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RESEARCH PAPER

Formulation and Pharmacokinetics of Thermosensitive Stealth® Liposomes Encapsulating 5-Fluorouracil

Chantal Al Sabbagh • Nicolas Tsapis • Anthony Novell • Patricia Calleja-Gonzalez • Jean-Michel Escoffre • Ayache Bouakaz • Hélène Chacun • Stéphanie Denis • Juliette Vergnaud • Claire Gueutin • Elias Fattal

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ABSTRACT

Purpose We optimize the encapsulation and investigate the pharmacokinetics of 5-Fluorouracil (5-FU) delivered by thermosensitive stealth® liposomes (TSLs) designed to trigger drug release upon hyperthermia using focused ultrasound (FUS).

Methods 5-FU was encapsulated into liposomes made of 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine/cholesterol/1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-N-PEG_{2000} either as a free molecule or complexed with copper-polyethylenimine. Heat-triggered drug release was evaluated using either a water bath or FUS. Formulation cytotoxicity was assessed on HT-29 cell line by MTS assay. Pharmacokinetics and biodistribution of 5-FU were evaluated in HT-29-tumor bearing mice.

Results 5-FU was easily encapsulated using the lipid hydration method (encapsulation efficacy of 13%) but poorly retained upon dilution. 5-FU complexation with copper-polyethylenimine improved 5-FU retention into liposomes and allowed to obtain an encapsulation efficacy of 37%. At 42°C, heat-triggered 5-FU release from TSLs was 63% using a water bath and 68% using FUS, within 10 min, whereas it remained below 20% for the non-thermosensitive formulation. The MTS assay revealed that formulation toxicity arose from 5-FU and not from the excipients. In addition, 5-FU complex encapsulation into TSLs induces a reduction of the IC_{50} from 115 down to 49 μM. Pharmacokinetics reveals a longer circulation of encapsulated 5-FU and a more important body exposure, although tumor passive targeting is not significantly higher than free 5-FU.

Conclusions Complexation of 5-FU with copper-polyethylenimine appears an interesting strategy to improve 5-FU retention into TSLs *in vitro* and *in vivo*. TSLs allow heat-triggered release of the drug within 10 min at 42°C, a reasonable time for future *in vivo* experiments.

KEY WORDS 5-Fluorouracil · copper-polyethylenimine complex · focused ultrasound · pharmacokinetics · thermosensitive stealth® liposomes

ABBREVIATIONS

[2-^{14}C]-5-FU	^{14}C-radiolabeled 5-Fluorouracil
5-FU	5-Fluorouracil
5-FU-Cu-PEI	5-Fluorouracil-copper-polyethylenimine complex
CHOL	Cholesterol
Cu	Copper (II) acetate monohydrate
Cu-PEI	Copper-polyethylenimine complex
d_H	Hydrodynamic diameter
DLS	Dynamic light scattering
DPPC	1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine
DSPE-PEG_{2000}	1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-N-PEG_{2000}
EDTA	Ethylenediaminetetraacetic acid
EPR	Enhanced permeation and retention
FBS	Fœtal bovine serum
FDA	Food and drug administration
FUS	Focused ultrasound
g	Gravitational acceleration
HPLC	High performance liquid chromatography
HT-29	Human colorectal adenocarcinoma cells

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C. Al Sabbagh • N. Tsapis • P. Calleja-Gonzalez • H. Chacun • S. Denis • J. Vergnaud • C. Gueutin • E. Fattal (✉)
Univ Paris-Sud, Institut Galien Paris-Sud, LabEx LERMIT, Faculté de Pharmacie, 5 rue J.B. Clément, 92296 Châtenay-Malabry, France
e-mail: elias.fattal@u-psud.fr

C. Al Sabbagh • N. Tsapis • P. Calleja-Gonzalez • H. Chacun • S. Denis • J. Vergnaud • C. Gueutin • E. Fattal
CNRS UMR 8612, Institut Galien Paris-Sud, LabEx LERMIT, 5 rue J.B. Clément, 92296 Châtenay-Malabry, France

A. Novell • J.-M. Escoffre • A. Bouakaz
Université François-Rabelais de Tours, Inserm, Imagerie et Cerveau UMR U 930, Tours, France

IC_{50}	Half maximal inhibitory concentration
LTSLs	Low temperature sensitive liposomes
MTS	CellTiter 96® AQueous One Solution Cell Proliferation Assay
NTSLs	Non thermosensitive liposomes
PBS	Phosphate buffer saline
PDI	Polydispersity index
PEG_{2000}	Polyethylen glycol 2 KDa
PEI	Polyethylenimine, ethylendiamine branched
psi	Pound per square inch
T_m	Phase transition temperature
TSLs	Thermosensitive liposomes
TTSLs	Traditional thermosensitive liposomes
UV/VIS	Ultraviolet/visible light domains
ζ-potential	Zeta potential

INTRODUCTION

Liposomes with sizes below 1 μm have been extensively investigated as drug delivery carriers for anti-cancer chemotherapy. In particular, three anthracycline-loaded liposomes Myocet®, Daunoxome®, Doxil®/Caelyx® and anti-metabolite-loaded liposomes Depocyt® were approved by the FDA for tumor treatment. Many additional liposome-based anti-tumor nanomedicines are currently in clinical trials or in early- or late-stage preclinical development (1). Their development relies mainly on the enhanced permeation and retention effect (EPR effect) allowing the passive extravasation and intratumoral accumulation of nanomedicines in solid tumors (1,2). Passive targeting of tumors however remains limited since the proportion of nanomedicines usually reaching the tumor site is below 10% of the injected dose (3). In addition, the extent of tumor vascularization and its porosity depend on the tumor type and its stage of development (1). Finally, although observed in the clinics, the EPR effect seems to be limited to certain patients (4) and other strategies should be considered to increase nanomedicine accumulation in solid tumors.

Among strategies to increase drug concentration in tumors, ultrasound-mediated drug delivery can be considered. The idea is to trigger drug release by application of ultrasound waves locally on the tumor as liposomes circulate to increase drug concentration in the tumor (5). Ultrasound-mediated drug delivery can be triggered either by cavitation which leads to chemical disruption of the carrier (6) or by mild hyperthermia (up to 42°C) on thermosensitive carriers (7,8). Thermosensitive liposomes (TSLs) were first suggested by Yatvin *et al.* in the late 1970s (9). Liposome thermosensitivity arises from the property of some lipids to possess a phase transition temperature (T_m) higher than physiological temperature. Above the phase transition temperature, lipids exhibit a conformational change leading to liposome rupture and release of the encapsulated drug. ThermoDox® (Celsion corporation), a temperature-sensitive PEGylated liposomal doxorubicin, is already in phase III for unresectable hepatocellular carcinoma, and in earlier phases of clinical trials for other tumor types (ClinicalTrials.gov; Identifier: NCT00617981).

Among anti-tumor molecules, 5-Fluorouracil (5-FU) remains for more than 50 years the most effective chemotherapeutic agent in the treatment of colorectal cancer and forms the central component in the FOLFIRI regimen in combination with irinotecan, and in the FOLFOX regimen in combination with oxaliplatin (10,11). However, 5-FU suffers from several drawbacks: a short plasmatic half-life after intravenous bolus administration (11.4 min), drug clearance from plasma within 1 h as a consequence of a very rapid metabolism by the dihydropyrimidine dehydrogenase or uracil reductase enzymes (12). In addition, 5-FU treatments lead to the development of drug resistance by tumor cells, thereby requiring high doses that lead to severe side effects, including gastrointestinal, haematological, neuronal and dermatological effects, pancytopenia, and cardiotoxicity (13). To overcome these limitations, 5-FU has been encapsulated into liposomes to prolong its circulation time, reduce the associated side effects, improve its therapeutic index and efficacy, and favor drug accumulation into tumors thanks to the enhanced permeation and retention effect (14–18).

Our goal is to optimize the formulation of PEGylated TSLs encapsulating 5-FU. PEGylated lipids will confer stealth properties to the formulation, leading to a prolonged systemic circulation (19). As a thermosensitive lipid, we have chosen dipalmitoylphosphatidylcholine (DPPC) because it exhibits a phase transition temperature T_m of 41.5±0.5°C (20), in the adequate range for thermosensitive formulations. Cholesterol (CHOL) was also included to promote *in vivo* stability (20–22). We have compared formulations encapsulating 5-FU either free or as a ternary complex with copper (II) and low molecular weight polyethylenimine (5-FU-Cu-PEI) as suggested before (17). 5-FU release upon mild hyperthermia was performed using either a water bath or focused ultrasound (FUS). Formulations toxicity on HT-29 colorectal carcinoma cells was evaluated. Finally, the pharmacokinetics and biodistribution of the optimized formulation and controls were determined using radiolabeled 5-FU in HT-29-tumor bearing mice. To the best of our knowledge, this work constitutes the first evaluation of a thermosensitive 5-FU formulation.

MATERIALS AND METHODS

Materials

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC, T_m=41.5 ±0.5°C) was purchased from Genzyme (Liestal, Switzerland)

and 1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-N-PEG_{2000} (DSPE-PEG) from Avanti Polar Lipid Inc. (Alabaster, Alabama, USA). Cholesterol (CHOL), 5-Fluorouracil (5-FU, purity ≥99%), Copper (II) Acetate monohydrate (Cu), polyethylenimine, ethylendiamine branched (PEI, MW = 800 g/mol) and phosphate buffer saline (PBS) were obtained from Sigma (St Quentin-Fallavier, France). [2-^{14}C]-5-FU was supplied by Moravek Biochemicals, Inc. (Brea, California, USA). Hionic-Fluor and Ultima Gold Scintillation fluids were purchased from Perkin Elmer (Perkin Elmer, Inc., Waltham, Massachusetts, USA). Sephadex G-50 size exclusion gel was purchased from Pharmacia AB, Laboratory Separation Division (Upssala, Sweden). Solvents used, were of analytical grade. All chemical substances and solvents were used without further purification. Water was purified using a RIOS/Milli-Q system from Millipore (Molsheim, France).

5-FU Liposomes

5-FU-Loaded Liposomes Preparation

Liposomes were composed of DPPC/CHOL/DSPE-PEG. Two liposomal formulations were produced: DPPC/CHOL/DSPE-PEG (90:5:5 mol%) and DPPC/CHOL/DSPE-PEG (65:30:5 mol%). The molar percentage of DSPE-PEG was fixed at 5% since it has been shown that this ratio leads to satisfyingly stealth liposomes (19). Liposomes were prepared by the lipid hydration method followed by extrusion and ultracentrifugation to remove non-encapsulated compounds (23). Briefly, lipid mixtures either of 0.12, 0.4 or 0.8 mmol of total lipids, were dissolved into 10 mL of chloroform in either a 50 or 100 mL round-bottomed flask. The solvent was evaporated to dryness under reduced pressure for 40 min at 45°C to form a thin lipid film. Multilamellar liposomes were formed by hydration of the lipid film at 50°C with 10 mL of either 12 or 53.8 mM 5-FU solution (10 mM PBS, 138 mM NaCl, pH 7.4) to yield an initial drug to lipid ratio either of 12:12 mM, or 12:40 mM, or 53.8:40 mM or 53.8:80 mM. The resulting mixture was stirred vigorously followed by extrusion, ten times for 5 mol% CHOL or 6 times for 30 mol% CHOL, since this formulation is very long to extrude, or less depending on the lipid concentration. Extrusion was performed at 60°C, above the transition temperature of DPPC, under nitrogen pressure, between 508 and 871 psi depending on CHOL mol%, through a stack of two 100 nm polycarbonate filters (Isopore, Millipore, Molsheim, France). Liposomes were stored at 4°C and characterization was carried out the day following preparation. Freshly prepared liposomes were centrifuged for 4 h at 4°C and 40,000 rpm (150,000 g) in a Beckman ultracentrifuge (Optima LE-80K, Beckman Coulter, USA) in order to remove non-encapsulated 5-FU. Both supernatant and pellet were used for further studies.

