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TITLE

Microfluidics Production of Lysolipid-Containing Temperature-Sensitive Liposomes

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Microfluidics, liposomes, thermosensitive, lysolipid, LTSLs, staggered herringbone micromixer, cholesterol-free, indocyanine green (ICG), doxorubicin loading (DOX).

SUMMARY

The protocol presents the optimized parameters for preparing thermosensitive liposomes using the staggered herringbone micromixer microfluidics device. This also allows co-encapsulation of doxorubicin and indocyanine green into the liposomes and the photothermal-triggered release of doxorubicin for controlled/triggered drug release.

ABSTRACT

The presented protocol enables a high-throughput continuous preparation of low temperature-sensitive liposomes (LTSLs), which are capable of loading chemotherapeutic drugs, such as doxorubicin (DOX). To achieve this, an ethanolic lipid mixture and ammonium sulfate solution are injected into a staggered herringbone micromixer (SHM) microfluidic device. The solutions are rapidly mixed by the SHM, providing a homogeneous solvent environment for liposomes self-assembly. Collected liposomes are first annealed, then dialyzed to remove residual ethanol. An ammonium sulfate pH-gradient is established through buffer exchange of the external solution by using size exclusion chromatography. DOX is then remotely loaded into the liposomes with high encapsulation efficiency (> 80%). The liposomes obtained are homogenous in size with Z-average diameter of 100 nm. They are capable of temperature-triggered burst release of encapsulated DOX in the presence of mild hyperthermia (42 °C). Indocyanine green (ICG) can also be co-loaded into the liposomes for near-infrared laser-triggered DOX release. The microfluidic approach ensures high-throughput, reproducible and scalable preparation of LTSLs.

INTRODUCTION

LTSL formulation is a clinically relevant liposomal product that has been developed to deliver the chemotherapeutic drug doxorubicin (DOX) and allows efficient burst drug release at clinically

attainable mild hyperthermia (T ≈ 41 °C)[1]. The LTSL formulation consists of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), the lysolipid 1-stearoyl-2-hydroxy-*sn*-glycero-3-phosphatidylcholine (MSPC; M stands for "mono") and PEGylated lipid 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG_{2000}). Upon reaching the phase transition temperature (T_m ≈ 41 °C), the lysolipid and DSPE-PEG_{2000} together facilitate the formation of membrane pores, resulting in a burst release of the drug[2]. The preparation of LTSLs primarily uses a bulk top-down approach, namely lipid film hydration and extrusion. It remains challenging to reproducibly prepare large batches with identical properties and in sufficient quantities for clinical applications[3].

Microfluidics is an emerging technique for preparing liposomes, offering tunable nanoparticle size, reproducibility, and scalability[3]. Once the manufacturing parameters are optimized, the throughput could be scaled-up by parallelization, with properties identical to those prepared at bench scale[3–5]. A major advantage of microfluidics over conventional bulk techniques is the ability to handle small liquid volumes with high controllability in space and time through miniaturization, allowing faster optimization, while operating in a continuous and automated manner[6]. Production of liposomes with microfluidic devices is achieved by a bottom-up nanoprecipitation approach, which is more time and energy efficient because homogenization processes such as extrusion and sonication are unnecessary[7]. Typically, an organic solution (e.g. ethanol) of lipids (and hydrophobic payload) is mixed with a miscible non-solvent (e.g. water and hydrophilic payload). As the organic solvent mixes with the non-solvent, the solubility for the lipids is reduced. The lipid concentration eventually reaches a critical concentration at which the precipitation process is triggered[7]. Nanoprecipitates of lipids eventually grow in size and close into a liposome. The main factors governing the size and homogeneity of the liposomes are the ratio between the non-solvent and solvent (i.e. aqueous-to-organic flow rate ratio; FRR) and the homogeneity of the solvent environment during the self-assembly of lipids into liposomes[8].

