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Loading drugs into liposomes by temperature up-down cycle procedure with controllable results fitting prediction by mathematical and thermodynamic process

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ABSTRACT

Liposomes are a useful carrier for delivering drugs but rarely make a poorly water-soluble drug (PWSD) realize its therapeutic potential. A key barrier lies in that, by conventional methods, PWSD is mainly loaded just in liposome bilayer membranes, which rarely provide sufficient room to accommodate drugs satisfying clinical therapy. In this investigation, a novel procedure of temperature up-down cycle (TUDC) was developed for loading PWSDs into the liposome interiors instead of bilayer membranes to hold enough agents. In particular, the TUDC procedure renders PWSDs such as curcumin (Cur) entrapment purposely controllable, as evidenced by the encapsulation efficiency (EE) of Cur varies nearly from 0% to 100% in response to changes the determinant factors of the procedure. In addition, several mathematical equations that could calculadoxorubicin
te the loading efficiency by TUDC were established and proved, when combined with thermodynamic process, able to successfully predict the loading results through including thermodynamic parameters, such as temperature and deduced drug solubility, thus remarkably cutting down the laborious experiments and enhancing liposome development efficiency. Cryo- TEM, SAXS, XRD and DSC tests proved that TUDC is feasible to load a PWSD into PEG-liposomes but rendering the drug in the amorphous state. Thus, the novel TUDC procedure and the established mathematical and ther-modynamic process may provide a useful tool to promote the development of liposome products.

1. Introduction

Liposomes are a useful carrier for delivering drugs since as a DDS (drug delivery system) they possess numerous advantages, such as diverse encapsulation for various drugs, versatile biodistribution- and pharmacokinetic-directed surface modifications, and high safety profiles [1–3]. Notably, decades of efforts have conquered many obstacles to preparing drug-delivering liposomes and paved the way for making clinical products, such as Active Ingredient®, Marqibo® and Shingrix® [4–8], as well as numerous vaccines which are thought the final game-changing tool to end the present devastating COVID-19 pandemic [9].

Pegylation, modification of an entity with a PEG (polyethylene glycol) polymer to stabilize particulate entities in vitro and in vivo, is regarded as one of the milestone achievements in liposome field [10,11,12]. Other key achievements in liposome field include the efficient liposome preparation and drug-loading procedures, such as thin lipid film dispersion, reverse phase evaporation, dehydration-rehydration, freeze-thawing as well as ethanol-injection microfluidic techniques [13–17]. In particular, the transmembrane pH gradient drug loading method, which is inspired to explore by the initial observation of the proton-induced non-equivalent transmembrane distribution of agents in liposomes by Deamer's group [16,17]. The method was later successfully developed by Cullis and coworkers for encapsulation of anticancer drugs in the mid-1980s [18] and further adapted as transmembrane NH_4^+ (ammonium sulfate) gradient method by Barenholz's group for loading Active Ingredient into liposomes, contributing significantly to industrial production of Active Ingredient® (marketed PEG-liposomal Active Ingredient) [18-20].

However, transmembrane ion gradient method is only applicable to the amphiphilic molecules that may exist at a high concentration in an

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aqueous medium, especially, at an elevated temperature [21]. Yet, it does not work on encapsulation of the poorly water-soluble drugs (PWSDs) and non-dissociating drugs, which also represent a high proportion of therapeutic agents [22]. Generally, PWSDs have a hydrophobic or lipophilic property and therefore, by conventional methods, are loaded into liposomes, not in the aqueous interiors, but in the bilayer membranes [5], which, unfortunately, have too little room to hold drugs with the sufficient amount for therapy. Moreover, at present, the PWSD-carried liposomes are, in most cases, made with formulation optimization through "blind" experimental tests, because the crucial factors influencing encapsulation often remain unknown and are often sought by arduous "trial and error" processes [6]. Therefore, it is desirable to develop a method capable of entrapping enough PWSDs in liposomes, and better, with a theoretical prediction on EE based on mathematical calculation of the influencing elements [4]. Realization of this can remarkably enhance the development efficiency of liposome products [23].

This report describes a novel procedure termed the temperature up-down cycle (TUDC) that is capable of entrapping PWSDs in the aqueous interior of liposomes. Curcumin (Cur) is a polyphenol compound of (1E,6E)-1,7-bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione with a molecular weight (MW) of 368.38 Da (Fig. 1) [24]. Cur shows a versatile biological activity and has been formulated as nanocarrier-based therapeutic medicine against diseases including cancer [25]. Cur is a light-sensitive but thermostable PWSD [26], whose solubility increases proportionally with temperature [27], was therefore taken as a model drug. Experimental results demonstrated that TUDC procedure renders drug entrapment in liposome interiors purposely controllable, as evidenced by the encapsulation efficiency (EE) of liposomes for a PWSD Cur which varies nearly from 0% to 100% in response to changes made to the determinant factors of the TUDC procedure. In addition, several mathematical equations that could calculate the drug entrapment by TUDC procedure were established and further proved, when combined with thermodynamic process, able to successfully and conveniently predict the loading results through including the thermodynamic parameters. Thus, the novel procedure of TUDC and the established mathematical and thermodynamic process provide an alternative useful tool to promote liposome development.

2. Materials and methods

2.1. Materials

Curcumin (95% purity) was generously provided by Shijiazhuang Pharmaceutical (Shijiazhuang, China). EPC (egg phosphatidylcholine, a MW of 770) and DSPE-PEG$_{2000}$ (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-(amino(polyethylene glycol)$_{2000}$) (ammonium salt), an average MW of 2805) were purchased from Avanti Polar Lipids (Alabama, USA). High performance liquid chromatography (HPLC) grade methanol was purchased from Tianjin Shield Fine Chemicals Co., Ltd. (Tianjin, China). Other reagents and chemicals were of analytical grade and provided by local merchants. Pure water was self-made using Ultrapurified Milli-Q water apparatus (Millipore Merck, Shanghai, China).

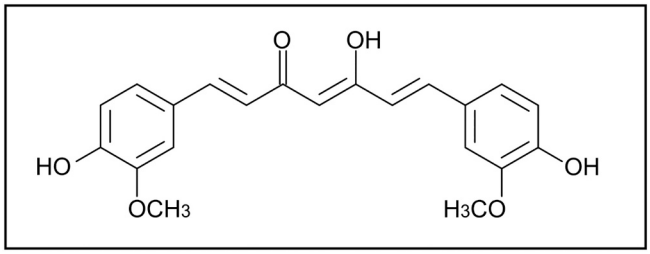

Fig. 1. Molecular structure of curcumin.

2.2. Determination of Cur solubility in water at different temperatures

The aqueous solubility of Cur was measured by separation of saturated solution using a 10-mL jacketed extruder (Jacketed Extruder GJE-10 mL, Genizer LLC, Los Angeles, USA). Briefly, an excess amount of Cur was put in a 10-mL extruder which was subsequently added with 5 mL distilled water. Then the extruder was filled with pure N_2 gas and stirred at 120 rpm (rounds per min) for 24 h with a mini electromagnetic stirrer, meanwhile the extruder was kept at the predetermined temperature (0, 15, 25, 35 or 50 °C) by continuously circulating water through the jacketed hollow cavity using a constant temperature water-circulating tank. After that, the saturated solution was driven out by N_2 gas at an elevated pressure and was collected, after discarding the initial 0.5 mL filtrate, in a glass tube. An accurate volume of the collected solution was removed of water by N_2 stream, and the left Cur was dissolved in an appropriate volume of methanol and quantified by UV–Visible spectrophotometry at 405 nm wavelength using a UV–Visible spectrophotometer (UV-3600, Shimadzu, Japan) [27].

