Electronic Supplementary Information

A novel combined method of thin-film evaporation and supercritical carbon dioxide technique to prepare fluorescent siRNA-liposome

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Experimental section

Materials
SiRNA was purchased from Sangon Biotech (Shanghai) Co., Ltd., folic acid was purchased from Aladdin industrial co. (Shanghai, China), 10,12-pentacosadiynoic acid (10,12-PCDA) was obtained from Alfa Aesar (Tianjin, China), N-(3-Dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC) was purchased from Adamas Reagent Co. Ltd (Shanghai, China), Egg phosphatidyleholine (EPC) and Dodecylamine were purchased from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China), Pyrene (98%) was purchased from Sigma-Aldrich Co. LLC. (Shanghai, China), Docetaxel (DTX) was applied by Knowshine Pharmachemicals Inc. (Shanghai, China), Dulbecco’s modified eagle medium (DMEM), fetal bovine serum (FBS) and 0.25% trypsin were purchased from Invitrogen corporation, (New York, USA). Minimum essential medium (MEM) was purchased from Thermo Fisher Scientific Inc. (Beijing, China). Fluorescent nucleic acid probe KGM025R, penicillin–streptomycin and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Keygen Biotech Inc. (Nanjing, China). All other reagents and solvents were obtained from Shanghai Lingfeng Chemical Reagent Co. Ltd and used without further purification.

TE-scCO$_2$ method
A balanced amount of EPC, PCDA, DTX and siRNA (molar ratio of EPC to siRNA was 103:1) were dissolved in chloroform methanol mixture (chloroform : methanol = 9:1) in a lightproof flask. Then the organic solvents in the mixture were removed using a vacuum rotary evaporator, and a thin uniform translucent film was formed on the flask walls in a dark environment. Afterwards, the film was irradiated by UV light for 3 min, and then an appropriate amount of double distilled water, which was pre-treated with diethyl pyrocarbonate and steam sterilizing, was added into the flask to dissolve the film. The resulted liposomal suspension was transferred to a stainless steel autoclave, and incubated under temperature of 37 ℃ and pressure of 18 MPa for an hour in scCO$_2$. The organic solvents were removed after depressurization, and a transparent liposome solution was obtained. The liposomal solution was further stirred overnight to achieve self-assembly and to remove remaining CO$_2$ in the liposome solution at room temperature and pressure. At last a clear and stable liposome solution was obtained.

Thin-film hydration method
EPC, PCDA and FAD (weight ratio 8:1:0.8) were dissolved in chloroform methanol mixtures (chloroform : methanol = 9:1). The organic solvent was removed under a vacuum rotary evaporator to yield a thin uniform translucent film, and the film was further dried under nitrogen flow. An appropriate amount of double distilled water was added to dissolve the lipid bilayers. Above processes were carried out under dark condition to avoid PDA polymerization. Then the liposome suspension was exposed to UV irradiation (254 nm) for few minutes, and was heated to 65 ℃ under strong stirring for 10 min. The resulted liposomal suspension was cooled to room temperature and further incubated for 4h. For entrapment of DTX, DTX methanol solution was added to the chloroform methanol mixtures at beginning.

Ethanol injection method
EPC was dissolved in ethanol to obtain lipid solution. FAD, PCDA and DTX were dissolved in as few amount as possible mix solvents (chloroform : ethanol = 9:1). This mixed solution was added slowly to lipid ethanol solution to ensure no components separate out. Lipid ethanol solution was then injected to siRNA-water solution under vigorous stirring at 50 ℃. The suspension was kept at 50 ℃ overnight to let ethanol volatilize. Resulted transparent solution was stood at room temperature for 4 hours before characterization.

**Characterization of liposomes**

**Evaluation of size distribution and zeta potential**
The Z-average particle sizes and zeta potentials of FDSL was measured by using a dynamic laser light scattering technique (zeta-sizer ZEN 3600, Malvern instruments Ltd, UK). The concentration of lipid was kept constant at 0.1 mmol/L in double distilled water.

**Determination of siRNA encapsulation efficiency**
Into a total volume of 2.0 mL liposome solution was added 0.4 µL of KGM025R from a DMSO stock solution. Fluorescence was measured on FLSP 920 spectrofluorometer, with excitation and emission wavelengths of 535 and 615 nm, respectively. Percent entrapment was calculated as 100% minus the fluorescence signal of KGM025R divided by the fluorescence following the addition of 20 µL of 100 mmol/L Triton X-100 to release siRNA from the liposome. The result was corrected for the effect that Triton X-100 has on the fluorescence of KGM025R.

**Determination of the micropolarity**
Ethanol solutions of pyrene (5 µmol/L) were added to the lipid solutions before thin film formation. The amount of pyrene solution varied with respect to the volume of liposome solution that to be prepared. But in general, the concentration of pyrene should be kept at 5 nmol/L at last. After the preparation of liposome solution, fluorescence measurements were performed with a FLSP 920 (Edinburgh, UK) spectrofluorometer using excitation at 336 nm and fluorescence scanning from 360 to 520 nm. The ratio $I_1/I_{III}$ corresponding to the peaks at 373 nm (I) and 383 nm (III) was calculated.

**Transmission electron microscope (TEM)**
The morphology and size of FDSL was observed via a transmission electron microscope (TEM, JEM-1400EX JEOL Ltd, Japan). Briefly, a drop of sample suspension was placed onto a carbon-coated copper grid and then air-dried. After that, the grid was negatively stained with 2% phosphotungstic acid for 1 min and air-dried before observation.