Efficient fluid mixing in microfluidics is therefore essential to the preparation of homogeneous liposomes, and various designs of mixers have been employed in different applications[9]. Staggered herringbone micromixer (SHM) represents one of the new generations of passive mixers, which enables high throughput (in range of mL/min) with a low dilution factor. This is superior to traditional microfluidics hydrodynamic mixing devices[8,10]. The SHM has patterned herringbone grooves, which rapidly mix fluids by chaotic advection[9,11]. The short mixing timescale of SHM (< 5 ms, less than the typical aggregation time scale of 10–100 ms) allows lipid self-assembly to occur in a homogenous solvent environment, producing nanoparticles with uniform size distribution[3,12].

The preparation of LTSLs with microfluidics is, however, not as straightforward compared to conventional liposomal formulations due to the lack of cholesterol[8], without which lipid bilayers are susceptible to ethanol-induced interdigitation[13–15]. Until now, the effect of residual ethanol presents during the microfluidics production of liposomes has not been well understood. The majority of the reported formulations are inherently resistant to interdigitation (containing cholesterol or unsaturated lipids)[16], which unlike LTSLs are both saturated and cholesterol-free.

The protocol presented herein uses SHM to prepare LTSLs for temperature triggered-release drug delivery. In the presented method, we ensured the microfluidics-prepared LTSLs are nano-sized (100 nm) and uniform (dispersity < 0.2) by dynamic light scattering (DLS). Furthermore, we encapsulated DOX using the transmembrane ammonium sulfate gradient method (also known as remote loading)[17] as a validation of the integrity of the LTSL lipid bilayer. Remote loading of DOX requires the liposome to maintain a pH-gradient in order to achieve high encapsulation efficiency (EE), which is unlikely to happen without an intact lipid bilayer. In this presented method, distinctive from typical microfluidic liposome preparation protocols, an annealing step is required before the ethanol is removed to enable the remote loading capability; i.e. to restore the integrity of the lipid bilayer.

As mentioned previously, hydrophilic and hydrophobic payloads can also be introduced to the initial solutions for the simultaneous encapsulation of payloads during the formation of LTSLs. As a proof-of-concept, indocyanine green (ICG), an FDA-approved near-infrared fluorescent dye, which is also a promising photothermal agent, is introduced to the initial lipid mixture and successfully co-loaded into the LTSLs. An 808 nm diode laser is used to irradiate the DOX/ICG-loaded LTSLs and successfully induce photothermal heating-triggered burst release of DOX within 5 min.

All the instruments and materials are commercially available, ready-to-use, and without the need for customization. Since all the parameters for formulating LTSLs have been optimized, following this protocol, researchers with no prior knowledge of microfluidics could also prepare the LTSLs, which serves as the basis of a thermosensitive drug delivery system.

PROTOCOL

1. Equipment setup

1.1. Assemble the syringe pumps and SHM as follows.

1.1.1. Connect the “To Computer” port of the secondary syringe pump (Pump 02, for aqueous solution) to the “To Network” port of the master syringe pump (Pump 01, for ethanol lipid solution) using Pump to Pump network cable (**Figure 1, yellow**).

1.1.2. Connect the “To Computer” port of the master pump to the “RS232 Serial” port of the computer using PC to Pump network cable (**Figure 1, blue**).

1.1.3. Connect tubing to each of the inlets and outlets of the SHM using a nut and ferrule. Convert the terminal of the tubing for both inlets to female Luer using another nut and ferrule and a union assembly. Longer tubing of the inlets allows easier attachment to the syringes (**Figure 2**).

1.2. Set up the **pump control** software.

1.2.1. Assign the address of the master syringe pump and secondary syringe pump to “Ad:01”

and “Ad:02”, respectively, using the “Setup” button of the syringe pump. This only needs to be done for the first time.

1.2.2. Open the **pump control** software on the computer. The two syringe pumps should be detected automatically, followed by a beeping sound. Otherwise, click **Pumps** and **Search for pumps** to update the connection. (**Figure 3**).

1.2.3. Assign **Diameter** to 12.45 (mm) by choosing “HSW Norm-Ject 5 cc (Dia=12.45)”.

1.2.4. Assign **Rate** to 0.25 mL/min for Pump 01 (ethanol lipid solution) and 0.75 mL/min for Pump 02 (aqueous solution). The flow rates correspond to a total flow rate (TFR) of 1 mL/min and aqueous-to-ethanol flow rate ratio (FRR) of 3.

