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Encapsulation of Doxorubicin in Thermosensitive Small Unilamellar Vesicle Liposomes

Jean-Louis Merlin

The optimisation of the formulation of thermosensitive, doxorubicin-containing small unilamellar liposomes is described. The liposomes were first strictly defined in terms of size distribution and size stability and a quality level was defined. The suspension contained more than 95% vesicles with a maximal diameter of 50 nm and kept this level for a minimum of 24 hours. Several lipid mixtures were tested in defined thermal conditions usable for *in vitro* experiments: 43°C in fetal calf serum-containing medium. The mixture yielding the best differential thermal stability (DTS) defined as the difference of release between 37°C and 43°C exposures was found to be a dipalmitoylphosphatidylcholine/distearoylphosphatidyl–choline/cholesterol mixture in 5:4:2 molar ratio yielding 72% DTS. These thermosensitive liposomes were evaluated betwen pH 6.00 and 8.00 since hyperthermia-induced lethality was reported to be enhanced by pH variations. Their release capacity was not altered by any pH variations. Incorporation of doxorubicin within these liposomes was then performed. The release kinetics at 37° and 43°C were determined. It is proposed to use this formulation in *in vitro* experiments on tumour cells, although a decrease of DTS was evident.
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INTRODUCTION

SELECTIVE LOCALISATION of antitumour drugs is a goal that may result in better control of cancers. Most antitumour agents interact with non-malignant tissues reducing the therapeutic effectiveness when given systemically. Liposome-encapsulated drugs appear to represent an interesting alternative opportunity [1, 2]. The combination of liposome-mediated drug delivery and hyperthermic treatment is an original approach and synergistic applications have been demonstrated on tumour models with encapsulated methotrexate [3, 5], cisplatin [6] or bleomycin [7, 8], hyperthermia playing the double role of treating the tumour and triggering the local efflux of the drug from the liposomes. To our knowledge, no experimentation has been reported with doxorubicin encapsulated in thermosensitive liposomes.

Our experiments were designed to evaluate a series of liposomes prepared with dipalmitolyphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), and cholesterol in order to optimise the thermosensitivity. The molar ratios of these

components were adjusted from the formulation which was originally proposed in the literature [5, 3] in order to reach a maximal differential thermal stability between physiological (37°C) and hyperthermic (43°C) temperature in serum-containing media. Size distribution analysis was performed in order to define a "quality level" of the liposome suspensions which were prepared. In addition, the effect of pH—which possibly enhances the effect of hyperthermia [9]—on the stability of the liposomes was investigated. Encapsulation and the release kinetics of doxorubicin were then performed.

MATERIAL AND METHODS

Liposome preparation

Egg yolk phosphatidylcholine (PC), DPPC, DSPC and cholesterol were obtained from Sigma as products of higher purity (99%) and were used without further purification. Doxorubicin was obtained from Laboratoires Roger Bellon (Neuilly, France). Sephadex gels were obtained from Pharmacia. 6-carboxyfluorescein (6-CF) was obtained from Kodak and was purified to remove lipophilic impurities by gel filtration on Sephadex LH-20 as described [10]. All other chemicals used were reagent grade and purchased from Prolabo (Paris). Liposomes were prepared according to Bangham's procedure [11] with slight modifications. Briefly, dry lipids were dissolved in chloroform; the organic solvent was then evaporated under reduced pressure at 40–45°C using a rotary evaporator. The film was flushed with nitrogen in order to remove any trace solvent. The dried lipid film was then rehydrated at 50°C with 1 ml of 150 mmol/l NaCl, phosphate-buffered saline (PBS) at pH 7.4 to reach a final lipid concentration of 35 μmol/l (20 mg/ml) for 1 hour under slight agitation.

For the determination of the thermal stability kinetics, self-quenched (100 mmol/l) purified 6-CF was added to the PBS. Doxorubicin was encapsulated by rehydration of the dry lipid film with a 1.7 μmol/l solution in PBS. In all cases the suspension was then submitted to ultrasonic disruption using a 20 kHz, 500 W sonicator (Sonics & Materials, Bioblock, Strasbourg) fitted with a 3 mm conical titanium probe.