2.3. Loading of PWSDs into liposomes by TUDC

All the experiments involving Cur were performed in a dark condition since the drug is sensitive to light. To diminish incorporation of Cur into bilayer membranes of liposomes, cholesterol was employed at a high fraction to prepare liposomes using the lipid thin film-dispersion technique according to a previous report by Papahadjopoulos et al. [28], but with a little modification. Briefly, an appropriate amount of EPC/CHO (5:3, mol/mol) was dissolved with 2-fold chloroform in a rotary flask, which was then evaporated out under a sub-atmospheric pressure to obtain a thin film using rotatory evaporator (RV-10, IKA-Werke GmbH & Co. KG, Germany) at a rotation rate of 120 rpm (round per min) at 40 °C. Then, the lipid film lining the flask was eliminated of trace solvent under vacuum overnight in the chamber of a freeze-dryer (Scientz-10 N, Ningbo Scientz Co., Zhejiang, China). After that, under protection of N_2 gas, the lipid film was warmed for 10 min and subsequently hydrated with water (or 88 mM sucrose solution for freeze-drying) for 30 min, using the rotatory evaporator with temperature continuously kept at 40 °C and rotation at 120 rpm to form primary liposomes. Subsequently, using an ice/water bath to keep the temperature under 30 °C, the coarse liposome suspensions were subjected to 40 cycles of sonication at 50 W in a pulse mode of 3-second-work on-2-second-work off with a probe-type sonicator (SCIENTZ-IID, Ningbo, Zhejiang Province, China).

DSPE-PEG$_{2000}$ was used for stabilization of liposome suspensions and was attached to liposomes by post-insertion method as previously described by Allen et al. [29]. Briefly, DSPE-PEG$_{2000}$ was weighed in a vial and fully dissolved in chloroform, which was then completely removed by a N_2 gas flush under rotation and subsequent vacuum evaporation overnight to form lipid film. Next, the extruded liposomes were added into the vial with mole ratio of EPC/DSPE-PEG$_{2000}$ of 65:5, and the mixture was stirred at 120 rpm for 1 h at 50 °C in a water bath to accomplish PEG insertion.

For drug loading by TUDC procedure, the temperature change range was set between 0 °C (273.15 K) and 50 °C (323.15 K) (or 65 °C for comparison). An aqueous suspension of the PEG-liposomes was placed in a N_2 gas-filled vial, which then was supplemented with PWSD (Cur) with an appropriate amount to just form a saturated Cur solution at 50 °C (or 65 °C) or with a huge surplus. Under an electromagnetic stirring at 120 rpm, the mixture was heated to 50 °C (or 65 °C), which was kept constant for 1 h to make drug fully dissolve. Then, the vial with mixture was cooled under stirring at 60 rpm at 0 °C in a water-ice bath for 0.5 h to precipitate Cur. Then, this temperature up-down process was cyclically carried out till Cur was accumulated in liposomes reaching the aimed level. After entrapment, samples were passed through Sephadex-G50 to remove free Cur from liposomes.

2.4. Characterization of Cur-PEG-liposomes

The size and distribution and zeta potential of liposomes were determined at 25 °C by DLS (dynamic light scattering) and ELS (electrophoretic light scattering), respectively, using Zetasizer Nano ZS-90 (Malvern Instruments Ltd., UK).

Unencapsulated Cur was separated from Cur-PEG-liposomes by centrifugation at 3000 rpm for 10 min at 4 °C using Sephadex G-50 1-mL-mini-column centrifugation method according to a previous report [30]. Encapsulated Cur and lipids in Cur-PEG-liposomes were quantified by HPLC using Shimadzu LC-20A HPLC system (Kyoto, Japan) equipped with SIL-20 AC Autosampler, CBM-20A system controller, and detectors of SPD-20A UV/VIS [31] or Essential ELSD-16 (evaporative light scattering detector) [32]. The EE (the ratio of encapsulated drug to total drug) and EC (the ratio of encapsulated drug to total liposome lipid) of Cur-PEG-liposomes were determined by quantification of the encapsulated drug and liposome lipids.

Cryo-TEM technique was used to visualize the general morphology and fine structure of drug-loaded liposomes by TUDC (Tecnai G2 Spirit, FEI, Oregon, USA) [8]. The hydrophobic carbon grid was converted to hydrophilic nature by glow discharge and was loaded with 5 μL of the sample which was made to evenly spread on the grid. The sample-holding grid got instant frozen through being submerged in liquid nitrogen at −196 °C for 10 min and transferred to a cryo-holder the ultralow temperature maintained using a liquid nitrogen storage box. The frozen sample on the grid was then inserted in the cavity of microscope for imaging in the ultralow temperature with operation voltage set at 200 kV.

SAXS was employed to testify the bilayer membranes of liposomes with the scattering vector q defined in the range of 0 to 0.3 using a SAXS system (SAXSpoint 2.0, Anton Paar, Austria). X-ray diffraction (XRD) was utilized to detect the crystallinity of Cur entrapped in liposomes by TUDC using an X-ray diffractometer (D-max 2400, Rigaku, Japan). For detection, to maintain the structure of liposomes and original amorphous or crystalline state of the entrapped drugs in liposomes, 0.2 mL of aqueous suspension of liposomes in a 5-mL vial was rapidly frozen in liquid N_2 and then lyophilized to get powder in a freeze-dryer (Scientz-10 N, Ningbo Scientz Co., Zhejiang, China). The monochromatic X-beams to irradiate samples were both produced by bombarding copper with electrons to have a wavelength of 1.54 Å.

DSC (differential scanning calorimetry) measurements were carried out with a DSC instrument (Q2000 TA Instruments, New Castle, DE, USA) for assay of the state of the drugs entrapped in liposomes. Also, to avoid drug state transformation, the samples had been rapidly frozen in liquid N_2 prior to freeze-drying (3% sucrose as a lyoprotectant). The sample powders including Cur, PEG-LPs, Cur-PEG-liposomes, and a mechanical mixture of Cur and PEG-LP, were sealed in the aluminum pans and detected in the temperature range of from −50 to 300 °C, at an increase rate of 10 °C/min.

Drug release feature at 37 °C of Cur-PEG-liposomes made by TUDC was sketched using the amount of drug liberated at different time points from liposomes that were suspended in different-pH media. To obtain the release parameters with minimal measurement errors, the Cur-loaded PEG-liposomes with highest EC made by TUDC were chosen for the tests. Briefly, an appropriate volume of Cur-PEG-liposomes was suspended in 5 mL 10 mM PBS (pH 7.4), 50 mM citrate buffer solution (pH 5.0), or 50 mM glycine-HCl buffer solution (pH 2.5) to obtain 10 mg/mL EPC final concentration in a 10-mL jacketed extruder (as mentioned in Determination of Cur solubility). Then the extruder was filled with pure N_2 gas and stirred at 120 rpm for 48 h with a mini electromagnetic stirrer, meanwhile the extruder was kept at 37 °C. At predetermined time points, 0.5 mL release medium was driven out by elevated N_2 gas pressure and collected for free drug measurement by UV–Visible spectrophotometry at 405 nm wavelength. Meanwhile, 0.5 mL buffer solution was supplemented to the samples in the cavity of 10-mL jacketed extruder. Finally, the drug release curves were plotted versus time using Excel software.

2.5. Mathematical model for prediction of loading capacity by TUDC

Dissection of the TUDC process derives the mathematical equations for calculation of the loading capacity of liposomes for a PWSD. In TUDC process, when temperature rises, PWSD solubility as well as liposome membrane permeability is increased, which, plus a continuous stirring, makes the drug dissolve and balanced between the exterior and interior of liposomes. During this process, the transient existence of the transmembrane drug concentration gradience drives drug transmembrane diffusion, engendering a (saturated) solution with drug concentration of C_{sh} (saturate concentration at high temperature) both outside and inside of liposomes. When temperature falls, PWSD solubility as well as liposome membrane permeability decreases, making drug precipitate and form a saturated solution with concentration of C_{sl} (saturate concentration at low temperature) inside and outside of liposomes. Drug precipitates are unable to cross liposome membranes rendering interior drug entrapped without drug leakage, which also occurs as a result of lack of transmembrane drug concentration gradience during cooling. After the first TUDC, when heated, while the exterior drug precipitates dissolve rapidly owing to stirring facilitation, the interior precipitates, very likely, stand still or just dissolve slowly due to lack of stirring within liposomes. Thus, repeated performance of TUDC cycle render drug successfully accumulated in liposomes.