1.2.5. Assign **Volume** to any values above 5 mL.

NOTE: The targeted infusion volume is set greater than the loaded liquid volume considering the void volume of the tubing.

1.2.6. Select **INF** (infusion) mode for both pumps.

1.2.7. Press **Set** to confirm the settings.

2. Prepare the LTSLs

2.1. Prepare a LTSL10 or LTSL10-ICG lipid mixture (see **Table 1**).

2.2. Withdraw 1 mL of lipid mixture and at least 3 mL of $(NH_4)_2SO_4$ solution using two 5 mL Luer lock syringes.

2.3. Install the two syringes onto the syringe pumps in the upright position by sliding the barrel flange of the syringe to the syringe retainer of the pump, and the plunger flange of the syringe to the pusher block of the pump (**Figure 4**).

2.4. Wrap the end of the heating tape to the syringes with the aqueous solution. Wrap the other end of the heating tape and temperature probe of the thermostat around the syringe with the lipid solution. It is helpful to practice this step with empty syringes in place in order to ease the assembly process (**Figure 5A**).

2.5. Connect the two syringes to the female Luer adaptors of the corresponding inlets of the SHM. Make sure the syringes containing the lipid mixture and $(NH_4)_2SO_4$ solutions are connected ethanol inlet and aqueous inlet, respectively. Adjust the plunger position to remove air bubbles from the syringes (**Figure 5B**).

NOTE: Ensure the syringes are still securely positioned onto the syringe retainer of the pumps.

2.6. Heat up the syringes to above 51 °C using the heating tape using a 10 s heating session. Allow the thermostat to update the temperature of the syringes. Repeat this step in the following steps to maintain the temperature during the infusion.

CAUTION: Turn off the heating tape after 10 s to prevent temperature overshoot and allow the thermostat to update the actual temperature. The heating tape should also be handled with care as its temperature rises very quickly. Heating continuously may damage the equipment and syringes, due to the time delay of the thermostat for updating the measured temperature.

2.7. Once the temperature is above 51 °C, run the syringe pumps by pressing **Run All** in the pump control software (**Figure 3**).

2.8. Ensure the fluid flow is free of air bubbles and any leakage. Dispose the initial volume (around 0.5 mL) of liquid from the outlet as waste.

NOTE: This initial waste volume is not definite and depends on the internal volume of the setup, which is the volume for fluid to travel from the syringes through the tubing and SHM to the outlet.

2.9. Collect the rest of the liquid as liposome samples into a microcentrifuge tube or bijou vial.

2.10. Pause/stop the infusion when the liquid in either of the syringes are almost empty.

NOTE: The pumps should be stopped manually, since the pumps may not accurately detect the position when the syringes are empty.

2.11. Place the collected liposome solutions in a 60 °C water bath to anneal for 1.5 h.

NOTE: This step is essential in enabling drug loading into the liposomes.

2.12. Transfer the solutions to dialysis tubes. Dialyze the solutions against 1 L of 240 mM $(NH_4)_2SO_4$ at 37 °C for at least 4 h to obtain purified liposomes.

NOTE: The protocol can be paused here. Liposomes at this step are at 5 mM of phospholipid. Purified liposomes can be stored at 4 °C.

2.13. To clean the SHM for repeated use, flush the SHM sequentially with deionized water, ethanol and dry with nitrogen gas.

3. Remote loading of DOX into LTSLs by transmembrane pH gradient

3.1. Exchange external buffer to HEPES-buffered saline (HBS) by using size exclusion chromatography (SEC) to establish a transmembrane pH gradient .

3.1.1. Add a total of 25 mL of HBS to the top of a SEC column to prepare the column. Allow all eluent to elute through the column and dispose the eluate.