The sonication time was adjusted to obtain liposome suspension with a defined size distribution. After centrifugation (1000 *g*, 10 min) to remove titanium particles, the suspension were gel filtered on Sephadex G75 to separate the non-encapsulated material (6-CF or doxorubicin) and sterilised by filtration on 0.22 μm polycarbonate membrane (Nuclepore, Serlabo, Paris). The liposome suspensions were kept in darkness under nitrogen atmosphere at 4°C. Size analysis was performed at 20°C on a Coulter N4 apparatus (Coultronics, Margency, France) based on photon correlation spectroscopy with right angle scattered light analysis. Size distribution process (SDP) mode was used to evaluate the size distribution of the vesicles, especially the weight–SDP mode which expressed the distribution as total weight ratios. The liposomes were defined as small unilamellar vesicles (SUV) when the suspension contained more than 95% of vesicles with a diameter less than 50 nm. The preparation procedure was optimised to reach this quality level and different DPPC/DSPC/cholesterol mixtures were compared in order to optimise the differential thermal stability, i.e. the difference between the 37°C and the 43°C leakage rates. For doxorubicin liposomes, the encapsulation rates were spectrofluorimetrically determined after disruption of the vesicles by addition of sodium deoxycholate. Excitation and emission wavelengths were, respectively, 485 and 565 nm.

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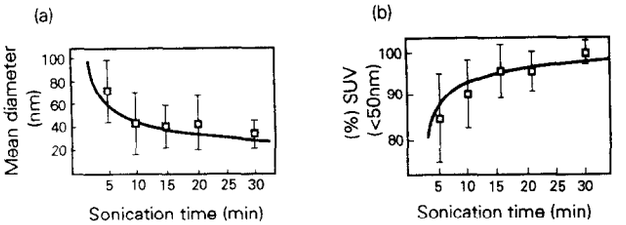

Fig. 1. Effect of the sonication time on the mean diameter (a) and the ratio of SUV, i.e. vesicles with diameter less than 50 nm (b) of DPPC/DSPC 6:3 liposome suspensions. Mean (S.D.) of triplicate experiments.

Determination of the vesicle stability

Thermal stability was performed by measuring the leakage of 6-CF from the liposomes incubated at 37 or 43°C in PBS with or without 15% fetal calf serum (FCS) (Gibco). Self quenched within the liposomes, the 6-CF was not fluorescent but developed an intense fluorescence when leaking out of the liposome and diluted into the dispersion medium.

These analyses were performed on a Shimadzu RF 5000 spectrofluorimeter (Roucaire, Velizy-Villacoublay, France) fitted with a thermostatable cell. Excitation and emission wavelengths were, respectively, 485 and 515 nm. Temperatures (37 and 43°C) were programmed and controlled (within 0.1°C) by a Haake PG20 controller (Roucaire, Velizy-Villacoublay, France). The fluorescence monitoring began when the experimental temperature was reached in the analytical cell as measured by an immersed thermocouple and was performed for 2 hours. Results were expressed as a percentage of the maximal values which were obtained by disruption of the liposomes by addition of 10 μl of a 4% sodium deoxycholate solution. The "zero" value was measured on an intact suspension at 4°C. The lipid crystal phase transition temperature was determined by submitting 6-CF-encapsulated liposomes to temperature increasing from 30 to 50°C at 1°C/min. Doxorubicin liposomes were tested for thermosensitivity by incubating the liposome suspension in a dialysis bag with a 10 000 dalton cut-off threshold (Spectrapor, PolyLabo, Strasbourg), immersed in a thermostatted water bath. Aliquots were taken every 10 minutes and assayed for doxorubicin concentration. Results were expressed as a percentage of the maximal value obtained after disruption of the vesicles.

pH stability was assessed with the same procedure. 100 μl of the 6-CF-liposome suspension was diluted in 4 ml of PBS with pH ranging from 6.00 to 8.00, at 37° and 43°C, with and without FCS. Stability in size of the suspensions was controlled over a 24 hour period of storage at 4° and 20°C, in darkness.