To set up a mathematical model, it is proposed that an aqueous dispersion of the liposomes possesses an exterior aqueous volume of V_{EL} containing Q_s amount of PWSD and a total interior aqueous volume, V_{IL}, of liposomes (the total volume of aqueous medium enclosed within all liposomes), which contains initially no PWSD. And the drug precipitates inside and outside liposomes both are argued to occupy only a marginal volume that is negligible, compared to the total aqueous volume V which equals the sum of V_{EL} and V_{IL}. Two conditions are considered: (1) Cur with the amount just forming a saturated concentration at top temperature of TUDC, e.g., 50 °C (323.15 K) (or 65 °C for comparison), was used for entrapment; (2) Cur with a huge surplus to forming a saturated concentration at top temperature of TUPC was used for entrapment. In both conditions, at bottom temperature, e.g., 0 °C (173.15 K), a PWSD has an aqueous concentration that is regarded as zero to be omitted.

Condition 1: It is supposed that the initially added drug amount Q_0 equals Q_s which is just sufficient to form a saturated solution with concentration of drug solubility C_s at the highest top temperature in TUDC process. Then, for the first, second, third, ……, and nth TUDC cycle, the drug entrapment each time with the amount of Q_1, Q_2, Q_3, ……, Q_{n-1} and Q_n, is calculated, correspondingly, as the following series of equations:

$$Q_1 = Q_s \frac{V_{IL}}{V} \tag{1}$$

$$Q_2 = (Q_s - Q_1)\frac{V_{IL}}{V} \tag{2}$$

$$Q_3 = (Q_s - Q_1 - Q_2)\frac{V_{IL}}{V} \tag{3}$$

$$Q_{n-1} = (Q_s - Q_1 - Q_2 - \ldots - Q_{n-2})\frac{V_{IL}}{V} \tag{4}$$

$$Q_n = (Q_s - Q_1 - Q_2 - \ldots - Q_{n-2} - Q_{n-1})\frac{V_{IL}}{V} \tag{5}$$

Then, E-5 − E-4 (Equation 5 − Equation 5) hits

$$Q_n - Q_{n-1} = -Q_{n-1}\frac{V_{IL}}{V} \tag{6}$$

namely,

$$Q_n = Q_{n-1}\left(1 - \frac{V_{IL}}{V}\right) \tag{7}$$

Therefore, each TUDC cycle generates an entrapment, whose value together constitutes a geometric series with a common ratio of (1 − V_{IL}/V), the last term in the above E-7.

As such, after the nth time performance of TUDC cycle, the total amount of entrapped drug (Q_{TED}) is:

$$Q_{TED} = \frac{Q_S\frac{V_{IL}}{V}\left[1 - \left(1 - \frac{V_{IL}}{V}\right)^n\right]}{1 - \left(1 - \frac{V_{IL}}{V}\right)} = Q_S\left[1 - \left(1 - \frac{V_{IL}}{V}\right)^n\right] \tag{8}$$

Given the aqueous solution contains liposomes which are spheres each with a diameter of D and totally consume MV (M, mol/L phospholipid concentration) phospholipid (PL) molecules, each of which has an interfacial area of A, molecular weight of W_{PL}, and length of L. Assuming N_A is Avogadro's constant, and its multiplying A is represented by an invariable I, i.e., I = N_AA. Then, omitting liposome membrane thickness, V_{IL} is calculated as Eq. (9), and after n times performance of TUDC cycle, the totally entrapped drug Q_{TED}, or entrapment amount (EA), is calculated as Eq. (11):

$$V_{IL} = \frac{MVN_AAD}{12} \tag{9}$$

thus,

$$\frac{V_{IL}}{V} = \frac{MN_AAD}{6} = \frac{MID}{12} \tag{10}$$

$$EA = Q_{TED} = Q_S\left[1 - \left(1 - \frac{MID}{12}\right)^n\right] \tag{11}$$

If the EC (entrapment capacity of liposomes) is defined as the entrapped drug-to-PL molar ratio, omitting the volume of lipids, then

$$EC = \frac{Q_{TED}}{Q_{PL}} = \frac{Q_S\left[1 - \left(1 - \frac{MID}{12}\right)^n\right]}{MV} = \frac{C_S\left[1 - \left(1 - \frac{MID}{12}\right)^n\right]}{M} \tag{12}$$

Therefore, in this condition 1, the drug entrapment capacity by TUDC procedure depends just on liposome concentration and size, TUDC cycle number, and drug solubility C_s.

If the EE (entrapment efficiency of liposomes) is defined as the entrapped drug-to-total drug ratio, then

$$EE = \frac{Q_{TED}}{Q_S} = \frac{Q_S\left[1 - \left(1 - \frac{MID}{12}\right)^n\right]}{Q_S} = 1 - \left(1 - \frac{MID}{12}\right)^n \tag{13}$$

Notably, by TUDC procedure, using saturated drug solution for entrapment by TUDC, the drug EE depends only on liposome concentration and size, and TUDC cycle number, but has nothing to do with the initially added drug.

Condition 2: Here, it is supposed that the initially added drug Q_0 is much more than the amount needed to form a saturated solution Q_s, even after n times TUDC cycle. Also, it may well be assumed that the volume of drug precipitates is neglected, then the drug entrapment each time Q_1, Q_2, Q_3, ……, Q_{n-1} and Q_n, remains constant and is calculated as following Eq. (14):

$$Q_1 = Q_2 = \ldots = Q_n = Q_S\frac{V_{IL}}{V} \tag{14}$$

Then, after the nth performance of TUDC cycle, total entrapped amount (EA) Q_{TED}, EC and EE are calculated to be Eqs. (15), (16) and (17), respectively:

$$EA = Q_{TED} = nQ_S\frac{V_{IL}}{V} = \frac{nQ_SMID}{12} \tag{15}$$

$$EC = \frac{Q_{TED}}{Q_{PL}} = \frac{nQ_SMID}{12MV} = \frac{nQ_SID}{12V} = \frac{nC_SID}{12} \tag{16}$$

$$EE = \frac{Q_{TED}}{Q_0} = \frac{nQ_SMID}{12Q_0} = \frac{nC_SMID}{12C_0} \tag{17}$$

Thus, in this condition 2, the drug EC by TUDC is relevant to drug solubility, liposome size, as well as TUDC cycle number, and also the initially added drug amount.

2.6. Thermodynamic process for calculation of drug loading by TUDC procedure

The mathematic calculation provides a prediction on TUDC drug entrapment and, therefore, is a useful reference for optimizing liposome formulation and preparation. Notably, the mathematical calculation needs a key drug parameter, solubility, of which the test at each temperature is a rather time- and energy-consuming process. Fortunately, the drug solubility is a parameter that may be derived from general thermodynamic equations, as deduced as follows.

For a PWSD, the interaction between the drug and solvent (water) molecules is rather weak and, here, is neglected in the analysis of the related thermodynamic process. When a drug dissolves to form a saturated solution at high temperature, it is a balanced process as follows.

$$D_n \leftrightarrow nD$$

Obviously, D_n may well be given the value of 1, while nD may be regarded as the saturated molar concentration of a drug, C_s, namely, the aqueous solubility. Then, the balance constant, K_d, is calculated according to Eq. (18).

$$K_d = C_s \tag{18}$$

Under a constant pressure, Gibbs-Helmholtz equation gives E-19.

$$\frac{d\left(\frac{\Delta G}{T}\right)}{dT} = -\frac{\Delta H^*}{T^2} \tag{19}$$

where, T is the absolute temperature, and ΔH* represents here the apparent partial molar enthalpy of solution of the solute.