**Determination of DTX encapsulation efficiency**
The docetaxel encapsulated in the liposomes was measured by HPLC (Agilent LC1200, America). A reverse-phase HPLC column (Agilent Eclipse XDB-C18, 4.6 mm* 150 mm, 5 µm) was used. Briefly, 2 mL of only DTX loaded FDSL solution were centrifuged, the precipitation was mixed with 5 mL of methanol for liposomes disruption and dissolved in mobile phase consisting of methanol, acetonitrile and deionized water (30:40:30, v/v) to 10 mL. The solution was filtered through 0.45 µm syringe filter before transferred into HPLC vial. The flow rate of mobile phase was set at 1.0 mL/min. The column effluent was detected with a UV/Vis detector at 230 nm under
25 °C. The amount of entrapped docetaxel was determined according to the calibration curve and the drug encapsulation efficiency was defined as the ratio between the amount of docetaxel encapsulated in the liposomes and that added in the liposomes preparation process.

**Calculation of drug loading amount (w/w percentage of incorporated drugs to lipids of formulated liposomes)**

In the above method of DTX EE determination, precipitation of centrifuged sample was obtained. The precipitation was dried to get the total mass of formulated liposomes. The drug loading amount could be calculated as

$$\omega = \frac{EE\% \times m_0}{m_0 - EE\% \times m_0} \times 100\%$$

EE% was the encapsulation efficiency of DTX or siRNA, $m_0$ was the total mass of formulated liposomes. Drug loading amount of DTX and siRNA were calculated respectively.

**UV and fluorescent property of FDSL**

UV absorption spectrum was recorded on a UV-2450 spectrometer (Shimazu, Japan). 2 mL liposome solution was placed in a 1 cm glass cuvette, and the scanning range was from 450 nm to 700 nm.

Fluorescent spectra were obtained by using a FLSP 920 spectrofluorometer with 1.5 mL FDSL in a 1 cm quartz cuvette. For emission spectrum, the excitation light was set at $\lambda_{ex}=540$ nm and scanning range was set from 570 nm to 800 nm.

**Cell culture and maintenance**

Human breast carcinoma Bcap-37 line was purchased from American type culture collection (ATCC, Virginia, America) and cultured in folate-free DMEM media, supplemented with 50 $\mu$g/mL penicillin, 50$\mu$g/mL streptomycin and 10% FBS and maintained in a humidified atmosphere containing 5% CO$_2$ at 37 °C. The medium was changed every 2 or 3 days before it turned yellow and the cells were passaged by trypsinization.

Hs578Bst cell line was purchased from Bioleaf Biotech Co., Ltd, (Shanghai, China) and cultured in folate-free MEM media. Other conditions were the same as used in Bcap-37 cell line.

**Comparative cytotoxicity studies of drug loaded liposomes**

Cell viability was tested using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay based on the cleavage of yellow tetrazolium salt MTT by metabolically active cells to form an orange formazan dye which was quantified using microplate reader (Biorad, USA, model 550). Cells were seeded in 96 well microliter plates at 1*10^4 cells per well, followed by overnight incubation. Supernatants from the wells were aspirated out and replaced with fresh aliquots of growth medium containing various liposomes with different DTX concentration and different components. The solutions were further incubated for 24h at 37 °C. Subsequently, MTT reagent (20 $\mu$L, 5mg/mL) was added to each well, incubated for 4h. Dimethyl sulphoxide (150 $\mu$L) was added in each well after aspirating out the supernatant and shaked for 10 min. Absorbance at 490 nm was recorded using microplate reader.

**Cell uptake and intracellular distribution studies of liposomes by LSCM and flow cytometry**
Bcap-37 and Hs578Bst cells were transferred separately to glass-bottom culture dishes at $1 \times 10^5$ cells per dish and incubated overnight at 37 ºC. The culture medium was replaced with FDSL and then incubated for 6 h at 37 ºC in medium. The culture medium was then removed and each dish was washed with cold PBS solution and the fluorescent images of the cells were observed using a laser scanning confocal microscopy (A1R, Nikon, Japan). The excitation wavelength was 561 nm, and emission scanning range was 600-680 nm. Then 100 mL trypsin was added to detach the cells. Detached cells were collected by centrifuging and resuspended in 500 mL culture medium and analyzed by the flow cytometer (FACS-Calibur, BD Biosciences).

**Particle sizes and zeta potentials of FDSL**

<table>
<thead>
<tr>
<th>Method</th>
<th>Z-average size (nm)</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thin film evaporation</td>
<td>191.1</td>
<td>-24.8</td>
</tr>
<tr>
<td>TE-scCO₂ method</td>
<td>170.6</td>
<td>-36.5</td>
</tr>
<tr>
<td>Ethanol injection method</td>
<td>78.4</td>
<td>-15.6</td>
</tr>
</tbody>
</table>

**Micropolarity of the membrane**

![Figure S1. Micropolarity of liposome membrane with pyrene as a probe.](image)

$I_1/I_{III}$ was calculated for blank liposome and FDSL separatively. Blank liposome showed a value of 1.388, while FDSL showed a value of 1.172

**Drug loading amount of DTX and siRNA**

The drug loading amount of DTX was 14.1 ±5.3%, and was 5.7 ±1.1% for siRNA.

**The UV-Vis spectrum of siRNA-water solution under UV irradiation**
The $A_{260}$ intensity and $A_{260}/A_{280}$ value of siRNA were both kept almost unchanged after UV irradiation for 10 min. Under experimental conditions in this work, UV irradiation did not cause obvious damage to siRNA.

**Figure S2.** UV-Vis spectrum of siRNA-water solution before and after UV irradiation