RESULTS

Small unilamellar vesicles

The evolution of the size distribution of DPPC/cholesterol 6:3 liposome suspensions was plotted against the sonication time (Fig. 1): the mean diameter of the suspension (Fig. 1a) decreased until a plateau was reached near 50 nm. Further duration of sonication did not influence the mean value but tended to homogenise the suspension, as expressed by a decrease in the standard deviation. SDP analysis results (Fig. 1b) further illustrated this evolution since the ratio of the 50 nm population increased with the sonication time, reaching 95% for 15 minutes.

Table 1. Size analysis results of DPPC/DSPC/cholesterol liposomes suspension

	DPPC/DSPC/cholesterol ratio					
	6:3:0	7:1:0	6:3:2	6:3:4	5:4:2	4:5:2
Mean diameter (nm)	42 (18)	44 (16)	48 (13)	42 (16)	46 (17)	45 (12)
% SUV < 50 nm	96 (6)	97 (8)	95 (6)	98 (4)	96 (7)	98 (5)

Suspension submitted to 15 min sonication.
Mean (S.D.) of triplicate experiments.

15 minutes sonication was selected for the following experiments with liposomes made of DPPC/DSPC/cholesterol mixtures. Size analysis on these suspensions (Table 1) showed that a 15 minute sonication was suitable to reach the quality level of 95% of vesicles with a diameter less than 50 nm.

Thermosensitivity

Several formulations were experimented upon for differential thermal stability (DTS) measurement between 37° and 43°C, in buffers containing or without 15% FCS (Fig. 2). Our goal was to obtain liposomes with the highest DTS value in 15% FCS buffer and exhibiting an exponential profile of leakage suitable to reach the best hyperthermal release in *in vitro* experiments.

The first formulation to be tested was the DPPC/DSPC 7:1 mixture which was first referenced as thermosensitive. Results (Fig. 2a) showed that these liposomes were quite stable in FCS-free buffer both at 37° and 43°C since no leakage was detected for 2 hours. When incubated in FCS buffer, 6-CF leaked out of these vesicles at a constant rate at 37° and 43°C, the latter leading to a maximal 90% leakage after 2 hours while a 70% value was

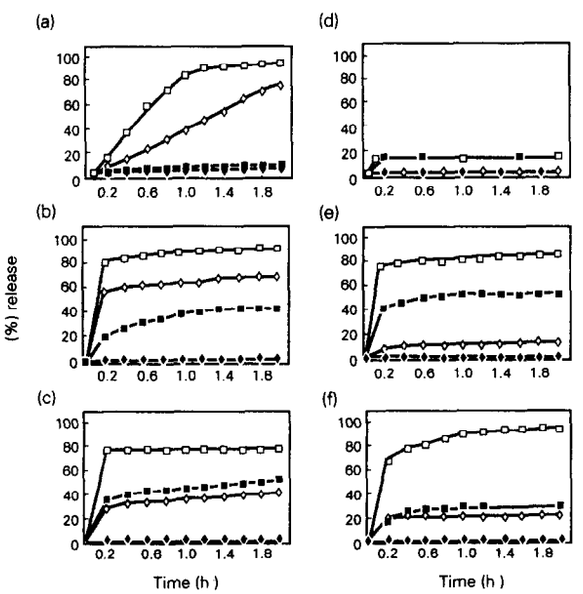

Fig. 2. Thermosensitivity of DPPC/DSPC/cholesterol liposomes at 37° or 43°C, in FCS-free or FCS-containing medium expressed by release kinetics of encapsulated self-quenched 6-carboxyfluorescein. -♦- 37°C, FCS-free; -◊- 37°C, 15% FCS; -■- 43°C FCS-free; -□- 43°C, 15% FCS. Ratios:(a) DPPC/DSPC/cholesterol 7:1:0, (b) 6:3:0, (c) 6:3:2, (d) 6:3:4, (e) 5:4:2, (f) 4:5:2.