3.1.2. Add 1 mL of dialyzed liposomes, prepared from step 2.12, to the column and dispose the elute.

3.1.3. Add 1.5 mL of HBS to the column and dispose the elute.

3.1.4. Add 3 mL of HBS to the column and collect the 3 mL of elute.

NOTE: The protocol can be paused here. Liposomes are collected at this step and are at 1.67 mM of phospholipid. Buffer exchanged liposomes can be stored at 4 °C.

3.2. Incubate LTSLs with doxorubicin (DOX) and purify LTSLs.

3.2.1. Add DOX solution in 1:20 DOX-to-phospholipid molar ratio into 1 mL of buffer-exchanged liposomes solution (1.67 mmol) contained in a bijou vial. This can be achieved by adding 48.4 µL of 1 mg/mL DOX solution (83.4 µmol).

3.2.2. Place the bijou vial in a 37 °C water bath for 1.5 h to allow DOX loading into the liposomes.

3.2.3. Mix 10 µL of the liposomes with 170 µL of HBS and 20 µL of 1% (v/v) Triton X-100 solution in a black 96-well plate. Repeat for three wells. These wells correspond to the "before purification" DOX content.

3.2.4. In case of preparing LTSL10-ICG, mix 40 µL of the liposomes with 160 µL of DMSO in a clear 96-well plate. Repeat for three wells. These wells correspond to the "before purification" ICG content.

3.2.5. Purify the liposome solution as described in step 3.1.

NOTE: To reuse the column for future purification, clean the column from free DOX by first adding 1 mL of diluted 0.5 M NaOH solution before performing step 3.1.1. Free DOX in red will turn violet-blue and elute through the column quickly.

3.2.6. Mix 30 µL of the purified liposomes solution with 150 µL of HBS and 20 µL of 1% (v/v) Triton X-100 solution in a black 96-well plate. Repeat for three wells. These wells correspond to the "after purification" DOX content.

3.2.7. In case of LTSL10-ICG, mix 40 µL of the purified liposomes solution with 160 µL of DMSO in a clear 96-well plate. Repeat for three wells. These wells correspond to the "after purification" ICG content.

3.2.8. Measure the DOX fluorescence intensity of the wells before (step 3.2.3) and after (step

3.2.6) purification, using a microplate reader (λ_{ex} = 485 nm, λ_{em} = 590 nm).

3.2.9. Calculate the encapsulation efficiency of DOX (DOX EE) by taking the ratio of the fluorescence intensities before and after purification.

$$DOX\ EE(\%) = \frac{\text{F. I. after purification}}{\text{F. I. before purification}} \times 100$$

3.2.10. Measure the ICG absorbance of the wells before and after purification, using a microplate reader (600 to 1000 nm).

3.2.11. Calculate the encapsulation efficiency of ICG (ICG EE) by taking the ratio of the absorbance at 792 nm before and after purification, taking into account the dilution factor (3 times) during the purification.

$$ICG\ EE(\%) = \frac{Absorbance_{\lambda\,=\,792\,nm}\ after\ purification \times 3}{Absorbance_{\lambda\,=\,792\,nm}\ before\ purification} \times 100$$

4. Dynamic Light Scattering (DLS)

4.1. Add 50 µL of liposomes solution (step 2.12) to 450 µL of deionized water in a disposable polystyrene cuvette, to achieve a final salt concentration of 10 mM, which is suitable for DLS measurements.

4.2. Place the cuvette inside the DLS instrument and perform the measurement according to the manufacturer's instructions.

4.3. Record the mean Z-average diameter and dispersity of three measurements for each sample.

5. Differential scanning calorimetry (DSC)

5.1. Concentrate 1 mL of the liposomes samples (step 2.12) with a centrifugal filter unit to 0.5 mL (final lipid concentration of 10 mM). Using a fixed-angle rotor, spin at 7500 x *g* for approximately 15 min.

5.2. Transfer 20 µL of $(NH_4)_2SO_4$ solution and liposomes samples to two respective DSC pans. Seal the pans with DSC hermetic lids using the DSC sample press kit.

