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Microfluidic self-assembly of a combinatorial library of single- and dual-ligand liposomes for *in vitro* and *in vivo* tumor targeting

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ABSTRACT

Precise engineering of nanoparticles with systematically varied properties (size, charge surface properties, targeting ligands, etc.) remains a challenge, limiting the effective optimization of nanoparticles for particular applications. Herein we report a single-step microfluidic combinatorial approach for producing a library of single and dual-ligand liposomes with systematically-varied properties including size, zeta potential, targeting ligand, ligand density, and ligand ratio. A targeting ligand folic acid and a cell penetrating peptide TAT were employed to achieve the optimal synergistic targeting effect. In 2D cell monolayer models, the single-ligand folic acid modified liposome didn't show any enhanced cellular uptake, while the incorporation of TAT peptide "switched on" the function of folic acid, and induced significant elevated cellular uptake compared to the single ligand modified liposomes, showing a strong synergistic targeting effect. The folic acid and TAT peptide dual-ligand liposome also demonstrated enhanced tumor penetration as observed using 3D tumor spheroid models. The *in vivo* study further confirmed the improved tumor targeting and longer tumor retention (up to 72 h) of the dual-ligand liposomes. Our work not only proved the versatility of this microfluidic combinatorial approach in producing libraries of multifunctional liposomes with controlled properties but also revealed the great potential of the optimized liposome formulation for synergistic targeting effects.

1. Introduction

Liposomes are spherical vesicles with an amphiphilic lipid bilayer enclosing an aqueous core [1,2]. Due to the advantages such as good biocompatibility and good stability, liposomes have gained an intense interest in various fields ranging from vaccine, drug delivery to diagnostics and imaging. From the first FDA-approved nano-drug Doxil [3] to those latest liposome formulations under clinical development [4,5], we have witnessed significant advances in developing lipid-based drug delivery systems for cancer diagnosis and treatment over the past decades. Recently, a lot of effort has been made to develop multifunctional liposomes [6–8]. Through a synergistic manner, these liposomes might address two or more challenges at the same time, such as overcoming multiple barriers to deliver drugs to specific sites, enhancing targeting specificity or increasing circulation time and tumor retention capability.

To achieve desired biological functions, liposomes are usually prepared with some specific ligands. Their biological function can be further enhanced by incorporating two or more ligands at the same time, such as using two targeting ligands or using an active targeting ligand and a cell-penetrating peptide. Ying et al. [9] developed dual-ligand liposomes using two active targeting ligands for transporting the drug across the blood-brain barrier and then targeting brain glioma. Takara et al. [10] used a specific ligand NGR and a cell-penetrating peptide oligoarginine to improve the cellular uptake efficiency through a synergistic effect. These dual-ligand liposomes [11] are promising candidates to achieve advanced properties and functions such as triggered release, better tumor targeting, and more efficient drug delivery. However, traditional methods for preparing liposomes are mainly based on bulk methods such as thin-film hydration, freeze-drying, detergent depletion and alcohol injection [12]. These methods always require post-processing steps to homogenize the size, thus often resulting in big batch-to-batch variations and poor quality control. To prepare more complex multifunctional liposomes, traditional methods might require further steps to conjugate or incorporate functional groups into the liposomes [13–15], thus leading to increased difficulties in controlling

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the properties and reproducibility of such liposomes, as well as the associated high manufacture cost and time consumed. In addition, as there are so many factors which can affect the *in vitro* and *in vivo* properties of multifunctional liposomes, it is essential to prepare a library of liposomes with varied compositions, thus allowing the systematic screening and evaluation. Therefore, it is critical to developing a new platform technology for investigating multifunctional liposomes in a simple, efficient, and reproducible way.

Microfluidic technology is a newly emerging method for the preparation of various nanoparticles due to its better manipulation of the synthesis process [16–18]. To prepare multifunctional liposomes in a single step, a microfluidic hydrodynamic flow focusing (HFF) approach has been developed [19,20]. This method can provide a well-controlled mixing of the organic solvent which contains the lipids and the aqueous buffer in the microfluidic device, thus precisely controlling the properties of the liposomes such as size, charge, and surface chemistry [21]. The method also allows combinatorial synthesis of libraries of liposomes with systematically-varied properties, which has been considered as an effective way to discover new drugs through high-throughput screening in the pharmaceutical industry. Moreover, this "micro-" preparation process can be easily scaled-up by using high aspect ratio or parallel integrated microfluidic devices [22,23], which is suitable for massive clinical applications and industrial production.

In this study, we used the hydrodynamic flow focusing method to generate a library of liposomes with varied properties, including different particle size, single ligand, dual-ligand and different ligand densities in a one-step manner. We employed a targeting ligand folic acid (FA) for active tumor targeting and a cell penetrating peptide (TAT) for efficient cell membrane translocation of the liposomes. Their biological functions were evaluated using two-dimensional (2D) cell monolayer, three-dimensional (3D) tumor spheroid models, and a tumor-bearing mouse model. The work offers a new strategy for preparing libraries of multifunctional liposomes, and the screening of them via various models allows the identification of the best formulation with the optimal biological functions. This strategy could pave the way for the liposome-based pharmaceutical applications.