According to ΔG = −RTlnK_d, where R is the gas constant (8.314 $J{\cdot}K^{-1}{\cdot}mol^{-1}$), the van't Hoff equation (Eq. (16)) was set up in 1887 to describe the relationship between solubility and temperature, and its differential equation is presented as follows.

$$\frac{d(lnK_d)}{dT} = \frac{d(lnC_s)}{dT} = \frac{\Delta H^*}{RT^2} \tag{20}$$

Notably, though several revision editions of van't Hoff equation were later proposed to make the plot more linear [33], to do so, additional parameters were introduced rendering the equation resolution much more complicated, while gaining little improvement. Considering C_s of PWSD is rather low, the initial van't Hoff equation for ideal solutions is here suitable for use, but in the integral form, to illustrate the solubility as a function of temperature. Integration of initial van't Hoff differential equation gives the following Eq. (21), where C′ is the constant of integration.

$$lnC_S = -\frac{\Delta H^*}{RT} + C' \tag{21}$$

$$C_S = e^{\left(-\frac{\Delta H^*}{RT} + C'\right)} \tag{22}$$

In the above **Condition 1**: Eq. (12) becomes the following Eq. (23).

$$EC_{lip} = \frac{\left[1 - \left(1 - \frac{MID}{12}\right)^n\right]}{M} e^{\left(-\frac{\Delta H^*}{RT} + C'\right)} \tag{23}$$

In **Condition 2**: Eq. (16) becomes the following Eq. (24).

$$EC_{lip} = \frac{nC_S ID}{12} = \frac{nID}{12} e^{\left(-\frac{\Delta H^*}{RT} + C'\right)} \quad (24)$$

Thus, the calculation of drug entrapment of TUDC process is able to be accomplished using the thermodynamic parameter temperature instead of drug solubility. Obviously, using temperature for calculation of drug entrapment by TUDC is a much more efficient step, compared to use of drug solubility which, if used, should be painstakingly tested at each temperature. The temperature between 0 °C (273.15 K) and 50 °C (323.15 K) is argued feasible for TUDC process, since this range avoids the freezing and boiling process, as well as a too high temperature, all of which may induce a severe damage to drug or liposomes.

3. Results

3.1. Solubility of Cur

The aqueous solubility (C_s) of Cur at different temperatures (0, 15, 25, 35, 50 °C) was very low but increased significantly with temperature, as revealed by the results listed in Table 1. Therefore, a saturated Cur solution may well be treated as an ideal solution.

According to van't Hoff equation (Eq. (21)), at a constant pressure, an ideal solution display a linear relationship between ln(C_s) versus 1/T. For a saturated Cur solution, regression of ln(C_s) versus 1/T by the least-squares method results in a linear Eq. (25) with R^2 of 0.9786, suggesting a linear relationship does exist (Fig. 2). From the linear equation, ΔH* which represents the apparent partial molar enthalpy of solution of the solute and the integration constant C′ were calculated to be 26.21 kJ·mol^{-1} and -6.314, as listed in Table 1.

3.2. Characteristics of liposome samples

3.2.1. Liposomes size by DLS

DLS test data on liposome samples revealed that PEG-liposomes, after Cur entrapment by 10-cycle TUDC with temperature change scope of 0 to 50 °C, only increased their size from the original of about 120 to 150 nm and PDI from about 0.1 to up to 0.3, suggesting TUDC procedure does not severely damage the pegylated liposomes (Table 2). In contrast, plain liposomes (without pegylation) dramatically changed their size to larger than 500 nm with PDI > 0.7 (data are not shown), indicating plain liposomes can hardly endure the TUDC conditions.

3.2.2. Cryo-TEM image of liposomes

The above results of liposome size by DLS are in good agreement with cryo-TEM images (Fig. 3), which indicate that the original pegylated liposomes had a good spherical shape (Panel a) but a relatively polymorphic one after TUDC (Panel b). However, plain liposomes (images of the original ones are not shown) which showed a similar appearance to PEG-liposomes, when subjected to TUDC, seemed to undergo fusion thus forming bulk lipid blocks (Panel c). The cryo-TEM images also evidence that a PWSD, such as Cur, was actually loaded by TUDC into the interior of liposomes, wherein the drug aggregated into sediments (dark spots in Panel b and c) as a result of drug concentration greatly exceeding its solubility. Notably, as far as the surface charge is concerned, all the prepared liposomes fell in the scope of a neutral state, since their absolute values of zeta potential were less than 5 mV and close to zero (Table 2), which is too small to contribute to the stability of liposomes.

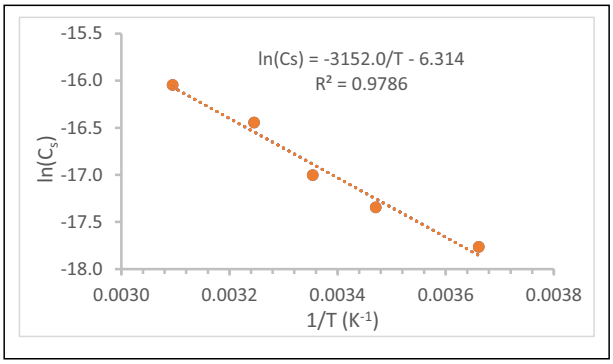

Fig. 2. The plot of the linear regression of the dependent variable of natural logarithm of Cur solubility versus the independent variable of reciprocal absolute temperature.

3.2.3. SAXS and XRD pattern of liposomes and drugs

SAXS was employed to investigate the maintenance of the unilamellar bilayer membranes of original PEG-liposomes. The SAXS pattern of PEG-liposomes subjected 10-cycle TUDC shows a peak at q of 0.137 Å^{-1} which is much wider and higher than that of the original PEG-liposomes, while the plain liposome SAXS pattern shows the widest and highest peak at the same position (Fig. 4a). These results demonstrate that, subjected to TUDC, perhaps, a fraction of unilamellar PEG-liposomes transformed into the oligolamellar ones, whereas the plain unilamellar liposomes changed to the multilamellar ones or lipid bulks, and all the liposomes had a single bilayer membrane with the thickness of about 4.6 nm. These are also in accordance with the displays in the cryo-TEM image and the size data of the DLS test, both of which clearly demonstrate the expansion, more or less, in volume of the corresponding liposomes.

The XRD patterns (Fig. 4b) indicate that raw Cur had a good crystal structure, as evidenced by the defined characteristic peak file of the drug. The XRD pattern of a physical mixture of Cur and PEG-liposomes demonstrate the presence of main characteristic peaks of both Cur or PEG-liposomes. By comparison, the XRD pattern of Cur-PEG-liposomes presents mainly the characteristic peaks of PEG-liposomes but almost no peaks of Cur. The results testified that Cur, or a majority of Cur, existed in the amorphous state after being loaded into liposomes by TUDC.

3.2.4. DSC thermogram of liposomes and drugs

DSC was employed to examine the state of liposomal Cur loaded by

Table 1
Solubility (C_s) of Cur at different temperatures (T), and calculated ΔH* and the integration constant C′ (mean ± SD) ($n = 3$).

$$lnC_S = -\frac{3152.0}{T} - 6.314 \quad (25)$$

T (K)	1/T (K^{-1})	C_s (μg·L^{-1})	C_s (mol·L^{-1})	Ln(C_s)	ΔH* (kJ·mol^{-1})	C′
273.15	0.00366	7.1	1.927E-08	−17.76		
288.15	0.00347	9.8	2.660E-08	−17.44		
298.15	0.00335	13.2	3.583E-08	−17.14	26.2	−6.314
308.15	0.00324	28.6	7.763E-08	−16.37		
323.15	0.00309	39.6	1.075E-07	−16.05		

Table 2
The formulation, average number diameter (AD), polydispersity index (PDI), zeta potential (ζ), the tested or calculated Cur EE and EC of liposomes, PEG-liposomes, or Cur-PEG-liposomes by made TUDC with temperature varying from 0 to 50 °C, or thin film-dispersion method (mean ± SD) ($n = 3$).