5.3. Measure the sample from 30 °C to 60 °C at a heating rate of 1 °C/min using a differential scanning calorimeter.

5.4. Analyze the data with appropriate software. Take the phase transition temperature (T_m) as the onset of the phase transition (melting peak), which is measured by the x-intercept of the tangent of the point of maximum slope.

6. Doxorubicin release

6.1. Preheat HBS at designated temperature (37 or 42 °C) using a water bath. Prepare an ice water bath for quenching the samples.

6.2. Add 100 µL of purified DOX-loaded liposomes (step 3.2.5) into 1.9 mL of HBS in a microcentrifuge tube. Place the tube into the water bath of the designated temperature.

6.3. Withdraw immediately 200 µL of samples from the tube and quickly place it in the ice water bath to quench any subsequent drug release. This sample corresponds to the initial (t = 0) time point.

6.4. Withdraw 200 µL of samples at subsequent time points (t = 5, 10, 15, 30, 60 min) and quickly place it in the ice water bath to quench any drug release.

6.5. Mix 50 µL of sample of each time point with 150 µL of HBS in a black 96-well plate. Measure the DOX fluorescence intensity using a plate reader.

6.6. Add 20 µL of 1% (v/v) Triton X-100 into random selected wells prepared in step 6.5. Measure the DOX fluorescence intensity of these wells using a plate reader. These values correspond to the fully released (t = ∞; 100% release) time point.

6.7. Calculate and plot the percentage of DOX released by interpolating the fluorescence intensity of each time points ($I(t)$), compared to the initial and fully released) value.

$$DOX\ Release(\%) = \frac{I(t) - I(0)}{I(\infty) - I(0)} \times 100$$

7. Laser Heating and Triggered Release

7.1. Set water bath temperature to 37 °C and allow the temperature to stabilize.

7.2. Add 200 µL of DOX loaded LTSL10-ICG ([ICG] = 10 µg/mL) to a clear 96-well plate, then place it in the water bath, keep the bottom immersed in water.

7.3. Set the current of the laser system to 2.27 A. Place the collimator of the laser system at 5 cm vertically above the surface of the 96-well plate, which corresponds to an energy flux of 0.5 W/cm^2 [**Figure 6**].

CAUTION: The laser system should be operated in compliance with relevant laser safety measures.

7.4. Switch on the laser and monitor the temperature using a fiber optic temperature probe every minute.

7.5. At the time points of 5 min and 10 min, withdraw 10 µL of the laser-irradiated LTSL10-ICG solution and mix with 190 µL of HBS in a black 96-well plate. Repeat for three wells. These wells correspond to the "laser-induced release" DOX content.

7.6. Mix 10 µL of the liposomes with 190 µL of HBS in black 96-well plate. Repeat for three wells. These wells correspond to the "0% released" DOX content.

7.7. Mix 10 µL of the liposomes with 170 µL of HBS and 20 µL of 1% (v/v) Triton X-100 solution in a black 96-well plate. Repeat for three wells. These wells correspond to the "100% released" DOX content.

7.8. Measure the percentage of DOX release as described in step 6.7.

REPRESENTATIVE RESULTS:

The preparation of LTSLs by microfluidics requires the lipid composition of DPPC/MSPC/DSPE-PEG_{2000} (80/10/10, molar ratio; LTSL10). **Figure 7A** (left) shows the appearance of as-prepared LTSL10 from step 2.9, as a clear and non-viscous liquid. LTSL10 formulation is developed from the conventional formulation, LTSL4 (DPPC/MSPC/DSPE-PEG_{2000}, 86/10/4, molar ratio) since LTSL4 forms a gel-like viscous sample, as indicated by the large amount of air bubbles trapped in the sample (**Figure 7A**; right).

DLS measurement of LTSL10 (**Figure 7B**, red) showed that the Z-Average diameter and dispersity of LTSL10 were 95.28 ± 7.32 nm and 0.100 ± 0.022, respectively, indicating the success of the experiment. **Figure 7B** (gray) also shows a suboptimal sample, which was prepared at 20 °C, where larger and more dispersed liposomes were obtained.

