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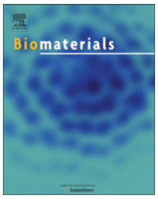

Crosslinked multilamellar liposomes for controlled delivery of anticancer drugs

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ABSTRACT

Liposomes constitute one of the most popular nanocarriers for the delivery of cancer therapeutics. However, since their potency is limited by incomplete drug release and inherent instability in the presence of serum components, their poor delivery occurs in certain circumstances. In this study, we address these shortcomings and demonstrate an alternative liposomal formulation, termed crosslinked multilamellar liposome (CML). With its properties of improved sustainable drug release kinetics and enhanced vesicle stability, CML can achieve controlled delivery of cancer therapeutics. CML stably encapsulated the anticancer drug doxorubicin (Dox) in the vesicle and exhibited a remarkably controlled rate of release compared to that of the unilamellar liposome (UL) with the same lipid composition or Doxil-like liposome (DLL). Our imaging study demonstrated that the CMLs were mainly internalized through a caveolin-dependent pathway and were further trafficked through the endosome-lysosome compartments. Furthermore, *in vivo* experiments showed that the CML-Dox formulation reduced systemic toxicity and significantly improved therapeutic activity in inhibiting tumor growth compared to that of UL-Dox or DLL-Dox. This drug packaging technology may therefore provide a new treatment option to better manage cancer and other diseases.

1. Introduction

Optimal treatment by many drugs often requires maintenance of the drug level for a prolonged time in order to achieve the therapeutic goals. While anticancer treatments require prolonged retention of highly concentrated cytotoxic drug levels to maximize antitumor effect, such requirement also poses the risk of systemic toxicity. Consequently, nanoparticle-based drug delivery systems, which can modulate the toxicity profile of anticancer drugs and improve drug circulation, have been widely viewed as a new treatment option for cancer therapeutics [1–3]. Many studies have demonstrated the accumulation of nanocarriers in the abnormal tumor microenvironment through the enhanced permeability and retention (EPR) effect as an advantage of nanoparticle-based drugs [4–6]. To date, approximately 20 therapeutic nanoparticles have received FDA approval for clinical use [2,3].

Liposomes are one of the most popular nanocarriers for delivering many biologically active substances [7]. Based on their ability to encapsulate both hydrophilic and hydrophobic drugs, liposomal formulations of anticancer drugs have been extensively evaluated for treating cancers [6,8,9]. Among the many benefits of liposomal delivery of anthracyclin, e.g., doxorubicin or daunorubicin, compared with the administration of the free drug, is reduced cardiac toxicity with remaining therapeutic efficacy to tumors. However, poor stability and limited drug loading have remained prohibitive obstacles for practical applications. Furthermore, inherent instability of lipid-based carriers in the presence of serum components results in the fast-burst release of the chemotherapeutic drugs, which has limited their utility for the delivery of anticancer agents [9]. To overcome these challenges, a PEGylated liposomal drug formulation was developed by using an ammonium sulfate

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gradient loading procedure, enabling stable drug entrapment into liposomes and their extended blood-circulation time [10]. Notably, the liposome encapsulating doxorubicin (Dox), Doxil/Caelyx, exhibited an improved safety profile by reducing cardiac toxicity and enhancing penetration and accumulation in solid tumors. Consequently, it has been used in the treatment of a wide range of cancers [11–13]. Indeed, such liposomal drug formulations do appear to improve accumulation of liposomes at the tumor site. However, slow and incomplete drug release could still lead to low drug bioavailability within tumor tissue, limiting, in turn, therapeutic activity [14–17]. Furthermore, a lack of controlled-release properties of encapsulated drug may lead to toxic side effects, such as palmar–plantar erythrodysesthesia that is thought to result from unwanted drug distribution to skin during prolonged circulation of liposomal Dox [18], thus requiring further development or improvement in liposomal drug formulation. Attempts were made to improve the drug release rates of Dox by altering liposomal lipid compositions, but this method led to uncontrolled and rapid drug release kinetics, which also lowered therapeutic efficacy [19]. Therefore, a strategy to improve liposome-based anticancer drugs should involve the development of a stable liposomal formulation with improved drug release from the carrier in a controlled and sustained manner, thereby enhancing bioavailability.

