5×10^4 cells/mL) and mixed with each other (v/v, 1;1). Afterwards, cells were transferred into flat-bottomed 96-well plates pre-coated with 2% agarose (200 μL/well). After an incubation period of 4 days, cellular aggregation occurred and MCTS were formed. The MCTS with diameter around 300 μm were included for experiments.

2.4. Animal model

BALB/c mice and New Zealand rabbits were purchased from Shanghai Lab. Animal Research Center. All animal experiments were conducted in strict compliance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (USA, 2011). In order to obtain H22 (murine liver cancer carcinoma cells) tumor xenograft mice, approximately 10^7 of H22 cells (a gift from Dr. Pengfei Cui in China Pharmaceutical University) were incubated subcutaneously to the flank region of the mice. Tumor volume (V) was determined by measuring length (L) and width (W), and calculated as formula: $V = L \times W^2/2$.

2.5. Preparation of ultra-small NLC (usNLC)

Glycerin monostearate (GM, 2 mg), phosphatidylcholine (PC, 3 mg), oleic acid (OA, 2 mg) and stearic acid-polyethylene glycol-folate (SA-PEG-FA, 3 mg) were added into 1 mL of ethanol and ultrasonicated (JY92-II, Ningbo Scientz Biotechnology Co., Ltd., China; 400 w, work 2 s and stand 3 s, 20 times) to obtain a transparent solution. To a preheated (70 °C) aqueous solution (10 mL), the mixture of lipid components was injected constantly (0.1 mL/s) *via* syringe under gentle agitation (500 rpm). The aqueous solution of PTX was allowed to agitate for another 10 min after the addition of lipids. Afterwards, the solution was centrifuged at 20,000 rpm (Allegra 64R, Beckman Coulter, USA) for 10 min to remove the un-encapsulated PTX and the supernatant was collected and stored in 4 °C for further use.

The chemical drug paclitaxel (PTX) or fluorescence probe was loaded into NLCs by physical encapsulation. Drug or fluorescence probe was dissolved with lipids in the organic phase, and proceeded as described above. After drug loading, the solution was centrifuged at 20,000 rpm for 10 min. The supernatant was collected and freeze-dried to obtain white powder. The powder was then dissolved in acetonitrile with appropriate heating, filtered through 0.45 μm membrane (Millipore, USA) and then subjected to high performance liquid chromatography (HPLC, 1100 series, Agilent, USA) analysis using a C_{18} analytical column (Agilent, USA) with a mobile phase of 50 : 50 water to acetonitrile and a detection wavelength of 227 nm. The drug loading content was calculated as follows:

$$\text{Drug loading content (\%)} = \frac{\text{Weight of PTX loaded NLC}}{\text{Weight of PTX loaded NLC}} \times 100$$

2.6. Preparation of large-sized NLC (lNLC)

GM (6 mg), OA (1 mg) and SA-PEG-FA (3 mg) were added into 1 mL of ethanol and heated to obtain a transparent solution. To a preheated (70 °C) aqueous solution (10 mL), the mixture of lipid components was injected *via* pipette under gentle agitation (300 rpm). The aqueous solution was allowed to agitate for another 10 min after the addition of lipids. Afterwards, the solution was centrifuged at 20,000 rpm (Allegra 64R, Beckman Coulter, USA) for 10 min to remove the un-encapsulated PTX and the supernatant was collected and stored in 4 °C for further use. The PTX or fluorescence probe was loaded into lNLCs as mentioned above.

2.7. Characterization of NLC

The particle sizes of NLCs were measured by Zetasizer Nano ZS90 (Malvern Instruments Ltd., UK, n = 3). The shape and surface morphology of NLC were observed by transmission electron microscopy (TEM, Hitachi 7700, Japan).

2.8. Drug release

The drug release profile of PTX loaded usNLC was investigated by dialysis method. Briefly, samples were placed into individual dialysis bag (MWCO: 7 KDa) and immersed in plastic tube filled with 25 mL of phosphate buffer (PBS, pH 7.4) containing 0.2% Tween80. The plastic tubes were fixed in a thermostatic shaker (HZQ-C; Haerbin Dongming Medical Instrument Factory, China) at 37 °C with a stirring speed of 100 rpm. At predetermined time intervals, 1 mL buffer solution within the tubes was removed and replaced with equal volume of fresh medium. The buffer solution was filtered through 0.45 μm membrane and then subjected to HPLC analysis as descried above.