Figure 7C shows that the DOX EE of LTSL10. DOX EE should usually be around 80%. LTSLs prepared by the conventional method of lipid film hydration with extrusion (LF) are included for comparison, prepared as described elsewhere[18]. DOX EE of LTSL4 (LF) and LTSL10 (LF) showed decent DOX loading of around 70% and 50%, respectively. Annealing of as-prepared LTSL10 (step 2.11) is essential to enable DOX loading. In the absence of the annealing step, low DOX EE (< 20%) was persistent, regardless of incubation temperature (20 °C to 42 °C) and duration (1 to 24 h). This indicated the failure of LTSL10 to maintain a transmembrane pH gradient, where DOX was instead loaded passively or by adsorption. By annealing the as-prepared LTSL10, DOX EE increased significantly to a mean of 85%, indicating the success of the remote loading of DOX and the presence of the transmembrane pH gradient.

Figure 7D shows the DOX release profile of LTSL10. At 37 °C, the release of encapsulated DOX over 60 min was about 10%. In contrast, at 42 °C, all of the encapsulated DOX was released within 5 minutes, demonstrating the temperature-sensitivity of LTSL10. Similar results were observed with LTSL10 (LF) as a control.

Figure 8 shows the phase transition temperature (T_m) of LTSL10 characterized using differential scanning calorimetry (DSC). Dotted lines as tangent of the point of maximum slope, are added as

a visual aid of the onset phase transition temperature (x-intercept of the tangent line). LTSL10 has a relatively broad phase transition with onset at 41.6 °C and peak at 42.6 °C. Similar results were observed with LTSL10 (LF), suggesting a minor difference between the preparation techniques. As a comparison, LTSL4 (LF) has a lower and sharper phase transition, in agreement with the literature[1].

Figure 9 shows the characterization of LTSL10-ICG. Effect of initial ICG concentration on size (**Figure 9A**) and loading efficiencies of DOX and ICG (**Figure 9B**) are categorized into three concentration ranges. At low ICG concentration (ICG-to-lipid molar ratio of 0.003; initial concentration of 60 µM ICG and 20 mM lipid), Z-average, dispersity and DOX EE were similar to LTSL10 without ICG loading; ICG EE was around 75%. The efficient co-loading of DOX and ICG into LTSL10 can be achieved at this ICG concentration. At intermediate ICG concentrations, while the size and dispersity of the samples were satisfactory, both DOX and ICG EE were reduced. In particular, the decrease in DOX EE indicated the disruption of the liposomal membrane and thus, the pH-gradient. At high ICG concentrations, samples were again gelled; DOX and ICG EE were both significantly decreased.

LTSL10-ICG was irradiated with near-infrared laser (section 7) to induce photothermal heating and triggered the release of DOX (**Figure 10**). Upon laser irradiation, the sample first heated up to 49.7 °C with a gradual reduction of temperature. Subsequent laser irradiation increased the temperature to 36.7 °C. Quantification of the released DOX indicated that a complete burst release of encapsulated DOX was achieved after the first heating cycle. This was as expected since the temperature reached above 42 °C, in agreement with the DOX release profile shown in **Figure 7D**. In contrast, LTSL10 without ICG cannot provide photothermal heating, and thus did not release DOX upon laser irradiation.

FIGURE LEGENDS

Figure 1: Photograph of the syringe pumps setup. The “To Network” port of the master pump (Pump 01) is attached to the “To Computer” of the secondary pump (Pump 02; yellow); the “To Computer” port of the master pump is attached to the RS-232 port of the computer (blue).

Figure 2: Photograph of the SHM setup. (**A**) Assembled view of the SHM setup. (**B**) Exploded view of the SHM setup. Inlets and outlets of the SHM are connected to tubing using a nut and ferrule. The tubing of both inlets is extended by a longer tubing with nut and ferrule on each end, terminated by a female Luer adapter using a union assembly.

Figure 3: Interface of the pump control software. The two syringe pumps should be detected automatically upon initiating the software; otherwise, click **Pumps** on the top left corner and **Search for pumps**. Parameters to be configured are highlighted in red boxes.