In this study, we investigated a previously reported liposomal formulation [20] as an anticancer drug nanocarrier and examined whether it could offer improved drug release, along with stable and sustainable delivery of cancer therapeutics. This liposomal formulation involves the creation of a robust multilamellar structure of the liposome by covalently crosslinking inter-lipid bilayers. As a nanocarrier platform for chemotherapy drug delivery applications, our study demonstrates that these crosslinked multilamellar liposomes (CMLs) can lower systemic toxicity and enhance therapeutic efficacy. In addition, the intracellular trafficking of CMLs was monitored and visualized to provide a better understanding of their delivery mechanisms.

2. Materials and methods

2.1. Cell lines, antibodies, reagents, and mice

B16 tumor cells (B16-F10, ATCC number: CRL-6475) and HeLa cells were maintained in a 5% CO_2 environment with Dulbecco's modified Eagle's medium (DMEM) (Mediatech, Inc., Manassas, VA) supplemented with 10% FBS (Sigma–Aldrich, St. Louis, MO) and 2 mM of L-glutamine (Hyclone Laboratories, Inc., Omaha, NE). The mouse monoclonal antibodies against clathrin, caveolin-1, EEA1, and the rabbit polyclonal antibody specific to trans-Golgi network (TGN38) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The mouse monoclonal antibody to Lamp-1 was purchased from Abcam (Cambridge, MA). Alexa488-goat anti-mouse immunoglobulin G (IgG) and Alexa594-goat anti-rabbit IgG antibodies were purchased from Invitrogen (Carlsbad, CA). Chloropromazine, Nystatin, and MβCD were obtained from Sigma–Aldrich.

All lipids were obtained from NOF Corporation (Japan): 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DOPG), and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[4-(p-maleimidophenyl) butyramide (maleimide-headgroup lipid, MPB-PE). ^{64}Cu was obtained from Washington University (St. Louis, MO) and the University of Wisconsin (Madison, WI). 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) and *N*-hydroxysulfosuccinimide (SNHS) were purchased from Thermo Scientific (Rockford, IL).

Female C57BL/6 mice, 6–10 weeks old, were purchased from Charles River Breeding Laboratories (Wilmington, MA). All mice were held under specific pathogen-reduced conditions in the animal facility of the University of Southern California (USA). All experiments were performed in accordance with the guidelines set by the National Institutes of Health and the University of Southern California on the Care and Use of Animals.

2.2. Synthesis of CMLs, ULs, and DLLs

Liposomes were prepared based on the conventional dehydration-rehydration method. 1.5 μmol of lipids of DOPC, DOPG, and MPB-PE at the molar ratio of the lipid composition of DOPC:DOPG:MPB-PE = 40:10:50, were mixed in chloroform, and the organic solvent in the lipid mixture was evaporated under argon gas and dried under vacuum overnight to form dried thin lipid films. The resultant dried film was hydrated in 10 mM Bis–Tris propane at pH 7.0 containing doxorubicin at a molar ratio of 0.5:1 (drugs:lipids), with vigorous vortexing every 10 min for 1 h, and then applied with 4 cycles of 15-s sonication (Misonix Microson XL2000, Farmingdale, NY) on ice at 1 min intervals for each cycle. To induce divalent-triggered vesicle fusion, $MgCl_2$ was added to make a final concentration of 10 mM. The resulting multilamellar vesicles were further crosslinked by addition of Dithiothreitol (DTT, Sigma–Aldrich) at a final concentration of 1.5 mM for 1 h at 37 °C. The resulting vesicles were collected by centrifugation at 14,000 g (12,300 RPM) for 4 min and then washed twice with PBS. For pegylation of CMLs, the liposomes were further incubated with 1 μmol of 2 kDa mPEG-SH (Laysan Bio Inc., Arab, AL) for 1 h at 37 °C. The particles were then centrifuged and washed twice with PBS. Nonencapsulated doxorubicin was removed by a PD-10 Sephadex gel filtration column, and then the final products were stored in PBS at 4 °C. Similarly, unilamellar liposomes (ULs) were prepared with the same lipid composition through rehydration, vortexing and sonication, as described above, except divalent-induced vesicle fusion and DTT crosslinking processes. The ULs were collected by centrifugation at 250,000 g for 90 min and then washed twice with PBS. Pegylation of ULs was carried out by incubation with 1 μmol of 2 kDa PEG-SH. Doxil-like liposomes (DLLs) were prepared using an ammonium sulfate pH gradient method as described [10]. Briefly, lipid film (HSPC:Cholesterol:DSPE-PEG_{2000} = 56:38:6) was rehydrated with 240 mM of ammonium sulfate buffer pH 5.4 with vigorous vortexing. Small unilamellar vesicles were prepared using sonication and extrusion at 60 °C through 100 nm polycarbonate filters 20 times using a mini-Extruder (Avanti Polar Lipids, Alabaster, AL). The DLLs were collected by centrifugation at 250,000 *g* (45,400 RPM) for 90 min, washed twice with PBS, and then resuspended with HBS pH 7.4 (20 mM HEPES, 150 mM NaCl) containing doxorubicin hydrochloride. The particles were then centrifuged and washed twice with PBS. Nonencapsulated doxorubicin was removed by a PD-10 Sephadex gel filtration column, and then the final products were stored in PBS at 4 °C.