Formulation[a]	TUDC cycle number	AD ± SD (nm)	PDI ± SD	ζ ± SD (mV)	EE ± SD (%)	EC (ppm) (mol ratio)	EE (%) prediction	EC (ppm) prediction
1: EPC/CHO (65:13, 65 mM EPC)	0	127 ± 6	0.12 ± 0.01		–	–	–	–
2: EPC/CHO/DSPE-PEG_{2000} (65:39:5, 65 mM EPC)	0	118 ± 9	0.10 ± 0.02	−4.5 ± 1.3	–	–	–	–
3: Cur + EPC/CHO/DSPE-PEG_{2000} (65:39:5, 65 mM EPC) (107.5 nmol Cur)	10	147 ± 18	0.26 ± 0.03	−3.8 ± 1.2	91.1 ± 10.7%	1.51	98.5	1.63
4: Cur + EPC/CHO/DSPE-PEG_{2000} (65:39:5, 13 mM EPC) (107.5 nmol Cur)	10	153 ± 20	0.30 ± 0.04	−5.7 ± 1.1	61.1 ± 7.7%	5.05	50.8	4.20
5: Cur + EPC/CHO/DSPE-PEG_{2000} (65:39:5, 65 mM EPC) (107.5 nmol Cur)	6	145 ± 12	0.21 ± 0.03	−4.4 ± 1.4	86.7 ± 9.5%	1.43	91.9	1.52
6: Cur + EPC/CHO/DSPE-PEG_{2000} (65:39:5, 65 mM EPC) (1.075 μmol Cur)	6	146 ± 11	0.22 ± 0.02	−3.8 ± 1.2	20.8 ± 2.3%	3.44	21.5	3.40
7: Cur + EPC/CHO/DSPE-PEG_{2000} (65:39:5, 65 mM EPC) (2.15 μmol Cur)	6	142 ± 12	0.23 ± 0.03	−5.5 ± 0.9	11.7 ± 1.7%	3.87	10.3	3.40
8: Cur + EPC/CHO/DSPE-PEG_{2000} (65:39:5, 65 mM EPC) (2.15 μmol Cur)	10	155 ± 19	0.33 ± 0.05	−5.4 ± 1.2	15.2 ± 1.9%	4.70	17.1	5.66
9: Cur + EPC/CHO/DSPE-PEG_{2000} (65:39:5, 13 mM EPC) (107.5 nmol Cur)	0	115 ± 10.5	0.11 ± 0.01	−5.9 ± 0.8	10.3 ± 3.3%	0.85	–	–

[a] The drug loading of formulations of from 3 to 8 was carried out with TUDC process, while formulation 9 drug loading was accomplished by conventional lipid thin film-dispersion method.

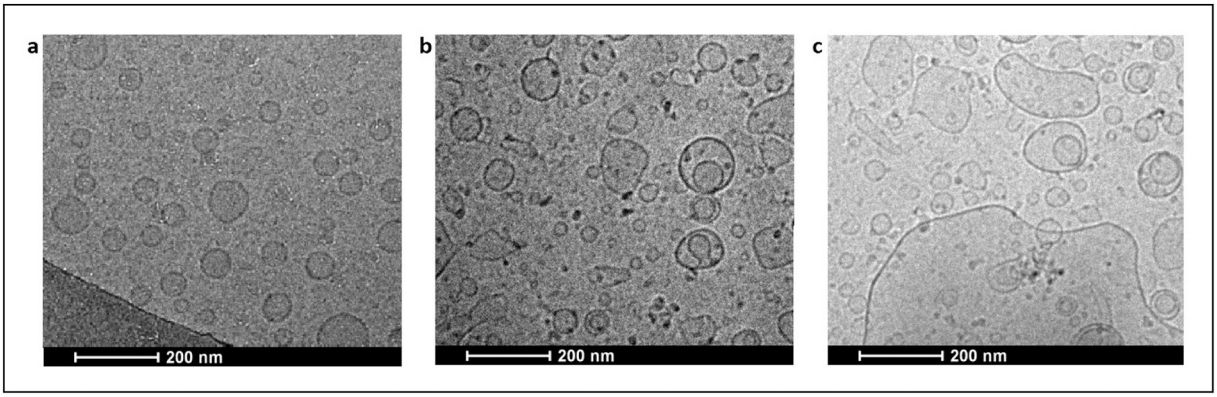

Fig. 3. Representative cryo-TEM images of liposome samples ($n = 3$). (a) An aqueous suspension of original PEG-liposomes; (b) PEG-liposomes subjected 10 cycles of TUDC with dark spots which are Cur sediments formed due to accumulation of Cur within liposomes; (c) Plain liposomes (without pegylation) subjected 10 cycles of TUDC are damaged into lipid bulk but also shows Cur sediments within liposomes.

TUDC and its interaction with liposome components using freeze-dried samples, which contained sucrose as a lyoprotectant. Since liposome EC for Cur is rather low, in order to highlight thermal behavior of Cur, Formulation 8 listed in Table 2 which engendered highest loading level was used to prepare samples. Representative DSC thermograms of Cur, PEG-liposomes, Cur-PEG-liposomes, and a physical mixture of Cur and PEG-liposomes, were obtained by testing samples from −50 to 300 °C (Fig. 5). DSC thermograms show that free Cur was a crystal and had a sharp endothermic peak at 170.4 °C corresponding to its melting point, which disappeared when loaded into liposomes but still stood when physically mixed with liposomes at a comparative quantity ratio. Moreover, the melting points of both Cur and sucrose in liposome samples were significantly widened, suggesting Cur entrapped in liposomes might exist in the amorphous state and, likely, be fixed in the vitreous matrix of sucrose. Notably, the endothermic peaks at around 47.5 °C, ascribed to DSPE-PEG_{2000}, in liposome samples showed little change, indicating Cur was unlikely intercalated within bilayer membranes. As such, it is argued that Cur loaded in liposomes by TUDC exists in the amorphous state, though it has been subjected to several cycles of cooling process.

3.2.5. Encapsulation efficiency (EE) and entrapment capacity (EC) of PWSD by TUDC

In the case where liposomes had a specific size and the temperature up limit of TUDC was set, the EE of Cur-loaded liposomes proved dependent mainly on TUDC cycle number, liposome concentration, and the initially added drug. As shown in Table 2, when the initial addition of Cur that was just sufficient to form a saturated solution (Formulations 3, 4 and 5), the drug was efficiently loaded into liposomes by controlling TUDC cycle number and liposome concentration, as demonstrated by the achievement of EE of >90%.

When the initial addition of Cur was much more than the amount that is sufficient to form a saturated solution (Formulations 6, 7 and 8), the entrapment efficiency (EE) was rather low and showed a value of less than 21% after 6 TUDC cycle number, meanwhile the loading capacity was increased depending just on TUDC cycle number. However, EE and EC of liposomal Cur loaded by TUDC process were both much higher than those achieved by conventional thin lipid film-dispersion method, as shown by Formulation 9 in Table 2.

