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Hyper-cell-permeable micelles as a drug delivery carrier for effective cancer therapy

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ABSTRACT

Although PEGylated liposomes (PEG-LS) have been intensively studied as drug-delivery vehicles, the rigidity and the hydrophilic PEG corona of liposomal membranes often limits cellular uptake, resulting in insufficient drug delivery to target cells. Thus, it is necessary to develop a new type of lipid-based self-assembled nanoparticles capable of enhanced cellular uptake, tissue penetration, and drug release than conventional PEGylated liposomes. Herein, we describe a simple modification of bicellar formulation in which the addition of a PEGylated phospholipid produced a dramatic physicochemical change in morphology, i.e., the disc-shaped bicelle became a uniformly distributed ultra-small (~12 nm) spherical micelle. The transformed lipid-based nanoparticles, which we termed hyper-cell-permeable micelles (HCPMi), demonstrated not only prolonged stability in serum but also superior cellular and tumoral uptake compared to a conventional PEGylated liposomal system (PEG-LS). In addition, HCPMi showed rapid cellular uptake and subsequent cargo release into the cytoplasm of cancer cells. Cells treated with HCPMi loaded with docetaxel (DTX) had an IC_{50} value of 0.16 μM, compared with 0.78 μM with PEG-LS loaded with DTX, a nearly five-fold decrease in cell viability, indicating excellent efficiency in HCPMi uptake and release. *In vivo* tumor imaging analysis indicated that HCPMi penetrated deep into the tumor core and achieved greater uptake than PEG-LS. Results of HCPMi (DTX) treatment of allograft and xenograft mice *in vivo* showed high tumoral uptake and appreciable tumor retardation, with ~70% tumor weight reduction in the SCC-7 allograft model. Taken together, these findings indicate that HCPMi could be developed further as a highly competent lipid-based drug-delivery system.

1. Introduction

Lipid-based drug delivery vehicles such as liposomes and micelles have been widely used for various biomedical applications, including cancer therapy [1–3]. The ability of lipids to self-assemble into diverse nanostructures in aqueous medium makes them attractive drug-delivery carriers [4,5]. In addition, diverse nanostructures can be formed depending on the composition and ratios of lipids used [6,7]. Furthermore, the efficiency of cellular uptake of lipid-based nanoparticles depends on the types of lipid used and the nanostructures formed therefrom [8,9]. Among lipid-based nanoparticles, PEGylated liposomes have been intensively used and their potential as drug-delivery vehicles demonstrated in preclinical and clinical trials; they have the ability to encapsulate diverse drugs (including hydrophilic and hydrophobic molecules) with relatively high efficiency and to circulate for long periods in the bloodstream [10–16]. However, the rigidity of liposomal membranes often limits drug release, and the hydrophilic PEG corona of the liposomes can hinder cellular uptake, resulting in insufficient drug delivery to target cells [17,18]. In addition, the larger size of liposomes hinders deep penetration of nanoparticles into the deeper core of tumor, yielding lower anti-tumor efficacy compared to smaller-sized micelles [19,20]. Thus, it is necessary to develop a new type of lipid-based self-assembled nanoparticles

Abbreviations: HCPMi, hyper-cell-permeable micelles; PEG, poly-ethylene glycol; PEG-LS, PEGylated liposomes; DTX, docetaxel; BC, bicelle.

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capable of greater cellular uptake and tissue penetration than conventional PEGylated liposomes, while retaining the benefits of lipid-based nanoformulations. Recently, bicelles, or disc-shaped nanostructures formed by self-assembly of a certain ratio of long-chain and short-chain phospholipids, have demonstrated superior transdermal drug delivery compared to other lipid-based nanoparticles such as liposomes or solid lipid nanoparticles [21–24]. Those findings suggest that the lipid compositions of bicelles are highly suitable for enhanced cellular uptake and penetration. Despite these favorable attributes, however, bicelles have not been used as drug-delivery carriers for systemic administration, as they are unstable, become aggregated shortly after injection, and are cleared from circulation due to lack of any "stealth" coating [25–27]. Herein, we report that the addition of a PEGylated phospholipid in the range of 4–10 wt % dramatically transformed bicelles from disc-like to uniform spherical structures, creating a nanocarrier system that is stable in serum, with rapid cellular uptake *in vitro* and deep tumor penetration and retention properties *in vivo*. The transformed lipid-based nanoparticles, which we termed hyper-cell-permeable micelles (HCPMi), demonstrated not only prolonged stability in serum but also superior cellular and tumor uptake compared to a conventional PEGylated liposomal system (PEG-LS). To evaluate the feasibility of HCPMi as a drug-delivery system, we chose to use the hydrophobic drug docetaxel (DTX), a microtubule inhibitor widely used in the treatment of various human malignancies [28]. DTX-loaded HCPMi showed higher toxicity *in vitro* and *in vivo* compared to PEG-LS and in mice elicited less toxicity than the parental drug DTX. We envision a wide variety of applications for a new class of HCPMi lipid nanoparticles in drug delivery, notably with hard-to-transfect cells.