HPLC Determination of 5-FU

Analyses were performed on a Waters HPLC system, equipped with Waters 1525 Binary HPLC pump, sample injector Waters 717 plus autosampler and Waters 2487 dual λ absorbance detector. The column used was a Waters μBondapak®C18 (Guyancourt, France), reversed phase, 300×3.9 mm i.d., 10 μm. The mobile phase consisted of 100% of 10 mM PBS. The pH was adjusted to 4.5 with perchloric acid. Chromatographic conditions were the following: Flow rate 1 mL/min, isocratic elution with column temperature maintained at 25°C, UV dual detection at 210 and 266 nm and an injection volume of 20 μL. The Breeze program (Waters Corp., Milford, Massachussetts, USA) was used as acquisition and analysis software. All measurements were run in duplicate and mean values were reported. Results were calculated from a linear regression of 5-FU generated by dissolving known amounts of 5-FU in PBS, pH 4.5, in the concentration range of 0.15–30 μg/mL.

5-FU was quantified in the pellet after solubilization in methanol, yielding the encapsulated 5-FU. Total 5-FU in the initial suspension was determined by solubilizing liposomes in methanol. In general, one volume of liposomes was solubilized with 9 volumes of methanol followed by tenth dilution with PBS. The effect of methanol concentration upon 5-FU peak was checked. The impact of the concomitant presence of lipid and 5-FU in samples was also determined by a calibration curve prepared by mixing 5-FU and solubilized liposomes. This was done in order to avoid matrix effects.

Determination of Phospholipid Concentration

The actual phospholipid concentration (DPPC and DSPE-PEG) after extrusion in the supernatant and in the total liposome suspension was determined using the Stewart assay for phospholipids where the ability of phospholipids to form a complex with ammonium ferrothiocyanate in organic solution is utilized. The resulting complex absorbs at 485 nm, thereby absorbance values were determined using a spectrometer (Lambda 25, UV/VIS Spectrometer, Perkin Elmer, USA) and translated into mmol of phospholipid according to a standard curve (24). Pellet lipids were determined by subtraction and total lipid amount was extrapolated according to the molar ratios of each formula. The same method was applied for 5-Fu-Cu-PEI-loaded liposomes after purification as described below.

Determination of Encapsulation Efficacy and Drug: Lipid Ratio

The concentrations of encapsulated 5-FU and total 5-FU in the suspension determined by HPLC were used

to calculate the percentage of encapsulated 5-FU according to:

$$\%\,\text{encapsulated}\,5\text{-FU} = \frac{C_{\text{encapsulated}}}{C_{\text{total}}} \times 100$$

The drug:lipid ratio was calculated as mmol of encapsulated 5-FU/mmol lipid in the purified liposome determined by the Stewart method according to:

$$\text{drug} : \text{lipid} = \frac{\text{Encapsulated}\,5\text{-FU}(\text{mmol})}{\text{Lipid}_{\text{tot}} - \text{Lipid}_{\text{supernatant}}(\text{mmol})}$$

5-FU-Cu-PEI-Loaded Liposomes

Copper and Polyethylenimine Concentrations Optimization

In the presence of Cu ions, PEI forms a dark blue cuprammonium complex that exhibits two maxima absorption peaks at 285 and 630 nm, respectively (25). On the one hand, to investigate the required concentrations of Cu and PEI for an optimal complexation, series solutions were prepared by varying Cu acetate concentrations within the range of 50–1500 mM in 10 mM PBS, the PEI concentration being constant at 1630 mM. These solutions were then appropriately diluted by 10 mM PBS. The complexation capacity was determined by recording the absorbance values at 285 nm. On the other hand, several complex solutions containing a fixed concentration of Cu acetate (600 mM) and increasing PEI amounts of 417 and 833 mM were tested to formulate liposomes as described above.

Cu-PEI Hydration Solution

The hydration solution was prepared by dissolving Cu acetate monohydrate by continuous stirring in PBS containing PEI at final concentrations of 417 mM (PEI) and 600 mM (Cu). The solution was heated at 60°C for 10 min and stored at room temperature until use. The osmolarity was measured using a micro-osmometer (Micro-osmometer Automatic Type 13 RS, Hermann Roebling MeBtechnik, Berlin, Germany).

5-FU-Cu-PEI-Loaded Liposomes Preparation

Two liposomal formulations were produced with the following composition: DPPC/CHOL/DSPE-PEG (90:5:5 mol%) and DPPC/CHOL/DSPE-PEG (65: 30:5 mol%). Liposomes were prepared as described previously. Briefly, lipid mixtures of 0.8 mmol of total lipids, were dissolved into 10 mL of chloroform in a 100 mL round-bottomed flask. After solvent evaporation, the thin lipid film was rehydrated at 50°C with 10 mL of Cu-PEI complex solution (600–417 mM). Following hydration, liposomes were extruded ten times at 60°C as described above. Free complex was separated from liposomes by exclusion-diffusion gel chromatography on a 1×30 cm Sephadex G-50 column pre-equilibrated with a 10 mM PBS (1776 mM dextrose, pH 7.4) and presaturated with empty liposomes. Liposomes were then reconcentrated using 0.5 mL Amicon Ultra centrifugal filters (cut-off 100 KDa, Millipore Corporation, Molsheim, France) for 80 min at 14,000 g using a tabletop Eppendorf centrifuge 5418 (Hamburg, Germany) to yield liposome concentrates of 80 mM total lipids. Afterwards, reconcentrated liposomes at 80 mM were mixed with a 1665 mM dextrose solution containing 107.6 mM 5-FU (pH 7.4), spiked with ^{14}C-5-FU, at equal volumes to maintain the 5-FU:lipid ratio 53.8:40 mM:mM. Mixture was then distributed over eppendorfs and incubated at 30°C, 1000 rpm for different times (0 to 48 h). At various time points, samples were withdrawn from the oven and stored at 4°C until analysis.

5-FU-Cu-PEI-Loaded Liposomes Characterization

To study 5-FU active entrapment into liposomes by complexation with Cu-PEI complex, liposome suspensions were transferred into 0.5 mL Amicon Ultra centrifugal filters and spinned for 80 min at 14,000 g, at 20°C using a Jouan centrifuge (MR22i, Thermo scientific, France). Subsequently, ultrafiltrates were collected and liposome concentrates were immediately recovered by reverse spinning for 2 min at 1,000 g. Ultrafiltrates contained unencapsulated 5-FU whereas concentrates contained both free and encapsulated 5-FU. Radioactivity of these separated fractions was measured using a scintillation counter (LS 6500 Multi-Purpose Scintillation Counter, Beckman Coulter, USA) and the encapsulated 5-FU concentration was calculated according to the following equation:

$$C_{\text{encapsulated}\,5\text{-FU}} = (C_{\text{total}} - C_{\text{free}\,5\text{-FU}})$$

All measurements were run in duplicate and mean values ± standard deviations are reported.

Determination of Encapsulated Cu-PEI Complex

The property of Cu-PEI complex to exhibit a second maxima absorbance peak at 630 nm (25) was used to quantify the Cu-PEI content of purified liposomes before and after 5-FU encapsulation, using a calibration curve at 630 nm of the 600–417 mM complex. 50 µL of liposomes were destroyed by adding Triton X-100 (2.5% v/v final concentration) in

2 ml PBS and sonication in a sonicator bath (Branson, USA) before absorbance reading at 630 nm.

Dynamic Light Scattering

Mean hydrodynamic diameter (d_H) and polydispersity index (PDI) of liposomes were determined using a Malvern Zetasizer Nano ZN (Malvern, UK, He-Ne laser 633 nm) based on quasi-elastic light scattering. Measurements were carried out at 20°C and intensity correlation functions were measured at a scattering angle of 173°. The d_H was obtained from the Stokes-Einstein relation. Viscosity of the suspension was taken into account using tabulated values from the literature. The ζ-potential of liposomes dispersed in 10 mM PBS (1776 mM dextrose) was measured. Prior to measurements, small aliquots of liposomes (50 μL) were diluted into 950 μL of milliQ water.

In Vitro Temperature-Dependent Drug Release from Liposomes

To determine 5-FU temperature-dependent release, purified liposomes at 5 and 30 mol% CHOL, were tested. 5-FU-loaded liposomes were purified by ultracentrifugation followed by pellet resuspension into iso-osmotic 10 mM PBS (64 mM dextrose, pH 7.4), while 5-FU-Cu-PEI-loaded liposomes were purified as described previously. For 5-FU-loaded liposomes, 200 μL of liposomes were diluted into 300 μL of 10 mM PBS (64 mM dextrose, pH 7.4) before exposure to hyperthermia, whereas for 5-FU-Cu-PEI-loaded liposomes, 25 μL liposomes were diluted in 375 μL 10 mM PBS (1776 mM dextrose pH 7.4). Releases induced by hyperthermia using a water bath or FUS were then compared.

In water bath experiments, temperature-dependent release studies were run from 25 to 49°C. For each temperature, heating was applied during 10 min in an eppendorf tube placed in a water bath. 5-FU time-dependent release was also carried out at 42°C for 5 to 30 min.

In FUS experiments, waves were generated from a single-element homemade transducer (47 mm diameter) focused at 48 mm with a center frequency of 1 MHz. The transducer was driven with an electrical signal generated by an arbitrary waveform generator (Agilent Technologies Inc., Santa Clara, California, USA) and amplified with a power amplifier (ADECE, Artannes sur Indre, France). Adapted from Mannaris *et al.* (26), the *in vitro* setup consisted of a sample holder (30 mm height, 6 mm inside diameter, 8 mm outside diameter; Fischer Scientific SAS, Illkirch, France) containing the solution of liposomes placed into a pure glycerol-filled polystyrene cuvette (45 mm height, 10 mm inside diameter, 12 mm outside diameter; Fischer Scientific SAS, Illkirch, France). Over the sides of the two containers placed in the front of the transducer, acoustical windows with 20-μm thick polyolefin heat shrink film (Rajashrink, Roissy, France) were placed to avoid ultrasound attenuation and reflection (27). The center of the double compartment (sample holder + cuvette) was immersed in a water tank maintained at 37°C and positioned along the propagation axis of the ultrasound beam. Thereby, liposomes were exposed to 1 MHz sinusoidal ultrasonic waves with a pulse repetition period of 1 ms, 400 cycles per pulse, corresponding to a 40% duty cycle. In a first set of experiments, different peak negative pressures ranging from 0 to 2 MPa were evaluated. For each acoustic pressure, ultrasound waves were applied during 10 min. In this range of pressure amplitude, the temperature elevation, measured by a calibrated thermocouple, varied from 0 to +12°C at the focus (6). In the second set of experiments, the pressure amplitude was fixed to 1.75 MPa while the total exposure time varied from 0 to 30 min. Thereafter, samples were cooled down in an ice bath and then stored at 4°C.