Figure 4: Photograph of the syringe pumps and installation of a syringe. (**A**) Syringe retainer bracket and syringe retainer thumbscrew (2, one on each side) of the syringe pump (yellow box). Pusher block, adjustment thumbscrew, and drive-nut button (white button on the left) of the syringe pump (green box). (**B**) Position of an installed syringe on the syringe pump.

Figure 5: Photograph of the assembly of syringes, heating element and SHM. (**A**) Assembly of syringes, heating tape, and thermostat. (**B**) Assembly of syringes, heating tape, thermostat, and SHM.

Figure 6: Photograph of the laser setup. (**A**) Photograph of the fiber coupled laser system during operation. (**B**) Position of the collimator 5 cm above the 96-well plate.

Figure 7: Characterization of LTSL10. (**A**) Photograph of (left) LTSL10 and (right) LTSL4, before dialysis. LTSL10 appeared as clear and non-viscous liquid, while LTSL4 was gel-like and viscous. (**B**) Z-average diameter and dispersity of 10 mM of LTSL10 prepared at 20 and 51 °C. Solid bars and open circles (○) indicate Z-average diameter and dispersity, respectively. (**C**) DOX EE of LTSL4 (LF; white), LTSL10 (LF; white), and LTSL10 before (gray) and after annealing (red). (**D**) DOX release of LTSL10 (circle) and LTSL10 (LF; cross) at 37 °C (black) and 42 °C (red). Data represent mean ± SD of at least three independent experiments. ***$p < 0.001$; two-tailed unpaired t-tests.

Figure 8: Thermal properties of LTSL10. Thermographs of LTSL4 (LF), LTSL10 (LF), and LTSL10 characterized by DSC. Dotted lines are added as a visual aid of the onset phase transition temperature. Data represent the mean of at least three independent experiments.

Figure 9: Characterization of LTSL10-ICG. (**A**) Z-average diameter (red) and dispersity (blue) of ICG-loaded LTSL10. (**B**) DOX EE (red) and ICG EE (green) of ICG-loaded LTSL10. Data represent the mean ± SD of at least three independent experiments.

Figure 10: Laser-induced photothermal heating triggered the release of DOX -loaded LTSL10 and LTSL10-ICG. (**A**) The temperature of irradiated samples and (**B**) DOX release of DOX-loaded LTSL10 (blue) and LTSL10-ICG (red) during the laser-induced photothermal heating. Data represent the mean ± SD of at least three independent experiments.

Table 1: Lipid mixtures, buffers, and stock solutions.

DISCUSSION

The presented protocol describes the preparation of low temperature-sensitive liposomes (LTSLs) using a staggered herringbone micromixer (SHM). The LTSL10 formulation enables temperature-triggered burst release of doxorubicin within 5 minutes at a clinically attainable hyperthermic temperature of 42 °C. Indocyanine green (ICG) can also be co-loaded for photothermal heating triggered the release of DOX. The method relies on: (i) self-assembly of phospholipids into liposomes under a homogenized solvent environment provided by the rapid, chaotic mixing of ethanol and ammonium sulfate solution in the SHM[11]; (ii) annealing of the liposomes to preserve the integrity of the lipid bilayer essential for DOX loading; and (iii) remote loading of DOX into LTSLs by an ammonium sulfate pH-gradient[17]. Since the equipment utilized in this protocol is commercially available off-the-shelf and the parameters are optimized, this approach is manageable for users without prior knowledge or microfluidic experience.

One of the most critical steps within the protocol is to ensure the whole assembly is properly secured and fluid can be properly infused (step 2.5). Since the reproducibility of the self-assembly of liposomes relies on a homogenized solvent environment, any instability, such as dislodgement of syringes or introduction of air bubbles, will disturb the stability of the fluid flow and result in suboptimal liposome size and dispersity. This is also the rationale behind step 2.8, where the volume, consisting of the fluid initially occupying the channel and before a stable flow is reached, should be disposed of.