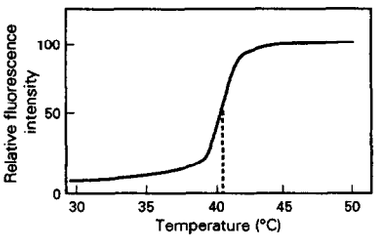

Fig. 3. Phase transition release of DPPC/DSPC/cholesterol 5:4:2 liposome encapsulated self-quenched 6-CF. Temperature gradient was applied from 30°C to 50°C at a rate of 1°C/min.

reached at 37°C (DTS 20%). After 1 hour the DTS was already 50% (85–35). The next formulation tested was prepared by enriching the mixture in DSPC (which must enhance the DTS of the liposomes, since its liquid-crystal phase transition temperature is 53°C against 41°C for DPPC).

Figure 2b illustrates the results obtained with DPPC/DSPC 6:3 mixture. It shows that even if a lower than 20% DTS value was achieved both at 1 and 2 hours, the leakage profile was different and tending to the desired exponential shape. Adding cholesterol to this mixture: DPPC/DSPC/cholesterol 6:3:2 (Fig. 2c), enhanced the stability in FCS buffer leading to DTS values of 40% and 35%, respectively, for 1 and 2 hours. Further addition of cholesterol (6:3:4) generated thermostable liposomes with DTS decreasing to 0% (Fig. 2d).

Examining these results, it appeared that the cholesterol/phospholipid ratio was to be near 2:9 mol/l and that further enhancement of the DTS would only occur with the modulation of the DPPC/DSPC ratio. The next mixture was therefore composed of DPPC/DSPC/cholesterol in 5:4:2 molar ratio. The results obtained with these liposomes (Fig. 2e) showed a significant increase in DTS reaching 70% even after a short thermal exposure of 30 minutes. Further relative enrichment in DSPC (4:5:2) did not lead to higher DTS values as illustrated in Fig. 2f.

Phase transition temperature

The phase transition temperature of DPPC/DSPC/cholesterol 5:4:2 liposomes was estimated by monitoring the leakage of encapsulated 6-CF throughout the vesicles when exposed to a temperature increasing from 30° to 50°C at 1°C/min rate as programmed on the temperature controller. Inertia of the system was controlled by immersing a thermocouple directly in the analytical cell and was found to be 2.0 (S.D. 0.1)°C. This value was deduced from the programmed values before drafting the results of triplicate experiments as percent leakage vs. temperature in the analytical cell (Fig. 3). The phase transition temperature was graphically estimated and found to be 41.2°C.

pH stability

Stability of DPPC/DSPC/cholesterol 5:4:2 liposomes was controlled in pH conditions varying from 6.00 to 8.00 in

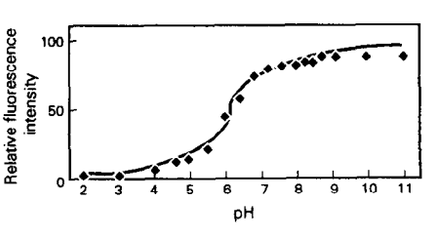

Fig. 4. Influence of pH on the fluorescence of 6-CF.

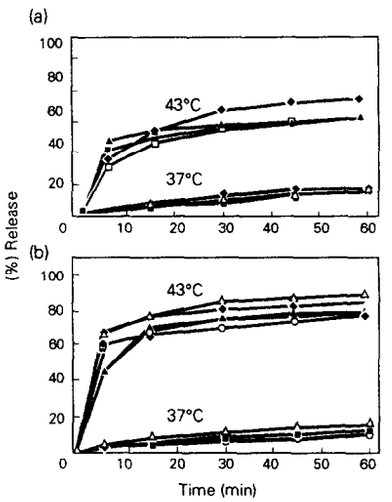

Fig. 5. pH sensitivity of DPPC/DSPC/cholesterol 5:4:2 liposomes at 37° or 43°C in FCS-free (a) or FCS-containing (b) medium. -◆- pH = 6.00, -◇- 6.5, -■- 7, -□- 7.40, -▲- 7.6 and -△- 8.00.

measuring the efflux of 6-CF throughout the vesicles. The fluorescence of 6-CF was first evaluated in the different pH conditions and found to be drastically altered between 6.00 and 8.00 (Fig. 4). In order to compare the results obtained at different pH values with the liposomes, the fluorescence intensities were corrected before being plotted as a percentage of the maximal value achieved with disrupted liposomes.