For analysis of released 5-FU after hyperthermia exposure, 5-FU-loaded liposomes samples were diluted into 1.5 mL 10 mM PBS (64 mM dextrose) and ultracentrifuged for 4 h at 4°C and 40,000 rpm (150,000 g). Supernatants were collected and 5-FU concentration was measured by HPLC. For 5-FU-Cu-PEI-loaded liposomes, samples were transferred into 0.5 mL Amicon Ultra centrifugal filters and spinned at 14,000 g, for 5 to 10 min, depending on the CHOL mol%, using a tabletop Eppendorf centrifuge 5418 (Hamburg, Germany). Subsequently, ultrafiltrates containing released 5-FU were collected and 5-FU was quantified by HPLC. Chromatographic conditions described above resulted in an overlap of peaks arising from Cu and 5-FU at 266 nm. Therefore, those conditions were optimized to obtain a single 5-FU chromatographic peak. For this purpose, EDTA was added to the mobile phase at 5 mM, pH 4.5 to sequester Cu from the 5-FU-Cu-PEI complex.

The percentage of released 5-FU was calculated according to the following equation:

$$\%\,\text{release} = \frac{M_s - M_c}{M_t} \times 100$$

Where M_s was the quantity of released 5-FU at a specific temperature, M_c the quantity of 5-FU spontaneously released from a control sample, corresponding to the control treated as samples without heating (stored at 4°C until dosage) and M_t the total quantity of 5-FU encapsulated into liposomes, obtained by solubilizing liposomes upon addition of methanol (90%, v/v). For each condition, the experiment was independently repeated twice.

Cells and Cell Culture

The HT-29 (human colorectal adenocarcinoma) cell line was purchased from American Tissue Culture Collection

(ATCC). McCoy's 5A modified medium containing sodium bicarbonate buffer (2.2 g/L) and L-glutamine was provided by Gibco life technologies (Saint Aubin, France). Fetal bovine serum (FBS), trypsine-EDTA and penicillin/streptomycin were supplied by Lonza Biowhittaker (Amboise, France). HT-29 cells were cultured as an adherent monolayer culture in 75 cm^2 culture flasks in McCoy's 5A modified medium supplemented with 10% decomplemented FBS and 0.5% penicillin/streptomycin at 37°C in humidified air with 5% CO_2 atmosphere. Cultures were passaged twice weekly by harvesting adherent cells by brief exposure to Trypsin-Versene® (EDTA) (1X) for up to 12 passages. Viability was assessed by CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) from Promega (Promega Corporation, Madison, USA). To determine cell viability, HT-29 cells were seeded at a density of 2,500 cells per well in a 96-well plates and incubated for 24 h, the time required to be in a log phase, before the addition of different samples. Serially diluted unloaded and purified loaded liposome samples, free 5-FU, free Cu-PEI complex and free 5-FU-Cu-PEI were added with 50 µL fresh media over cells. Then plates were incubated for 2 days. Thereafter, supernatants were aspirated, the MTS reagent was diluted to the 6th with fresh medium and added in each well (100 µL/well) and plates were incubated for additional 2 h at 37°C. Subsequently absorbance in each well was measured spectrophotometrically at 492 nm using a microplate reader (Multiskan MS Type 352, Labsystems, Finland) and the percentage of viable cells was calculated according to the following formula:

$$\%\, cell\ viability = \frac{(A_{treated} - A_{MTS})}{(A_{control} - A_{MTS})} \times 100$$

Where $A_{treated}$ is the absorbance of cells treated with the various samples, A_{MTS} is the absorbance of 100 µL of MTS reagent diluted with fresh medium in no cell well and $A_{control}$ is the absorbance of untreated cells (exposed to fresh medium). Experiments were run in triplicate.

Animals and Tumor Model

Female BALB/cAnNRj-Foxn1nu mice (aged 5–6 weeks, 20 g) were purchased from Janvier Labs (Saint-Berthevin, France). Experiments were conducted according to the European rules (86/609/EEC and 2010/63/EU) and the Principles of Laboratory Animal Care and legislation in force in France (Decree No. 2013-118 of February 1, 2013). Mice were housed in groups of 4 with access to water and food *ad libitum* and kept at a constant temperature (19-22°C) and relative humidity (45–65%). HT-29 cells were harvested from 75% confluent monolayer cultures and resuspended with PBS at 10^7 cells/mL. The colorectal tumor model was established by subcutaneous inoculation of 1×10^6 HT-29 cells in a volume of 100 µL of PBS into the right flank of the animal using 21G needles. The tumor volume was estimated by measuring two diameters with caliper; the volume was calculated as = 0.5 length (mm) × width $(mm)^2$ (Supplementary Figure 2). The experiments were performed when the tumor volume reached 150–250 mm^3.

Pharmacokinetic Study in HT-29-Tumor-Bearing Mice

Mice were randomly separated into four mice per group. 5-FU-TSLs (5% CHOL 5FU-Cu-PEI-loaded liposomes), 5-FU-NTSLs (30% CHOL 5FU-Cu-PEI-loaded liposomes) or free 5-FU (corresponding to 22 mg 5-FU/kg body weight, 1 µCi ^{14}C/150 µL, ~232 mg of lipids/Kg body weight) in PBS (888 mM dextrose, pH 7.4) were injected to tumor-bearing mice *via* tail vein injection (i.v.) using an injection volume of 150 µL/mouse. All the injected formulations were administered in dextrose medium (888 mM). At specified time points (5, 15, 30 min, 1, 2, 4, 24 h) after the injection, the mouse (four animals for each time point) was anesthetized with pentobarbital (109 mg/Kg body weight, injected intraperitoneally), blood was withdrawn *via* cardiac puncture (500–900 µL) and the mouse was humanely sacrified immediately after. Blood was collected into tubes containing 100 µL of sodium citrate and plasma was immediately separated from blood cells by centrifugation at room temperature for 20 min at 3,000 rpm and stored at −20°C until analysis. Afterward, for each time point, plasma samples were thawed and duplicated into two 20 mL scintillation vials (150 µL/vial). The plasma level of 5-FU was quantified by adding 10 mL of the scintillation fluid Ultima Gold followed by measuring ^{14}C radioactivity. Curve modeling was performed according to non compartmental model. The PK parameters including distribution half-life ($t_{1/2\alpha}$) in h, elimination half-life ($t_{1/2\beta}$) in h, peak concentration C_{max} in µmol.L^{-1}, time corresponding to peak concentration T_{max} in h, area under the concentration-time curve ($AUC_{0\text{-}4h}$, $AUC_{0\text{-}24h}$ and $AUC_{0\text{-}\infty}$) in µmol.h.L^{-1} and plasmatic clearance in L.h^{-1} were calculated.

Biodistribution Study in Tumor-Bearing Mice

The biodistribution (BD) of the three formulations mentioned above was followed only at time points 4 and 24 h after injection since liposomes exhibit a cumulative deposition in organs at earlier time points (28,29). After blood withdrawal, lungs, liver, spleen, kidneys, tumor and bone marrow were excised and mice were humanely sacrificed subsequently. Tissues samples were freed of blood after washing in isotonic saline solution and weighed after removing the excess of fluid. Two pieces of each organ of approximately 100 to 150 mg were minced and transferred into small pouches. Femur was removed and cleaned; bone marrow was flushed with 0.5 mL of isotonic saline solution, through 26G needle and syringe.

The femur was weighed before and after the flushing as a control of the bone marrow weight. Subsequently, all organs were kept at −20°C before measuring the radioactivity distribution. To quantify the tissular level of 5-FU, frozen organs parts were transferred into 20 mL scintillation vials and thawed at room temperature. Subsequently, organs were solubilized with 1 mL of Solvable and incubated overnight at 50 to 60°C. After solubilization was completed, 200 μL of 30% hydrogen peroxide was added to uncolour samples with an incubation at 50 to 60°C for 30 min. Afterwards, samples were cooled down and 10 mL of the scintillation fluid Hionic Fluor was added and were then counted for ^{14}C radioactivity. Radioactivity in bone marrow was quantified by thawing corresponding samples, transferring the whole volume into two 20 mL scintillation vials (250 μL/vial) and adding 10 mL of the scintillation fluid Ultima Gold. Control samples (5-FU-TSLs, 5-FU-NTSLs or free 5-FU) were run together with the corresponding mouse samples in duplicate. The results were represented as the quantity of 5-FU (μg) per gram of organ.

Statistical Analysis

All results are expressed as the mean ± standard deviation. Statistical analysis was performed with the two way ANOVA, followed by Sidak's multiple comparison test using GraphPad Prism version 6.01 for Windows (GraphPad Software, La Jolla California USA). A difference with $p<0.05$ was considered to be statistically significant.

RESULTS

Physicochemical Characterization of Liposomes

Liposomes made of DPPC/CHOL/DSPE-PEG at 90:5:5 mol% were chosen as thermosensitive formulations while DPPC/CHOL/DSPE-PEG liposomes at 65:30:5 mol% was used as the negative control. Liposomes with hydrodynamic diameters d_H between 80 and 140 nm, and PDIs below 0.15 were obtained. For an amount of CHOL of 5 mol%, d_H is around 80 nm and PDIs do not vary significantly as a function of the 5-FU:lipid ratio (Table I). However, as the percentage of CHOL increases from 5 to 30 mol% for a given 5-FU:lipid ratio, liposome d_H increases from about 80 nm to 110–140 nm (Table I). For an amount of CHOL of 30 mol%, the d_H increases as the initial lipid concentration increases. The initial 5-FU concentration does not significantly impact liposome diameter. For all conditions tested, the PDIs remained satisfying below 0.15, therefore ensuring a rather narrow size distribution.

Table I Liposome Hydrodynamic Diameters and PDI as a Function of the Initial 5-FU:lipid Ratio. Liposomes were Extruded Ten Times and Subsequently Diluted in PBS Prior to Measurement. Data are Expressed as Mean ± SD ($n=3$)

Liposome type	5-FU:lipid ratio (mM:mM)	CHOL (mol%)	Hydrodynamic diameter d_H (nm) (mean ± SD)	PDI (mean ± SD)
5-FU-loaded liposomes	12:12	5	78 ± 1	0.09 ± 0.01
		30	108 ± 10	0.07 ± 0.02
	12:40	5	78 ± 1	0.08 ± 0.01
		30	121 ± 25[a]	0.09 ± 0.03
	53.8:40	5	85 ± 4	0.07 ± 0.02
		30	123 ± 18[a]	0.08 ± 0.02
	53.8:80	5	82 ± 4	0.08 ± 0.01
		30	142 ± 11[a]	0.09 ± 0.01

[a] Liposomes were extruded five or seven times due to long extrusion cycles (4 h)

5-FU Encapsulation Efficacy

For a given 5-FU:lipid ratio, all formulations exhibit the same encapsulation efficacy of 5-FU. Only a mild increase of 5FU encapsulation was observed for 30 mol% CHOL. 5-FU encapsulation efficacy increases as lipid concentration raises: from around 4 to 25% as lipid concentration passes from 12 mM up to 80 mM. However, when 5-FU concentration increases from 12 to 53.8 mM while the lipid concentration is kept constant (40 mM), no encapsulation improvement could be obtained. To analyze the rate of association of 5FU, the total lipid amount of purified liposomes was determined. Figure 1 shows 5-FU:lipid final ratio (mmol:mmol). It can be observed that 5-FU:lipid ratio increases from 0.05:1 to 0.19:1, for 5 mol% CHOL formulations, and from 0.06:1 to 0.22:1 for 30 mol% CHOL formulation, as 5-FU:lipid initial ratio varies from 12:12 mM to 53.8:40 mM. One can notice that the drug:lipid final ratio is similar with 5-FU:lipid initial ratio of 53.8:40 mM and 53.8:80 mM. There is therefore no advantage in increasing lipid concentration. The formulation obtained with an initial ratio 53.8:40 mM therefore appears the more appropriate liposomal system for further studies. Following ultracentrifugation and pellet resuspension in 500 μL, this formulation containing 5 mol% CHOL yielded a final encapsulated 5-FU concentration of 3.6 mg/mL into 5 mol% CHOL liposomes.