2. Materials and methods

2.1. Materials

1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1-stearoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (ammonium salt) (Rh-DMPE), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (ammonium salt) (Rh-DSPE), 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (ammonium salt) (DMPE-PEG$_{2000}$), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG$_{2000}$) and mini-extrusion set were purchased from Avanti Polar Lipids (Alabama, USA). Uranyl acetate and docetaxel were purchased from Sigma-Aldrich (MA, USA). Staining solution with DAPI was purchased from Vectashield (MI, USA). All chemicals and reagents were purchased from Sigma-Aldrich unless stated otherwise. All animal experiments were performed according to the rules and regulations of animal care and handling procedures of the Korea Advanced Institute of Science and Technology (KAIST).

2.2. Preparation of bicelles (BC), hyper-cell-permeable micelles (HCPMi), and PEGylated liposomes (PEG-LS)

Conventional bicelles were formed with DMPC as the long-chain lipid and DHPC as the short-chain lipid, with a fixed **q** ratio of 3:1 (DMPC: DHPC) [24]. DMPC and DHPC were purchased in chloroform solution and mixed in a glass vial to obtain DMPC/DHPC at a final lipid concentration of 2 mg/mL. Chloroform was eliminated by a desiccator and the solution further dried in a lyophilizer to remove any traces. The lipid film was then re-hydrated with 1 mL phosphate-buffered saline (PBS) under constant stirring at ambient temperature. HCPMi was similarly formulated, with the addition of DMPE-PEG$_{2000}$ (2, 4, 6, 8 and10 wt %) in separate vials to form five distinct HCPMi. PEG-LS was formulated according to our previous publication with a slight modification [15], whereby DSPE-PEG$_{2000}$ was added to the composition at 10 wt % for parallel comparison with HCPMi. Re-hydration for liposome is similar to that for BC and HCPMi, with the addition of extrusion as the final step to produce liposomes of uniform size. Extrusion was performed with a hand-held mini extruder with a 100-nm polycarbonate membrane. The PEG-LS solution was extruded through the membrane at least 11 times for formation of 100-nm liposomes with narrow size distribution. Rhodamine-labeled BC and HCPMi were made with the addition of 0.5 wt % of Rhodamine-DMPE. The Rhodamine-labeled PEG-LS was made with the addition of 0.5 wt % of Rhodamine-DSPE. All formulations were then normalized for fluorescence intensity using a fluorospectrometer.

2.3. Dynamic light scattering (DLS) and zeta potential analysis

To determine the size and zeta potential of HCPMi and PEG-LS, 2 mg/ml solution of each formulation was made and transferred into transparent cuvettes, with care to avoid formation of bubbles. Their hydrodynamic diameter and zeta potentials were measured using the Zetasizer nano ZS90 (Malvern Instruments, UK). In addition, all HCPMi formulations with various PEG wt. % (2, 4, 6, 8 and 20%) and PEG-LS were characterized for their size and zeta potential, as recorded in Table S1.

2.4. Transmission electron microscopy (TEM) analysis

For TEM analysis, 10-μL aliquots of BC, HCPMi, and PEG-LS particles were applied to TEM-grade carbon-only mesh copper grids. Particles were left on the grid for at least 5 min at ambient temperature before filter paper was used to blot and remove excess sample. Each grid was washed five times with distilled water. The specimens were then negatively stained by applying 10 μL of 1% uranyl acetate and left at ambient temperature for 2 min. Washing steps were repeated three times with distilled water, then the specimens were left to dry and visualized by transmission electron microscopy (TEM) using a TECNAI F20 electron microscope (Philips Electronic Instruments, Inc., Mahwah, NJ) operated at 200 kV.

2.5. In vitro stability assay

Solutions of 2 mg/mL HCPMi and PEG-LS (900 μL each) were transferred to transparent DLS cuvettes. Fetal bovine serum (Gibco, 100 μL) was added to both cuvettes to obtain a final concentration of 10% serum and sealed with air-tight cap and parafilm to avoid contamination during incubation. The cuvettes were kept in a shaking incubator with constant temperature of 37 °C. The sizes of HCPMi and PEG-LS were recorded using DLS at the following time points: 0, 1, 2, 4, 8, 24, 48, 36, and 72 h.

2.6. In vitro cellular uptake

For cellular uptake experiments, Rhodamine-labeled HCPMi, and PEG-LS were used to visualize uptake via confocal microscope. SCC-7 and BxPC3 cells were grown to confluence on sterilized coverslips and then treated with either HCPMi or PEG-LS at a concentration of 100 μg/mL (after fluorophore normalization) for 1 h at 37 °C. Cells were then washed with PBS, fixed with 4% paraformaldehyde (PFA), and mounted on glass slides with Vectashield Hardset mounting medium with DAPI for imaging analysis using an Olympus Fluoview 1000 confocal microscope (Olympus