Results did not show any significant difference between the leakage kinetics obtained with pH varying from 6.00 to 8.00, at 37° or 43°C, in FCS-free or FCS-containing buffer (Fig. 5).

24 hour granulometric stability

The granulometric stability of DPPC/DSPC/cholesterol 5:4:2 liposome suspensions was evaluated during 24 hour storage at 4° or 20°C in darkness. These liposomes were found to be quite stable in these conditions, since no variation in mean diameter (Fig. 6a) or in SUV ratio (Fig. 6b) was noticeable. No difference was detected between 4° and 20°C storage conditions allowing light-protected room temperature storage of the suspensions.

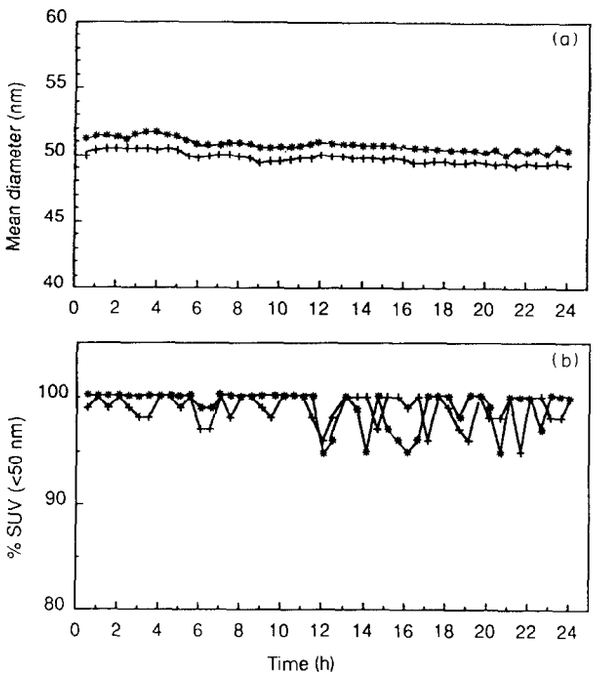

Fig. 6. Granulometric stability of DPPC/DSPC/cholesterol 5:4:2 liposomes stored in darkness for 24 h at 20° or 4°C. (a) Mean diameter of the suspension, (b) SUV ratio. + = doxorubicin-containing, * = empty.

Doxorubicin-containing thermosensitive liposomes

Encapsulation of doxorubicin in DPPC/DSPC/cholesterol 5:4:2 liposomes did not generate any particular problem. The characteristics of these liposomes (Table 2) were as follows: 15 minute sonication time yielded vesicles with a mean (S.D.) diameter of 56 (9) nm; the suspension being composed of 96 (5)% SUV. The encapsulation rate was 2.6 (0.9)% (n = 4). Differential thermal stability values were 38 (5) and 47 (8)%, respectively, after 30 and 60 minutes in FCS-containing medium.

DISCUSSION

Association of hyperthermia with liposome-mediated drug delivery is a very attractive combination. Indeed, it could be possible to associate a selective drug delivery to a local treatment of tumours and potentiate the drug effects. This would require formulation of thermosensitive vesicles exhibiting stability profiles allowing a quick release of the encapsulated compound in supranormal temperature conditions as well as simultaneous high stability at 37°C.

The first thermosensitive formulation of liposomes was proposed by Yatvin in 1978 [5] and further used in many studies [3, 4, 6, 8]. The liposomes were prepared with DPPC/DSPC (7:1 molar ratio) and were validated in estimating drug concentrations in blood from mice exposed to 90 second hyperthermic pulses [10]. *In vitro* release kinetics were established [6] and showed that these liposomes were maximally releasing their contents at 42°C. These studies demonstrated that the combination of hyperthermia and thermosensitive liposomes could offer a very interesting approach to tumour treatment since higher (four times) drug concentration were found in heated tumours.

Since it was clearly shown that the stability of small unilamellar liposomes is highly correlated to the cholesterol content [12, 13], our study was designed to further ameliorate the thermosensitivity of small unilamellar liposomes prepared with cholesterol in order to enhance their stability in biological fluids.