Temperature-Dependent 5-FU Release from 5-FU-Loaded Liposomes

5-FU release from liposomes composed of 5 or 30 mol% CHOL upon either water bath heating or FUS heating shows

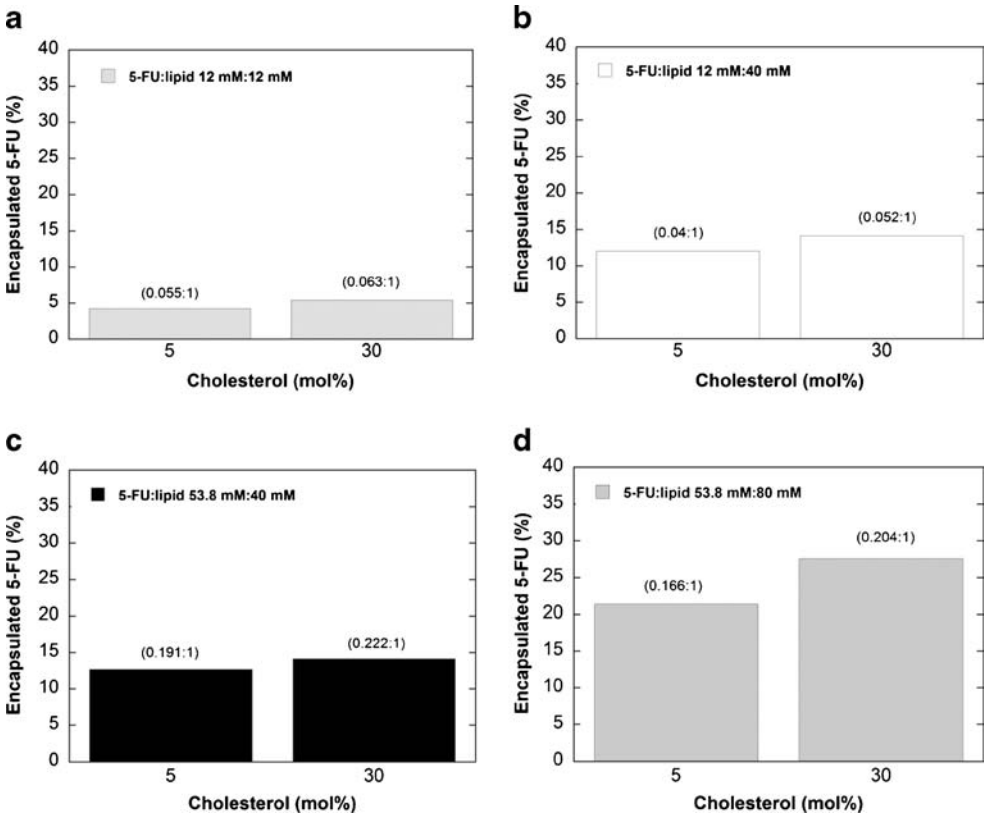


Fig. 1 5-FU encapsulation into liposomes containing 5 or 30 mol% CHOL as a function of drug to lipid initial ratio (mM:mM). Panels represent 5-FU encapsulation efficacy for (**a**) 5-FU:lipid 12:12 mM, (**b**) 5-FU:lipid 12:40 mM, (**c**) 5-FU:lipid 53.8:40 mM and (**d**) 5-FU:lipid 53.8:80 mM. The corresponding 5-FU:lipid final ratios (mmol:mmol) are given between *brackets* above the histograms. Data are expressed as mean ± SD ($n = 2–3$).

that 5-FU is quickly leaking out from the vesicles independently from any heating (Supplementary information, Figure S1). For these samples, as also observed with the control sample kept at 4°C, up to 90% of the encapsulated 5-FU is released.

Copper and Polyethylenimine Concentrations Optimization and Liposome Formulation

Given the poor retention of 5-FU in liposomes, an alternative strategy derived from the method described by Thomas *et al.* (17) was tested in which 5-FU was entrapped in the liposomes together with Cu-PEI forming a ternary complex. Briefly, a coordination complex of Cu with low molecular weight PEI is encapsulated within liposomes, which are further purified from the unencapsulated complex. Then, 5-FU is incubated with Cu-PEI loaded liposomes and ternary complex formation drives the encapsulation of the drug. The influence of Cu and PEI concentration and of the resulting Cu-PEI complex on liposomes formulation was investigated. The Cu acetate solution spectrum exhibits a maximum of absorption at 260 nm (Fig. 2) and appears turquoise blue. When PEI is added, the color turns into a deep blue and the absorbance maximum shifts towards longer wavelengths at 285 nm with a secondary maximum at 630 nm (Fig. 2). This shift indicates Cu complexation. When PEI concentration is fixed at 1630 mM and Cu concentration increases, absorbance increases for both wavelengths (285 and 630 nm) indicating that all Cu ions are complexed (Fig. 2).

Cu concentration was then fixed to 600 mM and PEI concentration varied between 167 and 1300 mM. For the high PEI concentration, a complex solution was prepared to hydrate a lipid film containing 5 mol% CHOL. The resulting solution was too viscous and liposomes extrusion was very slow and did not allow sufficient extrusion cycles to yield satisfying sizes. Formulations prepared with 417 and 833 mM PEI were extruded at least ten times and allowed to obtain liposomes below 200 nm (Fig. 3). In particular, the liposomes containing

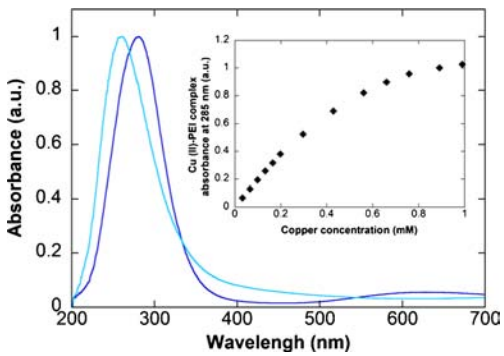


Fig. 2 Absorbance spectra of Cu acetate (*light blue*) and of Cu-PEI complex in PBS (*dark blue*). The inset shows the influence of Cu concentration on the absorbance of the Cu-PEI complex at 285 nm. The initial PEI concentration was 1630 mM, and the final PEI concentration after dilution was 1 mM.

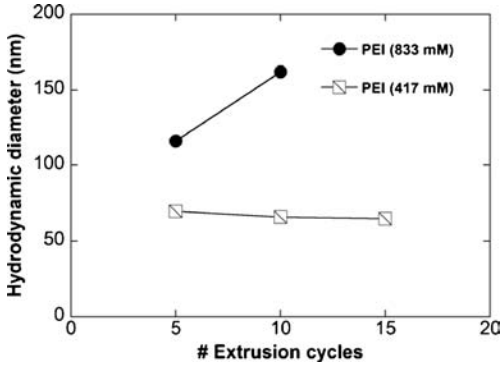


Fig. 3 Size of liposomes composed of 5 mol% CHOL encapsulating Cu-PEI complex at different polymer concentrations. Cu concentration was 600 mM.

Cu-PEI (600–417 mM) showed a good and stable size around 65 nm after ten extrusion cycles. This formulation was thus chosen as a compromise between optimal liposome size and a moderate PEI concentration. The resulting osmolarity was 1957 mosm/Kg reflecting the influence of the highly charged PEI.

Preparation and Physicochemical Characterization of Liposomes

Two liposomal formulations were produced using Cu-PEI (600-417 mM) as hydration solution: DPPC/CHOL/DSPE-PEG at 90:5:5 mol% as TSLs formulation and DPPC/CHOL/DSPE-PEG at 65:30:5 mol% as the non thermosensitive negative control. Table II summarizes liposome sizes and PDIs. Cu-PEI-loaded liposomes were dispersed into PBS (+1776 mM dextrose) following purification from the non-encapsulated Cu-PEI complex to yield an osmotic pressure identical to the Cu-PEI solution. The hydrodynamic diameter d_H is around 65 nm for 5 mol% CHOL and around 100 nm for 30 mol% CHOL with PDIs below 0.1. These values are smaller than those of unloaded liposomes dispersed in PBS but are similar to values obtained from unloaded liposomes dispersed in PBS and 1776 mM dextrose (Table II). As observed before, liposomes with 30 mol% CHOL are larger. Zeta potential values of Cu-PEI-loaded liposomes are less negative (around −6 or −3 mV) than those of unloaded liposomes (around −14 mV) (Table II).

5-FU Active Encapsulation into Cu-PEI-Loaded Liposomes

Since DPPC phase transition temperature T_m is 41.5 ± 0.5°C (30), to avoid the disruption of Cu-PEI-loaded liposomes during their incubation with 5-FU for active encapsulation, incubation temperature was set at 30°C and active entrapment was followed over time. The encapsulation kinetic is presented in Fig. 4. The initial phase of encapsulation was immediate reaching 28% for liposomes containing 30 mol% CHOL at 0 h, while no immediate encapsulation was observed for the 5 mol% CHOL formulation (Fig. 4). However, 5-FU was then speedily encapsulated within 2–4 h into both formulations with 39% of 5-FU encapsulated in the TSLs formulation and 44% in the control formulation, respectively, after only 4 h of incubation. A plateau is reached after 24 h incubation yielding to an encapsulated 5-FU concentration of 2.7 mg 5-FU/mL (Fig. 4).