2.3. Characterization of physical properties

The hydrodynamic size and size distribution of CMLs, ULs and DLLs were measured by dynamic light scattering (Wyatt Technology, Santa Barbara, CA). For cryo-electron microscopy imaging, the liposome samples were applied to the grid and plunge-frozen in liquid ethane using the FEI Mark III Vitrobot. CryoEM images were collected using a Tecnai T12 electron microscope (FEI Company) equipped with a Gatan Ultrascan 2k by 2k CCD camera.

2.4. In vitro drug encapsulation, release kinetics, and cytotoxicity

To study the loading capacity of Dox, Dox-loaded CMLs, ULs, or DLLs were collected and then washed twice with PBS, followed by lipid extraction of vesicles with 1% Triton X-100 treatment. Lipid concentrations of liposome suspensions were determined by phosphate assay [21]. Dox fluorescence (excitation 480 nm, emission 590 nm) was then measured by a Shimadzu RF-5301PC spectrofluorometer (Japan). To determine a half-time $t_{1/2}$ whereby 50% of entrapped Dox is released from liposomes, CMLs or ULs were incubated at 37 °C in 10% fetal bovine serum (FBS)-containing media, and the releasing media were collected to measure Dox fluorescence at regular time intervals. To obtain the release kinetics of Dox from liposomes, Dox-loaded CMLs, ULs or DLLs were incubated at 37 °C in 10% fetal bovine serum (FBS)-containing media, the releasing media were removed from CMLs, ULs, or DLLs incubated at 37 °C for quantification of Dox fluorescence every day, and fresh media were replaced for continuous monitoring of drug release.

B16 or HeLa cells were plated at a density of 5×10^3 cells per well in D10 media in 96-well plates and grown for 6 h. The cells were then exposed to a series of concentrations of Dox-loaded CMLs, ULs or DLLs for 48 h, and the cytotoxicity of Dox-liposomes was assessed using the Cell Proliferation Kit II (XTT assay) from Roche Applied Science, according to the manufacturer's instructions.

2.5. Confocal imaging

To label liposome particles with DiD lipophilic dyes, DiD dyes were added to the lipid mixture in chloroform at a ratio of 0.01:1 (DiD:lipids), and the organic solvent in the lipid mixture was evaporated under argon gas to incorporate DiD dyes into a lipid bilayer of vesicles. For the colocalization study with endocytic markers, HeLa cells were seeded on glass bottom dishes (MatTek Corporation, Ashland, MA) and grown at 37 °C overnight. The cells were then incubated with DiD-labeled CML particles for 30 min at 4 °C to synchronize internalization. After washing with PBS, the treated cells were then warmed to 37 °C to initiate particle internalization for the indicated time periods. The cells were fixed, permeabilized with 0.1% Triton X-100, and then immunostained with the corresponding antibodies specific to clathrin, caveolin-1, EEA1, TGN38, or Lamp-1 and counterstained with DAPI (Invitrogen).

Fluorescence images were acquired on a Yokogawa spinning-disk confocal scanner system (Solamere Technology Group, Salt Lake City, UT) using a Nikon eclipse Ti-E microscope equipped with a 60×/1.49 Apo TIRF oil objective and a Cascade II: 512 EMCCD camera (Photometrics, Tucson, AZ, USA). Image processing and data analysis were carried out using the Nikon NIS-Elements software. To