A second critical step for a successful experiment is the annealing step, to enable high DOX EE (step 2.11). In cholesterol-free liposomes, micelle-forming membrane components (i.e. MSPC and DSPE-PEG_{2000}) will accumulate at grain boundaries with a high degree of defects to accommodate a high membrane curvature[2]. These arrangements thermodynamically favor the formation and stabilize membrane pores, opened liposomes, or bilayer discs. The low DOX EE of LTSL10 without annealing suggested that porous structures existed even below T_m, resulting in the absence of the pH gradient required for DOX loading (**Figure 7C**). The premature formation of pores below T_m was not observed for LTSLs prepared by lipid film (LTSL4 (LF) and LTSL10 (LF)), where annealing is not required. Furthermore, cholesterol-containing formulations prepared by microfluidics also do not require annealing[8]. It is, therefore, speculated that the premature formation of pores is a combined effect of the presence of ethanol during the preparation and the lack of cholesterol in the lipid bilayer. Structural defects within the bilayer membrane have been reported to be eliminated by annealing the liposomes above T_m, allowing lipid molecules to redistribute homogeneously and defects to be corrected[19]. In addition, the annealing process is an irreversible process where annealed liposomes returning to a temperature below T_m do not recreate leaky vesicles[19], in agreement with the annealed LTSL10.

The nature of microfluidic preparation of liposome is a nanoprecipitation process, which requires two miscible solvents with distinctive solubility for the lipids: typically, ethanol (as a lipid solvent) and aqueous solution (as a lipid non-solvent). Thus, the presence of ethanol is unavoidable. Therefore, formulations that are sensitive or prone to alcohol-induced interdigitation[13], such as cholesterol-free liposomes[20], may require modification of the formulation or re-optimization of the protocol. As demonstrated with the preparation of LTSL4, the highly viscous gel was obtained, (**Figure 7A**), which was likely due to the formation of an interdigitated gel phase[15]. On the other hand, LTSL10, with its higher polymer concentration that prevents interdigitation[21], was prepared successfully. Consequently, an ethanol removal procedure also must be performed; here, it was removed simply by dialysis. While on-chip continuous purification techniques such as tangential flow filtration (capable of both ethanol removal and buffer exchange) have been developed[22, 23], their implementation (as one-chip or modular) are beyond the aim of this protocol. Nonetheless, in the future, we expect these modular or standardized designs to be optimized and increased in availability, streamlining the microfluidic production process.

Another limitation of the protocol is the sample loss due to the travelling distance of the liquids, namely the initial waste volume (step 2.8) as well as the last fraction of solutions that would be injected but wouldn't reach the outlet. These sample losses are almost unavoidable and may contribute to a significant portion of the preparation volume at bench-scale production,

especially when a small volume or precious samples are to be prepared. When necessary, the lipid recovery could be quantified by high performance liquid chromatography-evaporative light scattering detector method that enables rapid quantification of lipid concentrations[24]. However, once the process is optimized and scaled up, such as by using a larger syringe or fluid reservoir, the throughput could be further scaled up and sample losses would be less significant.

The main difference between this method and the existing preparation method is that liposomes are self-assembled in a controllable solvent environment in a high-throughput, continuous manner. The lipid film method is a batch manufacturing process and requires size homogenization. While very feasible at a bench-scale, it remains challenging to scale up for clinical production. Within existing microfluidic techniques, for instance, microfluidic hydrodynamic focusing, SHM offers a shorter mixing timescale[11] and a greater throughput (in the range of mL/min) with lower dilution factor; notwithstanding the preparation of LTSLs has not been reported using other microfluidic devices so far. The major advantage of our approach is the high-throughput, scalable production of thermosensitive liposomes.

Thus far, the microfluidics protocol offers continuous production of LTSL10 with drug loading capability. Payloads other than DOX and ICG are also viable. However, ethanol removal by dialysis, drug remote loading, and purification by SEC column remain as batch processes and are the bottlenecks of the overall formulation process. Future development could focus on utilizing microfluidic approaches (such as tangential flow filtration) to enhance the throughput of these downstream processes and increase the scalability of the protocol.

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DISCLOSURES

The authors have nothing to disclose.

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