3.2.6. Drug release feature of Cur-PEG-liposomes made by TUDC

To obtain the release parameters with minimal measurement errors, the Cur-loaded PEG-liposomes with highest EC (entrapment capacity), i.e., Formulation 4 in Table 2, were chosen for measurement of drug

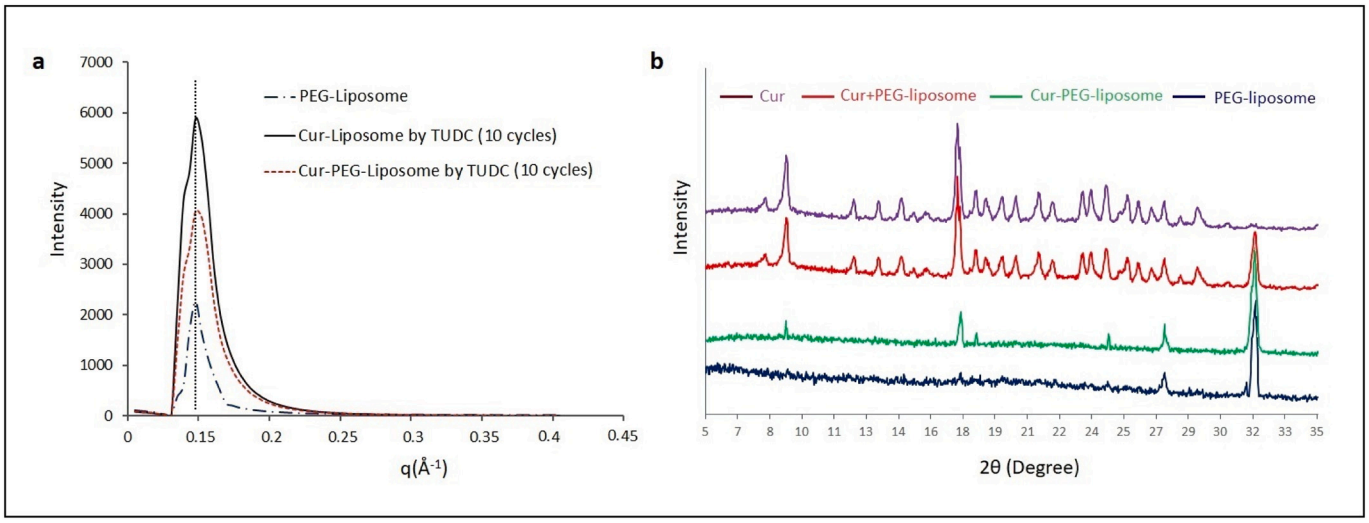

Fig. 4. The SAXS and XRD patterns of liposomes before and after Cur loading by 10-cycle TUDC ($n = 3$). (a) The SAXS patterns of PEG-liposomes were widened in a peak width and elevated in a peak height after Cur loading by TUDC, which were further intensified in the case of plain liposomes. (b) XRD patterns of Cur and liposome samples showed that raw Cur was in crystalline state but at least a large fraction transformed into the amorphous state after be loaded into liposomes through TUDC.

release. The drug release curves (Fig. 6) demonstrate that Cur was released from liposomes at a rather slow rate, showing the release fraction occupying only 8.6% of total entrapment after 48 h. But, in the investigation pH range of from 2.5 to 7.4, Cur release was somewhat influenced by medium pH values. For instance, Cur was released faster from liposomes in the medium with a high pH value than in the one with a low pH value. These results are reasonable, because the drug was to escape from liposomes it must dissolve in a medium at first. While the solubility of Cur is generally low in an aqueous medium at 37 °C underlying the slow release, it is increased proportionally with pH value [34]. Therefore, Cur dissolves to a larger degree and is released faster from liposomes in a high pH medium than in a low pH medium.

3.3. Mathematic calculation of drug entrapment of TUDC

In TUDC procedure, the 150 nm-sized liposomes were constructed with EPC/CHO/DSPE-PEG_{2000} (65:39:5) at an EPC concentration of 65 mM (mmol/L, about 5%, *w/v*). EPC is the main component and has previously been revealed of the average interfacial area per molecule of about 0.7 nm^2 [35]. Influences of CHO and pegylated lipids on liposome structure and size may well be omitted, because CHO is intercalated within bilayer membranes while DSPE-PEG_{2000} represents only a small fraction of total lipids and is just attached to out surface of liposomes. Then, the available data allow the above deduced mathematical model to be employed for theoretical calculation of liposome drug entrapment by TUDC procedure, as listed in Table 3. For comparison, the theoretically calculated Cur EEs and ECs of liposomes by TUDC procedure are also listed in Table 2. It can be seen that theoretically calculated EEs and ECs are in good agreement with the experimentally determined ones, suggesting the mathematical model may act as a tool for prediction of the drug entrapment of TUDC procedure. In particular, the model gives a clear view on the key determinants among influencing factors involved in TUDC process, and, therefore, may present a useful reference to optimizing liposome formulation.

3.4. Mathematic calculation of TUDC entrapment based on thermodynamic parameters

The mathematic calculation provides a prediction on TUDC drug entrapment based on a key drug parameter, solubility, which can be derived from general thermodynamic equations. Theoretically, if the solubilities of a drug at two different temperatures are accurately obtained by experimental test, the solubility of the drug at any temperature can be conveniently calculated using van't Hoff differential equation, as showed by Eq. (21). Thus, the solubility of Cur at 65 °C was deduced using Eq. (21) and used for estimation of the ECs obtained by TUDC procedure according to Formulation 5 and 6, except the temperature variation set of 0–65 °C, correspondingly, recorded as Formulation 5′ and 6′. The ECs for Cur by TUDC procedure using Formulation 5′ and 6′ were also gained by experimental test and were compared to the ones calculated using thermodynamic equations. The results are listed in Table 4, which shows that the ECs approached by the two different ways are in good agreement, suggesting the thermodynamically derived parameters are feasible to be included in mathematical equations for calculation of drug entrapment by TUDC procedure.

4. Discussion

Recently, numerous nanocarriers have been engineered as a DDS (drug delivery system) bearing heavy expectations to realize the targeted therapy for promising chemical entities [36,37]. Among these, liposomes prove a feasible and practical DDS, as evidenced by several marketed products. Despite of this, loading substantial drugs, especially, PWSDs, into liposomes still represents a big challenge for developing a liposomal drug. In this study, a temperature up-down cycle (TUDC) procedure was developed for loading of a PWSD (poorly water-soluble drug) into liposomes. As schematically described in Fig. 7, TUDC works as that, when temperature rises, a PWSD will, usually, dissolve and, if mixed with liposomes, then form a transmembrane concentration gradient as a driving force to propel the drug into liposome interiors till a balance. Then, when temperature falls, liposome membranes become less permeable restraining drugs from leakage; meanwhile, the drug is aggregating to form sediments of a large size that are unable to go the transmembrane movements, lading to drug entrapment within liposomes. As such, cyclic operation of TUDC process makes the drug successively accumulate in liposomes till hitting a large drug entrapment.

