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Inhomogeneous crystal grain formation in DPPC-DSPC based thermosensitive liposomes determines content release kinetics

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ABSTRACT

Thermosensitive liposomes (TSL) receive attention due to their rapid externally controlled drug release at transition temperature in combination with hyperthermia. This rapid release feature of TSL occurs when the liposome membrane is going through a phase change which results in numerous interfaces, at so-called crystal grain boundaries. Based on experience with TSLs, our group found that thermosensitive liposomes formulated by binary compositions of DPPC and DSPC at proper ratios are able to exhibit rapid release without incorporation of release-promoting components. The aim of this study was to understand the mechanism of rapid release from bi-component DPPC-DSPC based TSL. Based on the investigation of a series of TSLs formulated by different DPPC-DSPC ratios, and through the analysis of binary-phase diagrams of DPPC-DSPC TSLs, we conclude that inhomogeneous crystal grains are formed in bi-component TSL membranes rather than mono-component, thereby facilitating content release. The resulting inhomogeneous membrane pattern is affected by DPPC/DSPC ratio, i.e. this determines the number of interfaces between solid and liquid phases at transition temperature, which can be diminished by addition of cholesterol. At appropriate DPPC/DSPC ratio, substantive solid/liquid interfaces can be generated not only between membrane domains but also between crystal grains in each domain of the liposome membranes, therefore improving content release from the TSL at transition temperatures.

1. Introduction

Nanoparticle-mediated chemotherapy offers several advantages in tumor treatment, including reduced side-effects, prolonged circulation time and possibly improved intratumoral drug accumulation due to the enhanced permeability and retention (EPR) effect [1]. Especially lipid-based particles, liposomes, are successfully developed of which Doxil®/Caelyx® is one of most well-known and widely used. However, application of nanoparticles also introduces drawbacks, such as failure to adequately penetrate tumors [2]. The EPR effect is influenced by tumor microenvironment, tumor type and profile of nanoparticle, which all may hinder an optimal therapeutic effect of most conventional, passively-delivered liposomal formulations [3,4]. Important, and the key explanation for failure of Doxil® to surpass doxorubicin, is the slow drug release from the liposome, which limits therapeutic efficacy in spite of strikingly increased circulation time [5]. Hence, to obtain high local levels of free and bioavailable drug actively triggered release of encapsulated drug at the diseased site is a pursued possibility. One approach for local delivery is to use thermosensitive liposomes (TSL) and local hyperthermia (HT), in which the drug is rapid intravascularly released in the heated area, subsequently followed by massive uptake by tumor cells due to high concentration gradients.

The concept of thermosensitive liposomes was first introduced by Yatvin et al. [6], reporting a TSL formed by 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) alone or with 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), which generates content release at a phase transition temperature around 42 °C. Nevertheless, these TSL relatively slowly release their content limiting further application [7]. To enhance release from TSL, Needham et al. improved TSL composition by

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incorporating lysolipid (LPC) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-methoxy(PEG)-2000 (DSPE-PEG2000) in DPPC-based formulations. These LPC-containing TSLs show over 80% release in a matter of seconds at around 41 °C, achieving a rapid release profile necessary for intravascular delivery [8,9]. Currently, several different thermosensitive liposomal formulations have been reported [10].

The principle of TSL release is generally thought to result from phase separation at Tm causing interfaces or gaps in the bilayer enabling content release [10]. Ickenstein et al. proposed that lipids solidify into gel-phase domains in the membrane during cooling, and boundaries appear at adjacent domains due to spherical bending force [11]. Because of a high degree of disordered lipid-arrangement in domain boundaries, these regions possess lower melting points. This causes prior phase transition at domain boundaries, thus generating interfaces between gel/liquid-crystalline phases, which are in turn responsible for release of content [11,12]. Surfactant lysolipids tend to migrate to phase interfaces and form micelle-structures at phase transition, thus inducing nano-pores in membranes, which can be stabilized by PEG-linked lipids. Together they increase and enlarge the interfaces inflicting more rapid release [9,13]. Based on the same principle, Tagami et al. added Brij surfactants into DPPC-based TSL, which exerts comparable fast release in response to hyperthermia [14].

Most thermosensitive liposomes are formulated on the initially proposed matrix composed of DPPC and DSPC phospholipids [15–18]. Especially, in our group we have been working on DPPC-DSPC based thermosensitive liposomes for years and developed several PEG-DSPE-modified DPPC-DSPC based TSLs loaded with different drugs, showing desired temperature response [19–22]. In the follow-up study, we observed that TSLs formulated at proper DPPC/DSPC ratios exhibit rapid release at transition temperatures. However, this fast release is likely not explained by the defect mechanism of Ickenstein [11], and does not result from the nano-pore effect seen with lysolipid-based TSL as proposed by Needham et al. [9]. We speculate that apart from boundaries between individual domains as defective regions in membranes, other release regions and factors exist that influence content release from DPPC-DSPC based TSLs at transition temperatures. Therefore, in this study we designed DPPC-DSPC based TSLs, investigated rapid release at certain DPPC/DSPC ratios during phase transition, and elucidated the principle to achieve an optimal heat-triggered release DPPC-DSPC based liposome system.