5-FU-Cu-PEI-loaded formulations were then characterized in terms of size, polydispersity and ζ-potential. No differences could be observed as compared with Cu-PEI-loaded formulations (Table II). In particular, active encapsulation of 5-FU into Cu-PEI-loaded liposomes does not change importantly the surface charge. This supports the fact that 5-FU

Table II Overview of Liposome Formulations. Samples Were Diluted Either in 10 mM PBS or in 10 mM PBS + 1776 mM Dextrose for Size Measurement and in Deionized Water for Zeta Potential Measurement ($n = 3$). Data are Expressed as Mean ± SD ($n = 3$)

Liposome type	Hydration solution	CHOL (mol%)	5-FU:lipid initial ratio (mM:mM)	d_H (nm) (mean ± SD)	PDI (mean ± SD)	ζ-potential (mV) (mean ± SD)
Unloaded	PBS	5	0:80	87 ± 6	0.12 ± 0.05	−14.9 ± 5.9
		30		127 ± 12	0.09 ± 0.02	−17.6 ± 5.6
	PBS + 1776 mM dextrose	5		56 ± 1	0.12 ± 0.04	−15.2 ± 5.3
		30		97	0.1 ± 0.06	−13.1 ± 6.1
5-FU-loaded liposomes	5-FU/PBS	5	53.8:40	88 ± 6	0.06 ± 0.01	−13.4 ± 6.9
		30		122 ± 15	0.07 ± 0.02	−14.8 ± 6.3
	5-FU/PBS + 1776 mM dextrose	5		61 ± 3	0.15 ± 0.01	−15.7 ± 5.1
		30		74 ± 7	0.06 ± 0.02	−15.9 ± 5
Cu-PEI-loaded liposomes	Cu-PEI/PBS	5	0:80	66 ± 3	0.08 ± 0.00	−6.6 ± 4.5
		30		102 ± 13	0.07 ± 0.01	−2.9 ± 5.2
5-FU-Cu-PEI-loaded liposomes	Cu-PEI/PBS	5	53.8:40	65 ± 4	0.09 ± 0.01	−10.2 ± 5.2
		30		105 ± 8	0.06 ± 0.01	−4.9 ± 4. 1

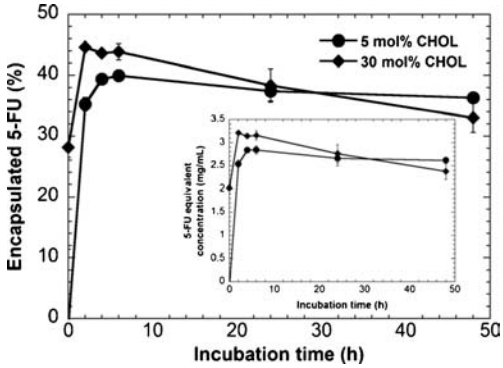


Fig. 4 5-FU active entrapment kinetics as Cu-PEI-loaded liposomes containing 5 or 30 mol% CHOL were incubated at 30°C with 107.6 mM 5-FU (10 mM PBS + 1665 mM dextrose, pH 7.4). Inset: Equivalent 5-FU final encapsulated concentration. Data are expressed as mean ± SD ($n = 2–3$).

does not interact with the lipid bilayer. After purification, liposomes were characterized in terms of 5-FU:lipid ratio and Cu:lipid ratio. The 5-FU:lipid ratio is similar around 1:2 for TSLs and negative control formulation, (Table III). Cu:lipid ratio is also approximately 1:2 for the TSLs formulation and slightly larger around 1.3:2 for the negative control formulation. The addition of 5-FU does not significantly modify these ratios. Finally, the 5-FU:Cu ratio is similar around 1:1 indicating that each 5-FU molecule is associated to one Cu (Table III).

In Vitro Temperature-Dependent 5-FU Release from 5-FU-Cu-PEI-Loaded Liposomes

5-FU release from liposomes upon exposure to hyperthermia was then performed using either a water bath or FUS. Figure 5a shows the release profile of 5-FU as a function of temperature after 10 min exposure to hyperthermia in a water bath. At 4°C, spontaneous release is low for both formulations (<1%). At 37°C, heating induces the release of 12±0.3% and 9±2% of 5-FU from 5 to 30 mol% CHOL formulations, respectively. As temperature increases from 37°C to 49°C, up to 70% of 5-FU is released from the 5 mol% CHOL formulation whereas only 33±2% is released from the 30 mol% CHOL formulation.

FUS was then applied for 10 min at various driving pressure and 5-FU release was determined (Fig. 5b). When no ultrasound is applied (0 MPa), as samples are maintained in a double compartment setup positioned in a water tank at 37°C (27), 5-FU release is equivalent to what was obtained previously in a water bath: 13±5% and 9±2% (Fig. 5a). Interestingly, as the acoustic pressure is increased from 0 to 1 MPa, up to 47±0.1% of 5-FU is released from 5 mol% CHOL formulation whereas only 12.6±16% is released from 30 mol% CHOL formulation. As the applied acoustic pressure increases to 1.25 MPa, 5-FU release yields 68±0% and 19±2%, respectively for 5 and 30 mol% CHOL. For higher acoustic pressures, 5-FU release from 5 mol% CHOL liposomes reaches a plateau close to 68%. On the other hand, 5-FU keeps being released with up to 32% at 2 MPa for 30 mol% CHOL.

Release kinetics at 1.75 MPa was then followed (Fig. 5c). Results indicate that, for both formulations, 5-FU is progressively released during the first 10 min before reaching a plateau. As observed before, 5-FU release from 5 mol% CHOL liposomes is higher than for 30 mol% CHOL liposomes (60% *versus* 20%).

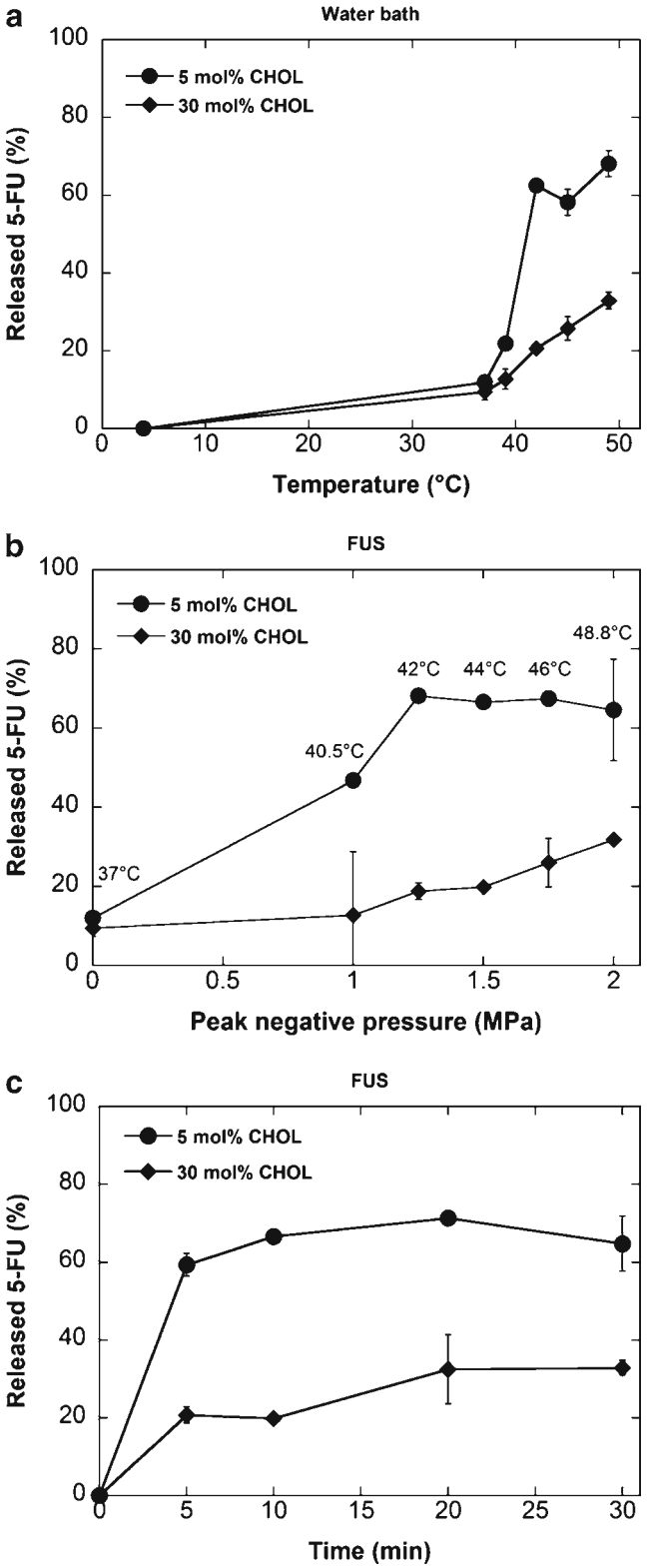
Cytotoxicity

Formulations were then evaluated in terms of cytotoxicity on HT-29 human colon cancer cells using the MTS assay that considers cell mitochondrial activity. Cell viability was evaluated as a function of 5-FU concentration, lipid concentration and Cu concentration (Fig. 6).

5-FU, at doses lower than 1 μM, has almost no inhibitory effect on HT-29 cells (Fig. 6a and b). Above 1 μM 5-FU, HT-29 cell viability decreases in a dose-dependent manner with an inhibitory concentration (IC_{50}) of 172±77 μM (Table IV). The 5-FU-Cu-PEI ternary complex exhibits typically the same behavior and the $IC_{50} = 115 \pm 67$ μM is similar as uncomplexed 5-FU (Table IV). When encapsulated into liposomes, the overall viability behavior is similar except that IC_{50} values are slightly lower: around 50 μM for 5 mol% CHOL liposomes and from 40 to 100 μM for 30 mol% CHOL liposomes. The effect of lipids on cell viability is presented in Fig. 6c and d. When liposomes are loaded with 5-FU or 5-FU-Cu-PEI, cell viability decreases. The IC_{50} is of 200 to 400 μM in the case 5-FU-loaded liposomes for 5 and 30 mol% CHOL and around 80 μM for 5-FU-Cu-PEI-loaded liposomes whatever the amount of CHOL. For unloaded liposomes and Cu-PEI loaded liposomes, cell viability always remains higher than 80% except for very high concentrations. Finally, cell

Table III 5-FU:lipid, 5-FU:Cu and Cu:lipid Ratios of Purified Liposomes After 24 h of Incubation with 5-FU

Formulation	Composition CHOL (mol%)	5-FU:lipid ratio (mmol:mmol)	5-FU:Cu ratio (mmol:mmol)	Cu:lipid ratio (mmol:mmol)
Cu-PEI-loaded liposomes	5	–	–	1.1:2
	30	–	–	1.44:2
5-FU-Cu-PEI-loaded liposomes	5	1.04:2	1.02:1	1.02:2
	30	1.12:2	0.92:1	1.22:2



◀ **Fig. 5** Release profile in PBS of encapsulated 5-FU-Cu-PEI from 5-FU-Cu-PEI-loaded liposomes upon heating for 10 min in (**a**) water bath at 37, 39, 42, 45 and 49°C after dilution by 10 mM PBS (1776 mM dextrose) (**b**) FUS setup at 1, 1.25, 1.5 1.75 and 2 MPa and (**c**) FUS setup at 1.75 MPa for 5, 10, 20 and 30 min. Temperature increment induced by FUS was measured at the focal distance using a thermocouple placed into the sample holder. Samples were compared to a control of each formulation kept at 4°C. Data are expressed as mean ± SD ($n = 2$).

viability was evaluated as a function of Cu concentration (Fig. 6e and f). In all cases, cell viability decreases in a Cu concentration-dependent manner. The IC_{50} of Cu-PEI solution is very high around 6 mM and decreases down to about 1.3 mM for 5-FU-Cu-PEI solution. For Cu-PEI loaded liposomes, the IC_{50} could not be determined but is above 500 μM independently of CHOL percentage. For 5-FU-Cu-PEI-loaded liposomes, the IC_{50} decreases down to 50 μM independently of the amount of CHOL.