Alteration of temperature to change liposome membrane permeability proves feasible and has been widely employed for loading drug into liposomes by, especially, the transmembrane ion gradient methods [19]. Notably, certain chemicals are labile to heat, and, therefore, when they are loaded into liposomes employing TUDC, the temperature up line may be set as low as possible given stability concerns. In addition,

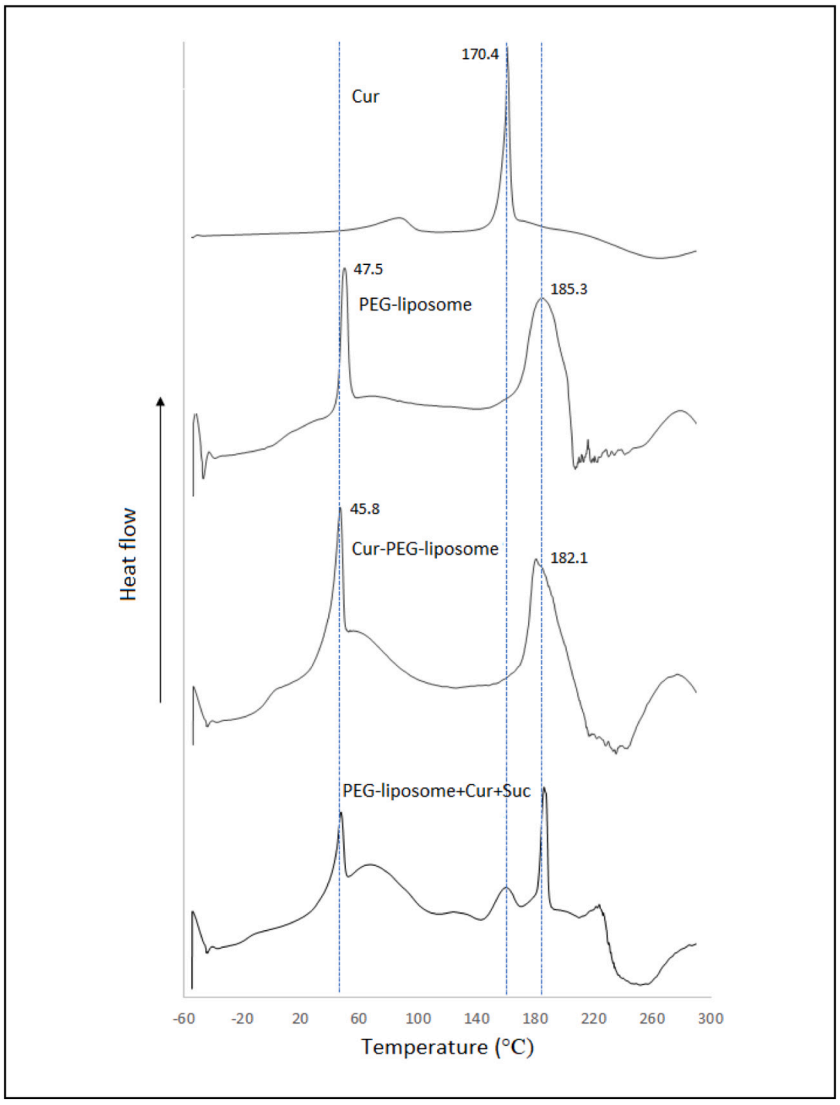

Fig. 5. DSC curves of Cur, lyophilized products of PEG-liposomes and Cur-PEG-liposome, and a physical mixture of PEG-liposomes + Cur + Suc (Suc, sucrose).

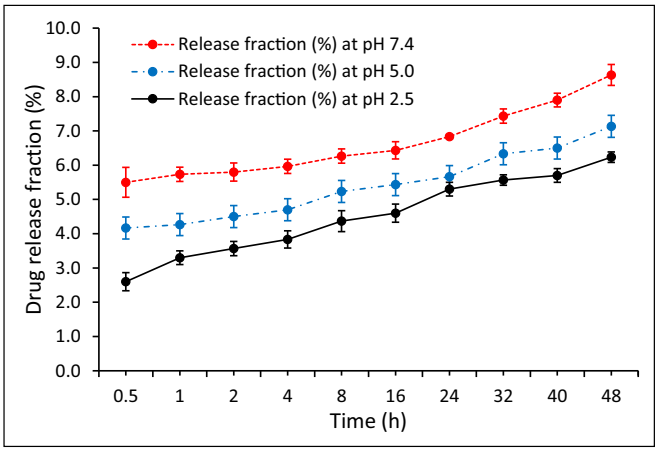

Fig. 6. The drug release feature of Cur-PEG-liposomes made by TUDC ($n = 3$). The release media were 10 mM PBS (pH 7.4), 50 mM citrate buffer solution (pH 5.0), and 50 mM glycine-HCl buffer solution (pH 2.5), and the liposome concentration was set as 10 mg/mL EPC in the release media.

phospholipids may also be damaged by high temperature which facilitates their hydrolysis and oxidation, as underlined in a previous report [38]. Therefore, it is suggested the experiments be carried out in the condition shielded from light and under protection by an inert gas.

Based on the features and thermodynamics of TUDC process, several mathematical equations (Eqs. (11), (12), (13), (15), (16), (17), (23) and (24)) have been deduced for theoretical calculation of the drug entrapment parameters, such EA, EE and EC. The experimental results demonstrated that the EE and EC of liposomes for a PWSD, Cur, obtained by wet lab TUDC are in good agreement with the theoretically calculated ones. This suggests that the established mathematical equations and the associated thermodynamic process work well and are able to be employed for prediction of the drug entrapment by TUDC procedure. Relatively accurate mathematical prediction is valuable for sifting from PWSDs the candidate that is eligible for the development of a liposome product, since it saves arduous experiments, which, furthermore, often bear numerous big deviations. Moreover, based on mathematical calculation, the drug entrapment parameters, such as EE and EC, can be purposely set with a proper value to allow optimizing liposome formulation and TUDC procedure with fewer factors and properly fixed parameters, such as liposome concentration and size, temperature range and cycle number. This will significantly enhance the development efficiency of the PWSD-containing liposomes [23].

Though mathematic calculation provides a reliable prediction on

Table 3
The parameters and mathematical calculation of EE and EC (drug/lipid mole ratio) of Cur-PEG-liposomes by TUDC procedure with temperature range of 0–50 °C.

Formulation	C_0 (mol/L) of Cur	C_s (mol/L) of Cur	M (mol/L) of EPC	D (dm) of Lip	N_A	A (dm^2) of EPC	n	Condition 1		Condition 2	
								EE = 1-(1-MID/12)^n	EC = C_sEE/M	EC = $nC_sID/12$	EE = $nC_sMID/12C_0$
3	1.075E-07	1.075E-07	0.065	1.5E-06	6.02E+23	7.0E-17	10	0.985	1.63E-06		
4	1.075E-07	1.075E-07	0.013	1.5E-06	6.02E+23	7.0E-17	10	0.508	4.20E-06		
5	1.075E-07	1.075E-07	0.065	1.5E-06	6.02E+23	7.0E-17	6	0.919	1.52E-06		
6	1.075E-06	1.075E-07	0.065	1.5E-06	6.02E+23	7.0E-17	6			3.40E-06	0.21
7	2.150E-06	1.075E-07	0.065	1.5E-06	6.02E+23	7.0E-17	6			3.40E-06	0.10
8	2.150E-06	1.075E-07	0.065	1.5E-06	6.02E+23	7.0E-17	10			5.66E-06	0.17

Table 4
Mathematic calculation of TUDC drug entrapment using thermodynamic parameter Cur-PEG-liposomes which were loaded with Cur by TUDC procedure with temperature range of 0–50 °C (Formulation 5 and 6) or 0–65 °C (Formulation 5′ and 6′).