2. Materials and methods

2.1. Chemicals and agents

1,2-Dipalmitoyl-sn-glycero-3-phosphcholine (DPPC), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-PEG_{2000} (DSPE-PEG) were provided by Lipoid (Ludwigshafen, Germany). Purified carboxyfluorescein (CF) was kindly provided by Dr. Lars Lindner and colleagues. PD-10 columns were obtained from GE Healthcare (UK). Cholesterol and other chemicals were purchased from Sigma Aldrich unless otherwise specified.

2.2. Preparation of liposomes

TSLs were composed of DPPC/DSPC/DSPE-PEG in a molar ratio of x / (100 − x) / 5 (x = 100, 80, 60, 40, 20, 0) by using the thin lipid film hydration method, followed by heated extrusion [19]. Briefly, 100 μmol of lipids was dissolved in methanol/chloroform (1/9 v/v) mixed solvent which was then evaporated at 40 °C, followed by nitrogen flush for 30 min to remove residual solvent. The resulting dried lipid film was hydrated with CF (100 mM, pH 7.4) solutions at 60 °C. Small unilamellar vesicles were obtained by extrusion through Nuclepore® (Whatman Inc., USA) filters with pore size of 100 nm on a Thermobarrel extruder at 65 °C (Northern Lipids, Canada). Unencapsulated CF was removed with a PD-10 column. Diameter (*Z*-average) and polydispersity index (PDI) were measured by using Zetasizer Nano-ZS (Malvern Instruments Ltd., UK).

2.3. Differential scanning calorimetry

Determination of TSL phase transition temperatures was done through differential scanning calorimetry (DSC) (NETZSCH Scientific Instruments Ltd. DSC200F). Six DPPC-DSPC based formulations were prepared as mentioned in Section 2.1 with or without CF loading. 30 mg of liposome with/without encapsulated CF in fetal calf serum (FCS) or in HEPES solution (pH 7.4), and the appropriate reference solution (HEPES solution), were added to the sealed aluminum container. The phase transition temperature range was measured over a temperature range of 30 to 70 °C at an interval of 5 °C/min increase. High purity nitrogen was used as carrier gas at rate of 10 ml/min.

2.4. CF-loaded TSL time- and temperature-dependent release

20 μl of 1 mM [lipid] CF-TSL suspension was added to 2 ml 100% FCS in a quartz cuvette at a series of determined temperature for 10 min. Real-time release of CF was detected with a water bath combined spectrofluorimetry (Ex. 493 nm/Em. 517 nm, Ex. slit 5 nm/Em. slit 5 nm) (Hitachi F-4500 Fluorescence Spectrophotometer, Japan). The average fluorescence intensity of the initial 5 s was recorded as I_0 of CF-TSL release, while fluorescence was measured as I_t at 10 min. After 10 min, detergent (10% Triton X-100) was used to disrupt all liposomes to measure maximal CF fluorescence, which was recorded as I_{max}. Release (%) = $(I_t - I_0) / (I_{max} - I_0) \times 100$.

2.5. Thermokinetic release of CF-loaded TSL

Time-dependent CF release curves obtained from Section 2.4, were fitted using three most common kinetic models (which are zero order, first order and Higuchi equations, respectively, see below), to determine the best-fitting profile of release kinetics and corresponding release rate [23].

$$\text{Zero order}: M_{t.} = M_0 + k_0\, t$$

$$\text{First order}: \ln(1 - M_t) = M_0 - k_1\, t$$

$$\text{Higuchi}: M_{t.} = M_0 + k_h t^{1/2}$$

where M_t is the amount of content released at time t. M_0 is the initial amount of release at time = 0. k_0, k_1 and k_h represent the release rate constant of zero-order, first-order and Higuchi, respectively. Here, M_t

Table 1
Characterization parameters of DPPC-DSPC based CF TSLs. Mean ± SD, $N \geq 3$.

TSL composition (mole)	Particle size (nm) (*Z*-average)[a]	Polydispersity index
DPPC/DSPE-PEG 100/5 (TSL 100)	117 ± 5	0.07 ± 0.01
DPPC/DSPC/DSPE-PEG 80/20/5 (TSL 80)	119 ± 3	0.05 ± 0.03
DPPC/DSPC/DSPE-PEG 60/40/5 (TSL 60)	113 ± 2	0.07 ± 0.02
DPPC/DSPC/DSPE-PEG 40/60/5 (TSL 40)	120 ± 4	0.04 ± 0.01
DPPC/DSPC/DSPE-PEG 20/80/5 (TSL 20)	115 ± 3	0.05 ± 0.02
DSPC/DSPE-PEG 100/5 (TSL 0)	119 ± 6	0.06 ± 0.02

[a] The *Z*-average of particle was reported by Zetasizer, which was measured based on Comulant model.