2. Materials and methods

2.1. Materials

TAT peptide with a terminated cysteine (Cys-TAT, CYGRKKRRQRRR, MW 1663) was synthesized by GenScript Corporation (Piscataway, NJ, USA). 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG_{2000}) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[folate(polyethylene glycol)-2000] (DSPE-PEG_{2000}-Folate) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000] (DSPE-PEG_{2000}-Mal) were obtained from Nanocs (New York, NY, USA). Cholesterol (CHO), histology mounting medium and Accumax solution were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mouse anti-human folate binding protein antibody was purchased from Abcam (Cambridge, UK). Alexa Fluor® 488 conjugate goat anti-mouse IgG secondary antibody, Hoechst 33342 and Alexa Fluor® 647 Phalloidin were purchased from Invitrogen (Carlsbad, CA, USA). Other chemicals and reagents were of analytical grade.

2.2. Synthesis of DSPE-PEG_{2000}-TAT

DSPE-PEG_{2000}-TAT was synthesized by conjugating TAT to the DSPE-PEG_{2000}-Mal through the Michael reaction. Briefly, DSPE-PEG_{2000}-Mal and Cys-TAT (molar ratio = 1:1.5) were mixed in chloroform/methanol (v/v = 2:1), and triethylamine was added as the catalyst. The reaction mixture was protected by nitrogen and then stirred in dark at room temperature for 24 h. The reaction process was monitored by thin-layer chromatography (TLC). After the DSPE-PEG_{2000}-Mal disappeared based on the TLC, the reaction mixture was evaporated by a rotary evaporation under vacuum. The residue was redissolved in chloroform and filtrated to remove the excess TAT peptide. The purified product was stored at -20 °C. ^{1}H NMR and MALDI-TOF mass spectrometry were used to verify the successful synthesis of the product.

2.3. Preparation of liposomes

To prepare PEG-Lip (55 mol% DMPC, 40 mol% CHO and 5 mol% DEPE-PEG_{2000}), FA-Lip (55 mol% DMPC, 40 mol% CHO, 4 mol% DEPE-PEG_{2000} and 1 mol% DSPE-PEG_{2000}-FA) , TAT-Lip (55 mol% DMPC, 40 mol% CHO, 4 mol% DEPE-PEG_{2000} and 1 mol% DSPE-PEG_{2000}-TAT) and FA-TAT-Lip (55 mol% DMPC, 40 mol% CHO, 3 mol% DEPE-PEG_{2000}, 1 mol% DSPE-PEG_{2000}-FA and 1 mol% DSPE-PEG_{2000}-TAT), different lipid materials were dissolved in $CHCl_3$. After mixing, the chloroform was removed by the rotary evaporator and the lipid film was then re-dissolved in anhydrous isopropanol (IPA). For the fluorescence-labeled liposomes, DiI or DiR was added to the IPA solution together with the lipids at a density of 5 μg and 50 μg/mL, respectively. The final lipid concentration was adjusted to 2.5 mM. For some cell experiments, liposomes with different single- and dual-ligand densities were also prepared, such as 0.5 mol% FA modified liposomes (0.5% FA) or 0.5 mol% FA and 1 mol% DSPE-PEG_{2000}-TAT modified liposomes (0.5% FA 1%TAT) which will be explained in the relevant figure captions.

Liposomes were formed by injecting the lipids into the microfluidic device where this lipids stream was intersected by PBS buffer from side channels. A total flow rate of 28.8 μL/min (corresponding to 0.4 m/s) was used, and flow rate ratios were varied from 3:1 to 16:1 for liposome preparation. The preparation process was conducted at room temperature.

2.4. Characterisation of liposomes

2.4.1. Dynamic light scattering (DLS)

The size, polydispersity index (PDI) and zeta-potential of the liposomes synthesized were determined using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK).

2.4.2. Transmission electron microscopy (TEM)

The morphology of FA-TAT-Lip was observed using a transmission electron microscopy (Jeol, Tokyo, Japan). Samples were dropped onto a copper TEM grid with a carbon film and air-dried at room temperature followed by negative-staining using 1% uranyl acetate.

2.4.3. In vitro stability in fetal bovine serum (FBS)

Liposomes in 96 well plate were incubated with an equal volume of FBS at 37 °C. At different time points (0, 1, 2, 4, 8, 12, 24, 48 h), the absorbance (A) at 680 nm was measured using a microplate reader. The relative turbidity at 0 h was set to be 1 and at other time points was calculated as $A_{sample}/A_{0\ h.}$

2.4.4. Quantification of FA on liposome surface

The amount of FA was quantified using a fluorescence spectrophotometer. FA-Lip or FA-TAT-Lip were concentrated using centrifugal filters (4000*g*, 10 min, 25 °C) to about 200 μL followed by the addition of the same volume of 1% Triton X-100 to de-emulsify the liposomes. Then the absorbance at 380 nm was recorded by UV–VIS. The amount of FA was calculated using a standard curve. The FA incorporation efficiency (FA IE) was determined as follows:

$$FA\ \ incorporation\ \ efficiency = \frac{The\ \ FA\ \ amount\ \ determined\ \ by\ \ spectrophotometer}{The\ \ theoretical\ \ FA\ \ amount}$$