Pharmacokinetics and Biodistribution in HT-29 Tumor-Bearing Mice

Before, exploiting 5-FU-TSLs as temperature activable drug delivery systems, their pharmacokinetics (PK) and biodistribution have been evaluated after i.v. injection and compared to 5-FU in NTSLs and free 5-FU. The plasmatic concentration of 5-FU after administration of 5-FU-TSLs, 5-FU-NTSLs and free 5-FU solution, was determined in HT-29-tumor-bearing mice. The PK profiles are depicted in Fig. 7a. From PK profiles the major PK parameters were calculated (Table V). The maximum detected plasma concentration C_{max} of 5-FU solution, 5-FU-TSLs and 5-FU-NTSLs were 54 $\mu mol.L^{-1}$ at 0.25 h, 75 $\mu mol.L^{-1}$ at 0.5 h and 84 $\mu mol.L^{-1}$ at 0.25 h post injection, respectively. These C_{max} values are of the same order of magnitude and occur approximately at the same T_{max}. After reaching their maximum concentration, drug levels decreased down to 0.2, 0.32 and 0.6 $\mu mol.L^{-1}$, respectively at 24 h (Fig. 7a). The drug plasmatic concentration decrease can be described with a biexponential time dependence. The first characteristic time corresponds to a rapid initial distribution phase and the second characteristic time corresponds to a slower elimination phase (Fig. 7a and b). The initial phase corresponds to a reversible distribution between the central and peripheral compartments. The second phase corresponds to an irreversible elimination from the central compartment *via* urinary excretion and/or hepatic biotransformation. The distribution phase of 5-FU administered in TSLs and NTSLs is longer than 5-FU administered as a solution in dextrose (Fig. 7b). A longer distribution half-life ($t_{1/2\alpha}$) of approximately 36 min (*i.e.* 0.6 h) is observed for both types of liposomes, whereas $t_{1/2\alpha}$ = 26 min (*i.e.* 0.44 h) for the 5-FU solution (Fig. 2b). We observe 5-FU elimination is longer when administered as a solution with $t_{1/2\beta}$ = 540 min (*i.e.* 9 h) than when administered as liposomes $t_{1/2\beta}$ = 336 min (*i.e.* 5.6 h). The $AUC_{0\text{-}24h}$ was calculated for all formulations. On one hand, the $AUC_{0\text{-}24h}$ of free 5-FU was 68 $\mu mol.h.L^{-1}$. On the other hand, liposomes formulations lead to higher values: 132 and

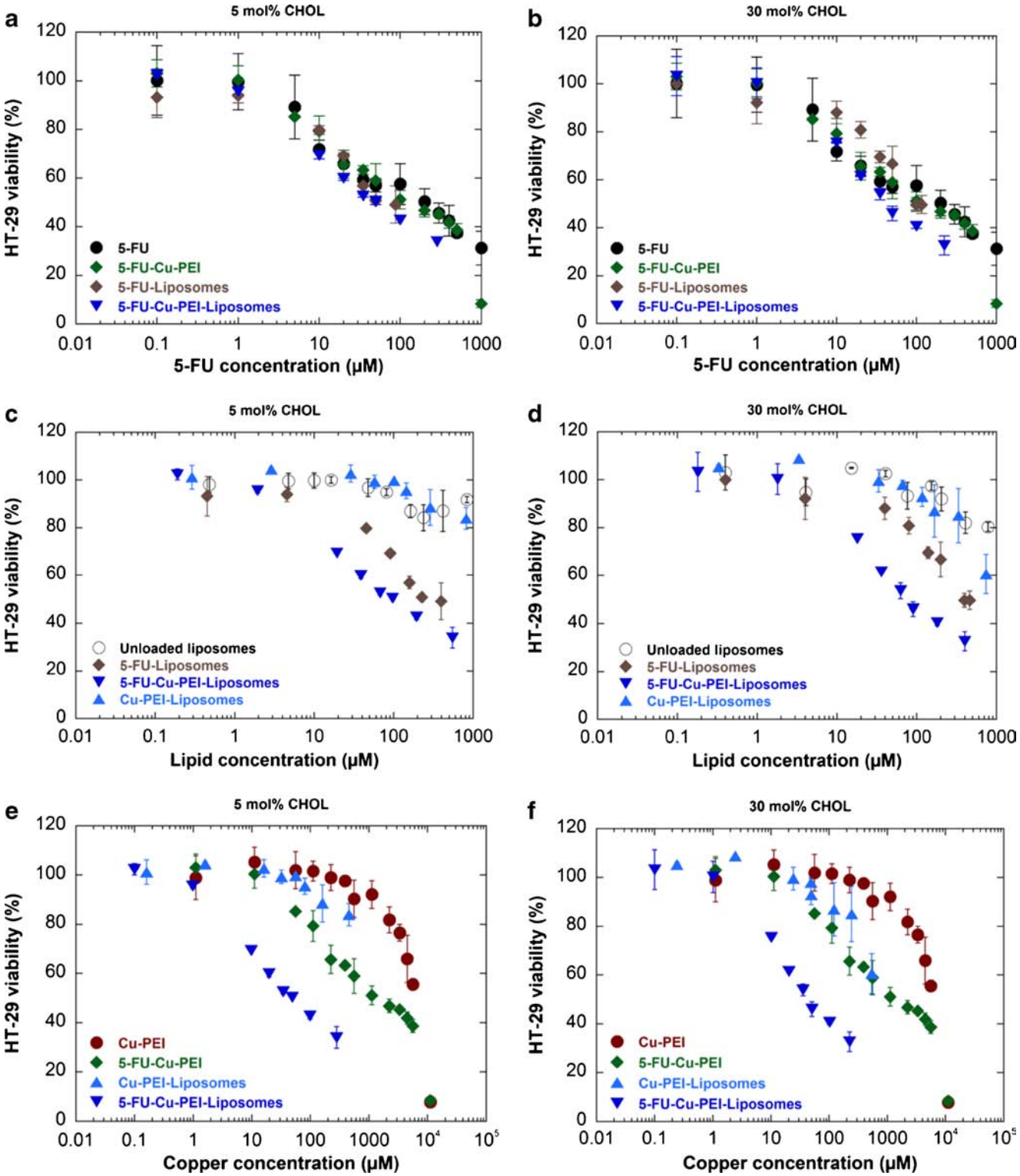


Fig. 6 HT-29 cell viability determined using the MTs assay after 48 h exposure to formulations and controls. Viability was plotted as a function of 5-FU concentration (**a** and **b**), lipid concentration (**c** and **d**) or Cu concentration (**e** and **f**). Each point represents the mean ± SD ($n = 3$) from three independent experiments, each performed in triplicate.

196 μmol.h.L^{-1} for TSLs and NTSLs, respectively. These AUC values are significantly different ($p \leq 0.05$).

In addition to the PK, 5-FU biodistribution was also determined at 4 and 24 h after administration (Fig. 8). In the

Table IV Inhibitory Concentration (IC_{50}) of the Different Formulations and Controls in Terms of 5-FU, Cu or Lipid Concentration on HT-29 Cells After 48 h Exposure as Revealed by the MTS Assay. NC = Does Not Contain CHOL. Data are Expressed as Mean ± SD (n = 3) from Three Independent Experiments, Each Performed in Triplicate

Considered toxic compound	Formulation	CHOL (mol%)	IC_{50} (μM) (mean ± SD)
5-FU	5-FU solution	NC	172 ± 77
	5-FU-Cu-PEI solution	NC	115 ± 67
	5-FU-loaded liposomes	5	49 ± 0.7
		30	98 ± 14
	5-FU-Cu-PEI-loaded liposomes	5	49 ± 6
		30	43 ± 6
Cu	Cu-PEI solution	NC	6225 ± 106
	5-FU-Cu-PEI solution	NC	1283 ± 742
	Cu-PEI-loaded liposomes	5	>460
		30	>538
	5-FU-Cu-PEI-loaded liposomes	5	48 ± 7
		30	45 ± 5
Lipids	Unloaded liposomes	5	>833
		30	>782
	Cu-PEI-loaded liposomes	5	>820
		30	>740
	5-FU-loaded liposomes	5	188 ± 48
		30	352 ± 46
	5-FU-Cu-PEI-loaded liposomes	5	96 ± 9
		30	73 ± 7

tumor, 5-FU concentration is around 0.5 µg/g tumor at 4 h when administered either free or in TSLs, and around 1 µg/g tumor when administered as NTSLs (*$p \leq 0.05$) (Fig. 8a). At 24 h post-injection, 5-FU concentration decreased and was not significantly different between the three groups. Drug concentration was also measured in the different organs of the reticuloendothelial system (RES): bone marrow, liver, spleen and lungs and in the kidneys to assess excretion. The 5-FU concentration in bone marrow at 4 h was identical irrespective to the administered formulation (Fig. 8b). The same finding was observed at 24 h but the overall 5-FU concentration in the bone marrow was lower. In the liver, 4 h after injection, as expected, 5-FU concentration was higher by a factor 2.6- (****$p \leq 0.0001$) and 1.9 (*$p \leq 0.05$), for NTSLs and TSLs groups respectively, compared to free 5-FU (Fig. 8c). 24 h post-injection, 5-FU concentration are not significantly different between the three groups. In the spleen, 4 h post-injection, 5-FU concentration was not significantly different between TSLs and free 5-FU, while NTSLs leads to a 2-fold increase (****$p \leq 0.0001$) of 5-FU concentration in the

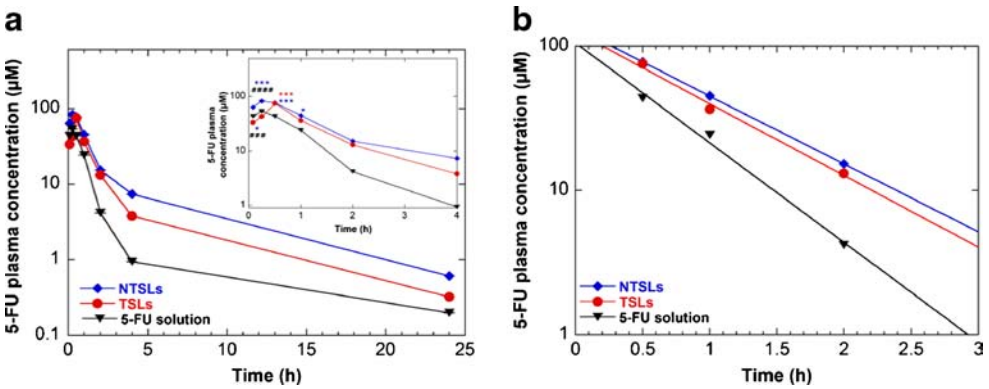


Fig. 7 (**a**) Plasma kinetics of 5-FU-TSLs, 5-FU-NTSLs and 5-FU solution. 5-FU was intravenously administered (22 mg 5-FU/kg body weight) into tumor-bearing mice. 5-FU plasma concentration (μM) is plotted. The blood clearance can be described with a biexponential time dependence. (**b**) Fitting of 5-FU concentration in plasma over the distribution phase. Data points and error bars are the average and standard deviation of the four mice. *, $p \leq 0.05$, ***, $p \leq 0.001$, significant difference compared to free 5-FU solution. $^{###}$, $p \leq 0.001$, $^{####}$, $p \leq 0.0001$, significant difference for liposomal formulations compared to each other.

Table V Pharmacokinetics Parameters for 5-FU When Administered Intravenously as Solution, TSLs and NTSLs. All Formulations Were Injected at 22 mg 5-FU/kg Body Weight into HT-29-tumor-bearing mice

	5-FU solution	5-FU-TSLs	5-FU-NTSLs
C_{max} (μmol.L^{-1})	54	75	84
T_{max} (h)	0.25	0.5	0.25
$t_{1/2\alpha}$ (h)	0.44	0.6	0.64
$t_{1/2\beta}$ (h)	9	5.59	5.55
$AUC_{0\text{-}4h}$ (μmol.h.L^{-1})	56	91	116
$AUC_{0\text{-}24h}$ (μmol.h.L^{-1})	68	132	196
$AUC_{0\text{-}\infty}$ (μmol.h.L^{-1})	71	135	201
Clearance (L.h^{-1})	0.046	0.025	0.017

spleen compared to free 5-FU (Fig. 8d). In the lungs, 4 h post-injection, 5-FU concentration does not differ between free 5-FU and 5-FU-TSLs (Fig. 8e). However, 5-FU-NTSLs leads to a 2-fold higher concentration compared to the other groups ($^{\#\#}p \leq 0.01$ and $^{***}p \leq 0.001$, respectively). Despite this observation, one should note that lung accumulation of 5-FU (solution or liposomes) is significantly ($p \leq 0.05$) lower compared to spleen uptake regardless from the formulation and the time point. In the kidneys, 4 h post-injection, 5-FU concentration is higher ($^{*}p \leq 0.05$) for 5-FU-TSLs than for free 5-FU and even more important ($^{****}p \leq 0.0001$) for 5-FU-NTSLs (Fig. 8f). This trend is further confirmed 24 h post-injection with 5-FU concentrations in kidneys more important for liposome formulation than for the free drug.