SUV were reported to be more suitable as drug delivery systems since they offered a better stability [14, 15]. To this main advantage can be added the possibility of SUV suspensions sterilised by filtration through 0.2 μm polycarbonate membranes. Since it was demonstrated that the size distribution of the liposome suspensions could affect their cellular interaction, a rigourous granulometrical quality level was defined and controlled by photon correlation spectroscopy. Only liposome suspensions which were composed of more than 95% of vesicles with a maximal diameter of 50 nm were further investigated. Their granulometrical stability during 24 hours ensured the quality level during the time of experimentation.

Table 2. Characterisation of doxorubicin-containing thermosensitive liposomes

Mean diameter (nm)	% SUV < 50 nm	Encapsulation rate (%)	DTS (%)
46 (18)	96 (5)	2.6 (0.9)	38 (5) 30 min 47 (8) 60 min

43°C hyperthermic temperature was selected in order to reach a high cytotoxicity level with 30 minute exposure times on cultured tumour cells (data not shown) and to potentiate the cytotoxicity of doxorubicin, which needs a minimal temperature of 43°C to be manifested [16]. DTS between 37° and 43°C in FCS-containing medium was determined for the "standard" formulation of thermosensitive liposomes [5] and was not found to be selective enough in our experimental conditions (17%).

Since the cholesterol content of liposomes is known to play a major role in their stability in serum containing media, i.e. culture media or biological fluids [12, 13], the formulation of thermosensitive liposomes might not be only based on liquid crystal transition phase temperature measurements of pure phospholipid mixtures but on *in vitro* assays evaluating their ability to exhibit a maximal differential thermal stability between physiological and hyperthermic temperature conditions.

We examined the DTS of a series of formulations which were deduced from the original one, but contained cholesterol in order to ensure the stability in FCS-containing media. As a result, the highest DTS value (72%) was reached with DPPC/DSPC/cholesterol 5:4:2 molar ratio liposomes only after 30 minutes of hyperthermal treatment.

Modification of extracellular pH can modulate the cytotoxicity induced in biological tissues submitted to hyperthermia [9]. It appeared thereafter important to control the effect of pH on the release capacity of DPPC/DSPC/cholesterol 5:4:2 molar ratio liposomes. As the relative fluorescence of 6-CF was drastically affected by pH variations, all results were expressed in relative fluorescence intensity. The release capacity of the thermosensitive liposomes was not found to be affected by any pH variations since no difference in the release of the encapsulated dye was detected.

Encapsulation of doxorubicin within SUV liposomes is of great interest since it was demonstrated to reduce its cardiotoxicity while enhancing the cytotoxicity [17].

Since 1986, several phase I and II studies have been reported which used liposome encapsulated doxorubicin in hepatocellular carcinomas [18], or, more recently, in advanced breast cancer [1, 2]. Less cardiac toxicities developed than with free doxorubicin at cumulative doses of 500–880 mg/m^2.

On the other hand, many studies established that 43°C hyperthermic treatment can potentiate the cytotoxicity of doxorubicin [16, 19, 20]. It appeared therefore interesting to encapsulate this compound in thermosensitive liposomes in order to associate these two modes of treatment, thus potentially enhancing the activity of doxorubicin and reducing the cardiotoxicity.

Experimentally, encapsulation of doxorubicin did not induce any difference in size distribution but lowered the differential thermal stability (47% vs. 82%) compared with the CF liposomes. This phenomenon can probably be attributed to the physico-chemical difference existing between the two compounds, 6-CF being quite hydrophilic while doxorubicin is amphiphilic and could thereby remain partly linked to the lipid bilayer of the liposomes and not diffuse through the dialysis membrane. However, the reported results validate the formulation of thermosensitive liposomes with a mixture of DPPC/DSPC/cholesterol in 5:4:2 molar ratio which yielded the best results in *in vitro* hyperthermal conditions at 43°C in FCS-containing buffered medium. Even if the release capacity is altered when doxorubicin is encapsulated, this formulation appeared usable in *in vitro* experiments since concentrations up to 2 nmol/l can be reached after 60 minutes at 43°C. Comparisons of free and encapsulated doxorubicin will only require an adjustment of the concentrations in regard to a release fraction which is reproducible in defined conditions. Such studies are in progress and will be reported.

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