Formulation	C_0 (mol/L) of Cur	C_s (mol/L) of Cur	M (mol/L) of EPC	D (dm) of Lip	N_A	A (dm^2) of EPC	n	EC (ppm)		
								tested	Condition 1: Eq. (23)	Condition 2: Eq. (24)
5	1.075E-07	1.075E-07	0.065	1.5E-06	6.02E+23	7.0E-17	6	1.43	1.48	
6	1.075E-06	1.075E-07	0.065	1.5E-06	6.02E+23	7.0E-17	6	3.44		3.32
5’	1.445E-06	1.445E-06	0.065	1.5E-06	6.02E+23	7.0E-17	6	2.53	2.29	
6’	1.445E-05	1.445E-06	0.065	1.5E-06	6.02E+23	7.0E-17	6	4.87		5.12

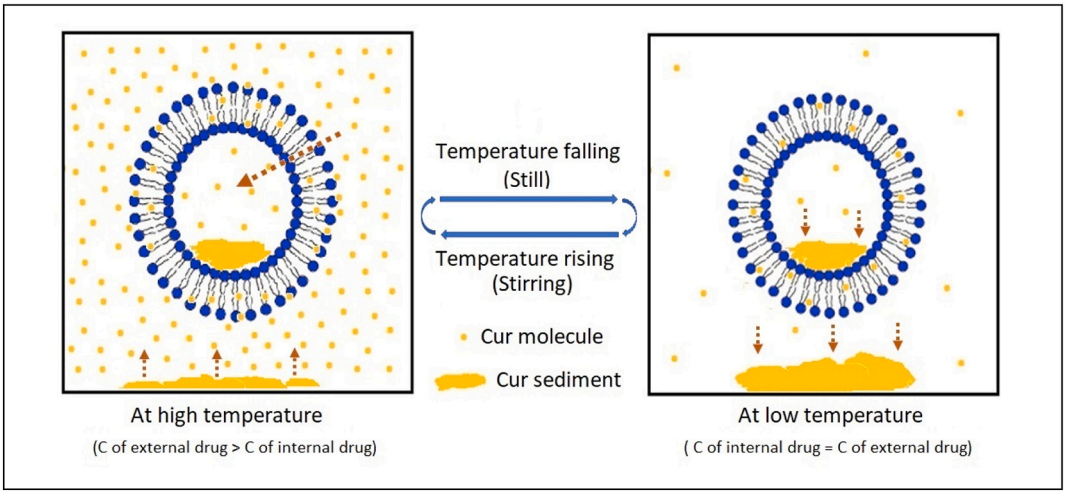

Fig. 7. Schematic description of loading drugs in liposomes by TUDC. Drugs that show a solubility rising proportionally with temperature in an aqueous medium will gradually accumulate in liposome interiors during the temperature up-down cycle procedure (TUDC) (C, abbreviation of concentration).

TUDC loading, it does not function without knowing a key parameter of drug solubility at the corresponding temperature. Actually, experimental test on drug solubility at different temperatures can finally realize the mathematic prediction on TUDC loading, however, the test of drug solubility is a rather time- and energy-consuming process [39]. Fortunately, the drug solubility may be theoretically treated as a thermodynamic parameter that may be worked out from the associated thermodynamic equations, such as van't Hoff equation. Thus, the mathematic prediction on TUDC loading is just conducted using the parameter of temperature instead of drug solubility, as shown by Eqs. (23) and (24). In theory, using the established mathematical equations (Eq. (12), (13), (16) and (17)) in combination with related thermodynamic parameters, the drug loading by TUDC procedure with any a temperature variation range can be conveniently calculated (with Eqs. (26) and (27)) and used as a reference to practical application. In practice, setting the temperature to range between 0 and 50 °C may be feasible for TUDC process, since, in this temperature scope, samples will not experience a freezing and boiling process, nor a too high temperature period, each of which may induce a severe damage to drug and liposomes. Notably, some PWSDs show an aqueous solubility that varies with temperature but does not comply with van't Hoff linear equation [33]. In these cases, calculation of TUDC drug entrapment should be based on certain specifically established thermodynamic equations instead of Eq. (21). Theoretically, according to Eq. (16), on the condition that the solubility of a PWSD does vary, in whatever relationship, with temperature, the mathematical model will always fit estimation of EC (entrapment capacity) of liposomes. However, if the PWSD solubility does not change proportionally with temperature, the procedure of TUDC should be adjusted for efficient entrapping the PWSD. For instance, if the solubility of a PWSD varies negatively with temperature, the drug will, theoretically but anti-intuitively, enter liposomes during temperature decrease process and precipitate in liposomes during temperature increase process of TUDC. Obviously, if a drug has a constant solubility toward temperature, the TUDC procedure established in this study does not effect.

It should be pointed out that the TUDC procedure is only practical for the PWSDs, especially, those that have a heavily temperature-dependent solubility, as can be seen from Eqs. (11)–(17), where C_s in fact should be

$C_{sh} - C_{sl}$ (C_{sh}, solubility at highest temperature; C_{sl}, solubility at highest temperature). For the PWSDs with a solubility that changes little with temperature [39], i.e., $C_{sh} - C_{sl}$ is too small, the TUDC procedure can hardly fulfil entrapment of sufficient drug in liposomes. Also, if a drug has a relatively high aqueous solubility at the bottom temperature (e.g., 0 °C) of TUDC process, it will have a low EE due to free transmembrane lost. Generally, the bottom temperature of TUDC may be set between the ice-forming and room temperature, while the top temperature may be set at a value between 50 and 80 °C, considering sample stability, operation practice as well as convenience.

Usually, common liposomes are an instable product and, when subjected to repeated temperature alteration, tend to go fusion, leading to carrier disassembly and content leakage [4]. As such, pegylation, which can confer a steric stabilization effect on labile carriers [12], was employed to ready liposomes for TUDC procedure. The experiment DLS size results (Table 2), cryo-TEM images (Fig. 3a and b) and SAXS pattern (Fig. 4a) showed that the size and structure of PEG-liposomes, even with different lipid concentrations, varied a little after different modes of TUDC process, suggesting pegylation may render the procedure feasible for loading of drugs into liposomes.

The XRD patterns (Fig. 4b) and DSC thermograms (Fig. 5) demonstrate that Cur entrapped in liposomes exists, in the presence of sucrose, very likely in the amorphous state. A PWSD that tends to crystallize as temperature falls may be more suitable for being loaded into liposomes by TUDC procedure, because crystal particles are more stable than the amorphous aggregates and, when subjected to the subsequent cycles of temperature rise, will not dissolve rapidly [40] to diffuse out of liposomes. Nevertheless, the experimental results of Cur prove the feasibility of using TUDC procedure to load into liposomes the drugs in the amorphous state.

It cannot be neglected on the cryo-TEM images in that they demonstrate some drug seem to be precipitated outside of liposomes, though the cryo-TEM images do display that the majority of drug precipitate is enclosed in liposome interiors. Considering that the analysis of EE through quantification of drug-to-lipid ratio, the authors argue that the liposome samples by TUDC contain little unencapsulated drugs. Also, it is argued that it cannot exclude the possibility that a few of drug may form precipitates outside of liposomes, due to leakage, during the rapid cooling process for preparing the cryo-TEM samples [41,42].

At present, transmembrane ion gradient method is regarded as the most efficient strategy for loading amphiphilic agents into liposomes [21]. But remote loading hardly works on encapsulation of PWSDs, which also represent a high proportion of therapeutic agents [22]. When loaded into liposomes by conventional method such as thin lipid film-dispersion method, PWSDs often show an EA of a trivial value, since it is entrapped in liposomes via bilayer membrane intercalation [5]. By contrast, the TUDC procedure renders a PWSD in liposome interior which can provide a relatively large room to accommodate enough drugs. Therefore, the novel TUDC procedure is rather feasible for making PWSD liposomes, which possess, especially, a controllable load; and, therefore, the procedure has a practical significance.

5. Conclusions

In this investigation, a temperature up-down cycle (TUDC) procedure has been successfully developed for loading PWSDs into liposomes interiors and proves able to render drug entrapment purposely controllable. In particular, several mathematical equations that can calculate the drug entrapment by TUDC procedure have been derived and can precisely predict the loading results through including the thermodynamic parameters, such as temperature and drug solubilities which can be obtained by deduction instead of by the laborious experimental test. Therefore, the novel procedure of TUDC as well as the established mathematical and thermodynamic equations may present a useful tool to the development of PWSD-delivering liposomes.

Author contributions

The authors state that the author contributions to this work are listed as follows: conceptualization, methodology and supervision by N Wang and T Wang; experimental investigation, software and data analysis, mathematical and thermodynamic model establishment by Y Qi, C Wang, R Qian, Minnan Chen, P Jiang, T Wang and N Wang; data validation and curation by N Wang and T Wang; writing and editing, N Wang and T Wang; resources, funding acquisition, N Wang. Authorship of this report is limited to those who have contributed substantially to the work.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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