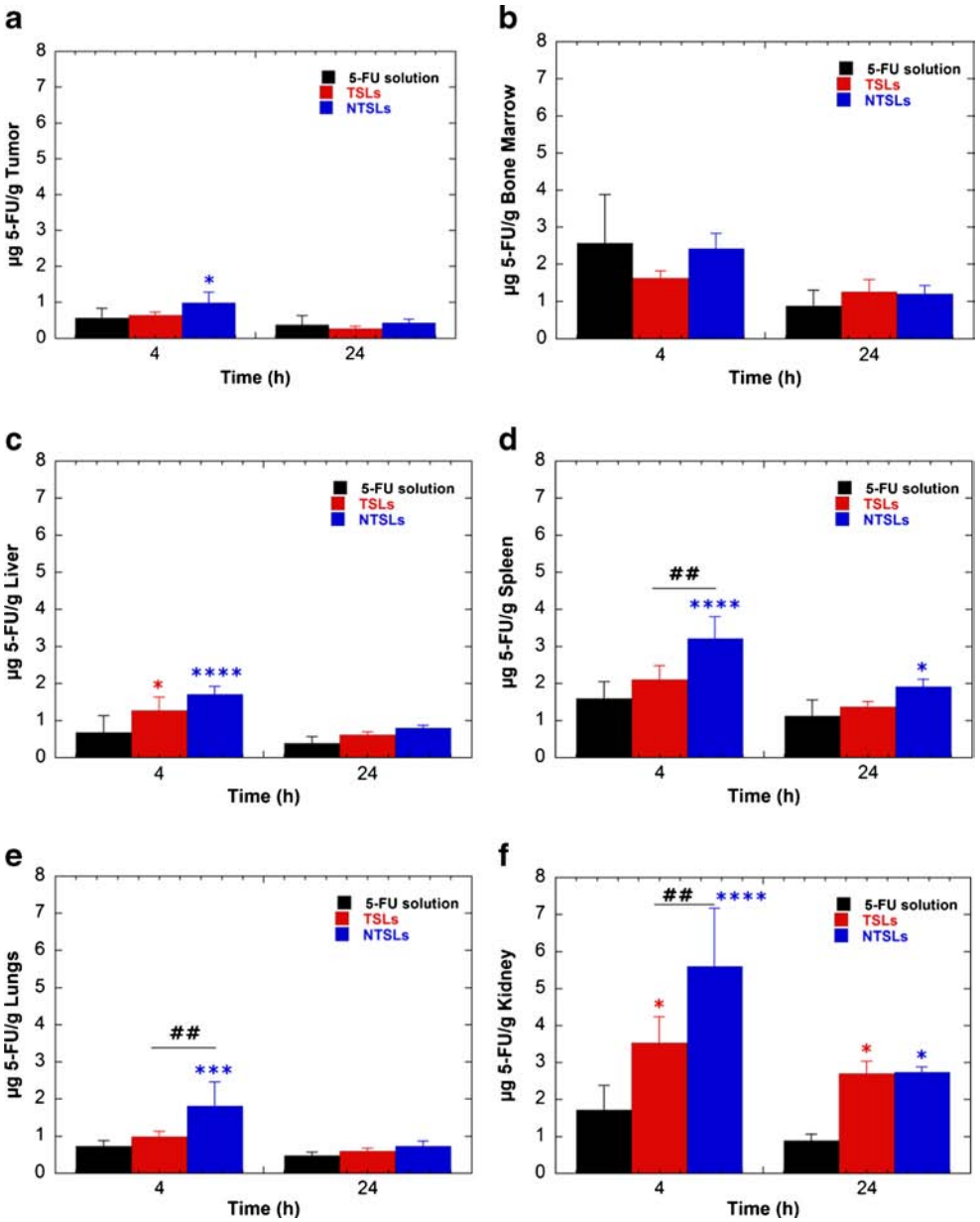


Fig. 8 Biodistribution of 5-FU at 4 and 24 h after intravenous administration of 5-FU (22 mg 5-FU/kg body weight) in different formulations into tumor-bearing mice. Data points and error bars are the average and standard deviation of the four mice. *, $p \leq 0.05$, ***, $p \leq 0.001$, ****, $p \leq 0.0001$, significant difference compared to free 5-FU solution at a definite time point. $^{\#\#}$, $p \leq 0.01$, significant difference compared to the second liposomal formulation at the same time point.

DISCUSSION

The objective of this study was to prepare thermosensitive 5-FU liposomes with optimum loading and stability and to evaluate their behavior after intravenous administration. Two different methods of encapsulation were considered to improve 5-FU encapsulation and retention: passive and active encapsulation. The release profile of 5-FU from TSLs and their negative control was studied under hyperthermia generated by either water bath heating or FUS heating. Finally, formulation cytotoxicity was evaluated. The requirements for a 5-FU TSLs formulation are first a sufficient drug-loading compatible with the dose needed for injection and second a good drug retention into the aqueous compartment until actual administration and application of hyperthermia.

Regarding passive encapsulation, an impact of CHOL content is observed as liposomes containing 5 mol% CHOL exhibit a smaller hydrodynamic diameter d_H compared to those made with 30 mol% CHOL. The larger size of 30 mol% CHOL liposomes can be explained by the higher rigidity of the lipid bilayer (22,31), that makes the extrusion process more difficult since these liposomes do not possess a gel to liquid transition temperature (Chantal Al Sabbagh, Anthony Novell, Jean-Michel Escoffre, Cédric Gaillard, Nicolas Tsapis, Elias Fattal and Ayache Bouakaz. *IN-VITRO* EVALUATION OF FOCUSED ULTRASOUND-MEDIATED THERMAL AND MECHANICAL EFFECTS ON THE CALCEIN RELEASE FROM TEMPERATURE-SENSITIVE LIPOSOMES. Submitted to International Journal of Hyperthermia). The rigidity of the lipid bilayer also explains the increase of hydrodynamic diameter (110 to 140 nm) as lipid concentration increases from 12 to 80 mM in vesicles made with 30 mol% CHOL. The higher the lipid concentration, the more viscous the suspension and the longer the extrusion process (up to 4 h for one cycle). One can notice that the presence of 5-FU passively encapsulated does not modify liposome hydrodynamic diameter regardless of the initial 5-FU concentration. Indeed, since 5-FU is very hydrophilic drug, it does not interact with liposome bilayer (14,15).

5-FU encapsulation efficacy depends strongly on initial 5-FU and lipid concentrations. Indeed, the higher the lipid concentration, the more numerous the liposomes, leading to a larger encapsulated aqueous volume and therefore a higher encapsulation of 5-FU (14,15). Increasing the initial concentration of 5-FU logically leads to an increase of the amount encapsulated in the aqueous compartment. Therefore, the higher encapsulation of 5-FU in 30 mol% CHOL liposomes prepared at high initial lipid concentrations (40 and 80 mM), compared to 5 mol% CHOL liposomes would arise from the higher encapsulated volume since the vesicles are larger in the presence of large amount of CHOL. 5-FU encapsulation seems to reach a plateau around 0.2:1 5FU:lipid ratio. Encapsulation is limited by 5-FU solubility (53.8 mM): as lipid concentration increases, the number of liposomes formed increases but the 5-FU:lipid ratio remains identical. Hence, 40 mM lipid initial concentration appears to be an optimum between good encapsulation and excipient cost. Passive encapsulation allows to yield a 5-FU concentration of up to 3.6 mg 5-FU/mL compatible with an efficient treatment *in vivo* on HT-29 xenograft tumors in mice (injection of 20–40 mg 5-FU/kg) (17,32). These results are in agreement with literature, where similar encapsulation efficacy of 5-FU has been reported for DPPC liposomes (14).

The results of temperature-dependent 5-FU release are in accordance with previous data available in literature demonstrating that 5-FU is poorly retained into unilamellar liposomes due to quick leakage (14,15,17,33). Various techniques have been suggested to improve 5-FU retention such as the formulation of stable multilamellar vesicles (15), lyophilisation of liposomes entrapping 5-FU (16), formulation of 5-FU into pH-sensitive niosomes (34), encapsulation of lipophilic derivatives of 5-FU (35), complexation of 5-FU with a Cu complex in order to enhance both of intraliposomal 5-FU loading and retention (17). Transition metal complexation of selected drugs has been widely used to enhance drug entrapment and/or drug retention into liposomes (36). Moreover, complexation of 5-FU by Cu-PEI exhibited higher efficacy than free 5-FU in *in vivo* (17). We therefore applied this strategy to formulate TSLs by active encapsulation.

As liposomes are loaded with the Cu-PEI complex, their size is not modified but the zeta potential decreases in absolute value (from −15 to −7 mV for 5 mol% CHOL and from −13 to −3 mV for 30 mol% CHOL liposomes) probably due to an adsorption of the positively charged complex onto negatively charged liposomes as reported by Thomas *et al.* (17). 5-FU active encapsulation does not significantly modify the zeta potential of the formulations. Even though incubation was carried out at low temperature, 5-FU active encapsulation is very quick, being somehow more rapid for 30 mol% CHOL liposomes than for 5 mol% CHOL liposomes. The difference of encapsulation kinetics remains to be fully explained. In addition, it reveals that the likely adsorption of Cu-PEI complex onto liposome surface does not prevent active encapsulation of 5-FU. Regardless their composition, dispersion of liposomes into 10 mM PBS+1776 mM dextrose results in a decrease of their d_H (Table II). This decrease does not arise from 1776 mM dextrose viscosity (3.317 cP at 20°C) (37) since it was taken into account for DLS measurements. The high dextrose concentration could probably modify the conformation of PEG chains at liposome surface by changing the osmotic pressure as it occurs with conterions (38), therefore decreasing their overall size.

Hyperthermia-mediated release experiments show that 5-FU retention is ensured by its complexation with Cu-PEI. The higher molecular weight of the ternary complex prevents its

leakage from liposomes (17). The thermosensitivity of the 5 mol% CHOL formulation as compared to the 30 mol% CHOL one, is verified using either a water bath or FUS. Indeed at 42°C, both modes of heating lead to massive release of 5-FU from the 5 mol% CHOL formulation whereas release from the 30 mol% CHOL formulation is equivalent to spontaneous release. FUS at 1 MHz and 1.75 MPa for 10 min constitute a powerful stimulus for TSLs by inducing a temperature increment of 9°C (*i.e.*, final temperature is 46°C) at the focal point (27). FUS is slightly more efficient than water bath heating with 68% release *versus* 62%. The additional release using ultrasound is in agreement with our previous results using calcein as a model molecule (Chantal Al Sabbagh, Anthony Novell, Jean-Michel Escoffre, Cédric Gaillard, Nicolas Tsapis, Elias Fattal and Ayache Bouakaz. *IN-VITRO* EVALUATION OF FOCUSED ULTRASOUND-MEDIATED THERMAL AND MECHANICAL EFFECTS ON THE CALCEIN RELEASE FROM TEMPERATURE-SENSITIVE LIPOSOMES. Submitted to International Journal of Hyperthermia). In addition, 5-FU release from TSLs is rather fast using FUS, since there is no benefit of applying FUS for longer than 10 min in terms of release. Usually, traditional TSLs (TTSLs) exhibit a slow drug release: only 10% of their drug load is released in 30 min when TTSLs are exposed to a temperature range of 43–45°C *in vitro* (21). Research therefore focused on low temperature sensitive liposomes (LTSLs) based on lysolipids designed to release their content in tens of seconds as temperature is increased. The ThermoDox® formulation is an example of LTSLs as it releases 60% of its encapsulated doxorubicin in the first 20 s of heating at 41.3°C *in vitro* (8). Our system can be considered an intermediate formulation between TTSLs and LTSLs. To the best of our knowledge, it constitutes the first thermosensitive 5-FU formulation.

At the supramolecular scale, the thermosensitivity arises from a gel to liquid crystalline phase transition of DPPC ($T_m = 41.5 \pm 0.5$°C) leading to conformational changes in the lipid bilayer resulting in permeability increase. The hypothesized mechanism involved in the enhancement of bilayer permeability is the formation of transient nanopores at the boundaries between solid and liquid crystalline domains in the bilayer (8). The enhanced permeability of the bilayer explains the release up to 68% of 5-FU. 68% release in 10 min is a good result compared to release rates previously reported regarding TSLs free of lysolipids (21,30). Moreover, it is sufficient to exhibit a therapeutic effect. Inserting 30% mol of CHOL into the lipid bilayer renders liposomes non thermosensitive, resulting in a less important release due to the weak permeability of the bilayer at 46°C. The contribution of inertial cavitation generated over the course of FUS application to induce bilayer rupture by mechanical stress is discussed. However, recent data described that drug release from TSLs results from thermal and mechanical constraints induced by FUS (6). We have previously published that 1.75 MPa, corresponding to a temperature of 46°C at the focal point, is the optimum peak negative pressure inducing a maximum calcein release from the same TSLs over 10 min (Chantal Al Sabbagh, Anthony Novell, Jean-Michel Escoffre, Cédric Gaillard, Nicolas Tsapis, Elias Fattal and Ayache Bouakaz. *IN-VITRO* EVALUATION OF FOCUSED ULTRASOUND-MEDIATED THERMAL AND MECHANICAL EFFECTS ON THE CALCEIN RELEASE FROM TEMPERATURE-SENSITIVE LIPOSOMES. Submitted to International Journal of Hyperthermia).

Many studies have demonstrated the potential of high-intensity focused ultrasound (HIFU) for localized heat-triggered drug release (39). *In vivo* studies showed that doxorubicin-loaded TSLs combined with ultrasound-induced mild hyperthermia resulted in higher concentration of the drug in the tumor (40,41). To our knowledge, the efficiency of ultrasound-induced hyperthermia for *in vivo* drug delivery was only evaluated for doxorubicin-loaded TSLs. Neither *in vivo* nor *in vitro* studies using 5-FU-loaded TSLs have been reported. Indeed, *in vitro* reports are scarce due to the difficulty to design an adapted and efficient setup (26). The results using 5-FU-loaded TSLs are however in good agreement with those obtained in previous *in vitro* work. Using a similar ultrasound setup, a maximal drug release of approximately 50% and 80% was reached when calcein-loaded TSLs (6) and doxorubicin-loaded TSLs (Escoffre *et al.*, PMB, 2013) (26) were assessed, respectively. In a recent study, a maximal calcein release of 60% was obtained *in vitro* using liposomes modified with thermosensitive polymer insonified in a water-filled dish (42). However, in this study, the calcein release was only attributed to the cavitation effect of ultrasound since no sufficient temperature elevation was measured in water.

Formulations were then considered in terms of toxicity focusing either on 5-FU, Cu or lipids as potential toxic compounds. Toxicity was assessed on a HT-29 cell line sensitive to 5-FU. HT-29 cells possess a doubling time of 24 h (data not shown), therefore 48 h exposure to formulations and controls were sufficient to assess toxicity and inhibition. 5-FU induces a dose-dependent inhibitory effect on HT-29 with an IC_{50} of 172 ± 77 μM. The IC_{50} values are in agreement with previously published ones on HT-29 cells after 48 h treatment (~300 μM) (43) (Fig. 6a and b). Cu-PEI seems to be well tolerated by HT-29 cells with a 6 mM IC_{50} in terms of Cu concentration, probably because low molecular weight PEIs are less toxic compared to higher molecular weight ones (44). These results are consistent with available data (17). When 5-FU is complexed with Cu-PEI, its inhibitory effect on HT-29 cells is very similar to free 5-FU with a similar IC_{50} (115 ± 67 μM): its binding does not alter its activity. Therefore the reduction of cell viability indeed arises from 5-FU. On one hand, unloaded liposomes and Cu-PEI-loaded liposomes do not reduce cell viability. On the other hand, 5-FU liposomes

and 5-FU-Cu-PEI liposomes lead to a decrease in cell viability probably because of a better cellular uptake of the liposomal form of either the free or the complexed drug. In agreement with previous data, liposomes favor the internalization of 5-FU either free or as a ternary complex by HT-29 cells reducing therefore 2 fold its IC_{50}.

When one considers the pharmacokinetics results, although formulations were administered intravenously, the maximum concentration does not occur immediately due to two reasons: first the injection site (tail vein) differs from the sampling site (cardiac puncture), second formulations were administered in dextrose at a high concentration leading to a high viscosity, therefore delaying their dilution in the bloodstream.

The longer distribution phase of 5-FU administered in TSLs and NTSLs compared to 5-FU administered as a solution confirms that liposome formulations circulate longer than the free drug. Values obtained for 5-FU solution are different from those obtained for 5-FU solution injected in saline for which the apparent distribution half-life was 12.9 ± 7.3 min (non-compartmental analysis) (45). The longer distribution half-life of 5-FU obtained here could arise from the concentrated dextrose medium in which 5-FU is injected. The elimination half-life of free 5-FU is probably longer since the free molecule does not interact with the immune system whereas liposomes, although stealth®, may be partially taken up by the reticuloendothelial organs.

The analysis of pharmacokinetics parameters reveal that $AUC_{0\text{-}24h}$ was not different than $AUC_{0\text{-}4h}$ for 5-FU solution. This indicates that 5-FU is already cleared within the first 4 h post injection, and thereby, beyond 4 h, 5-FU plasma concentration does not contribute to the exposure of the organism to the drug. While, for 5-FU-loaded liposomes, $AUC_{0\text{-}24h}$ was different than $AUC_{0\text{-}4h}$ showing that even during the elimination phase, 5-FU contributes to the whole body exposure. One should also note that values of $AUC_{0\text{-}24h}$ were not different than $AUC_{0\text{-}\infty}$ values, meaning that 24 h experiment is sufficient to assess 5-FU PK. Overall, as expected, liposomes lead to a prolonged exposition to 5-FU as compared to free drug. PEGylation was indeed efficient to promote liposome circulation. The difference between TSLs and NTSLs probably arises from their CHOL content. As shown in the literature (20–22), liposomes containing 30% CHOL are more stable in the bloodstream than those containing a lower concentration of CHOL. Clearance values are in agreement with previous findings with 5-FU being cleared faster when administered as a free molecule as compared with TSLs which are in turn cleared slightly faster than NTSLs (Table II). Although literature is abundant on different liposomes formulations of 5-FU, scarce data is reported about their PK. Thomas *et al.* (17) performed a PK study on non-PEGylated liposomes but did not calculate any PK parameters. Jin *et al.* (46) evaluated the PK of galactosylceramide liposomes encapsulating 5-FU and found a shorter distribution half-life of 28 min and a 3 to 5-fold lower AUC of 38 $\mu mol.h.L^{-1}$. Our TSLs and NTSLs formulations are therefore better in terms of exposure. The difference of AUCs and distribution half-lives between the free drug and the liposomes are not as impressive as what has been observed with Doxil® which might result from the different encapsulation mechanism but could be a benefit for release upon hyperthermia.

The higher 5-FU concentration in the tumor for NTSLs was likely due to passive tumor targeting due to the prolonged circulation of NTSLs as compared with the other formulations. Although TSLs circulate longer than the free drug, there was no significant difference in 5-FU accumulation in tumor at 4 h. This confirms the higher stability of NTSLs compared to TSLs as discussed above.

There was no significant difference between 5-FU-loaded liposomes liver capture at 4 h proving their stealthiness is identical. Despite their long circulation, NTSLs are better recognized than TSLs by spleen macrophages. Splenic uptake is often compared to a filtering or a sieving process, particularly effective to remove poorly opsonized antigens, therefore it does not depend on the nanoparticles surface properties (47). The spleen uptake of 5-FU-NTSLs compared to 5-FU-TSLs at 4 h is significantly higher by 1.5-fold in agreement with previous published data (28). Schroit *et al.* report that CHOL increases the uptake of PC liposomes by the RES (48). 24 h post-injection, 5-FU concentration globally decreases and is similar for free drug and TSLs and slightly greater for NTSLs. The greater accumulation of NTSLs in the lungs may arise from their larger size and higher rigidity (48). 24 h post-injection, 5-FU concentration in the lungs is similar for the 3 groups.

Drug concentration in the kidneys is in agreement with data that reports a short plasmatic half-life of 5-FU after i.v. bolus administration in patients and animals due to a clearance from plasma in 1 h as a consequence of a very rapid metabolism by dihydropyrimidine dehydrogenase or uracil reductase (45,12,49). Approximately 60–90% of the administered dose of 5-FU is excreted in urine within 24 h, primarily as alpha-fluoro-beta-alanine. Furthermore, 10% of the injected dose is excreted unchanged by urine (50). Therefore, free 5-FU concentration in kidneys at 4 h corresponds to the small amount that remains in blood during the elimination phase, and does not constitute a relevant comparison with 5-FU liposomes. Regarding liposomes formulations, the significant difference, in 5-FU concentration in kidneys at 4 h, between 5-FU-TSLs and 5-FU-NTSLs probably arises from the lower serum stability of TSLs compared to NTSLs, that induces an earlier 5-FU leakage in blood.

CONCLUSION

We have optimized the encapsulation and retention of 5-FU into TSLs made of DPPC/CHOL/DSPE-PEG (90:5:5 mol%)

by using a ternary complex of 5-FU-Cu-PEI. 1.75 MPa FUS applied for 10 min was sufficient to induce local and homogeneous mild hyperthermia (42°C) and therefore efficient and rapid release of 5-FU. CHOL insertion within lipid bilayers made of DPPC and 5 mol% of DSPE-PEG, allows to finely tune the thermosensitivity of liposome formulations. The 5-FU-Cu-PEI ternary complex does not seem to induce additional toxicity as revealed by the MTS assay on HT-29 cells. We have administered intravenously two optimized formulations of 5-FU-loaded TSLs and 5-FU-loaded NTSLs in HT-29 tumor-bearing mice and we have demonstrated improved pharmacokinetics and biodistribution of the anticancer drug 5-FU at short time points compared to the free drug solution. Both TSLs and NTSLs exhibited a longer blood circulation but only NTSLs improved 5-FU accumulation in tumor. Combination of TSLs with focused ultrasounds to induce local hyperthermia in the tumor will be tested for efficacy in the future.

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