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Development of liposomal capreomycin sulfate formulations: Effects of formulation variables on peptide encapsulation

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Abstract

Purpose: The aim of this work was the investigation of the effects of preparation variables on drug content for the development of capreomycin sulfate (CS) liposomal formulations as potential aerosol antitubercular agents.
Methods: Dipalmitoylphosphatidylcholine (DPPC), hydrogenated phosphatidylcholine (HPC) and distearoylphosphatidylcholine (DSPC) were used for liposome preparation. A freeze–thawing method was chosen for CS encapsulation. Peptide entrapment, size and morphology were evaluated by UV spectrophotometry, photocorrelation spectroscopy (PCS) and transmission electron microscopy (TEM), respectively. A 2^3 full factorial protocol was designed to evaluate the conditions for CS encapsulation improvement.
Results: Peptide content ranged between 1 and 8%. Vesicles showed a narrow size distribution, with average diameters around 1 μm and a good morphology. A mathematical model was generated for each liposomal system and check point analyses revealed good agreement between experimental and predicted values. DPPC liposomes were found to provide the highest CS content.
Conclusions: Peptide content was successfully increased by assessing formulation variable effects using a 2^3 factorial design that proved to be a time saving method helpful in developing new CS liposomal formulations for a possible application in aerosol antitubercular therapies.

Keywords: Capreomycin sulfate; Liposomes; Freeze–thawing; Factorial design

1. Introduction

Tuberculosis (TB) infections have been increasingly spreading in the last 10 years. This increasing incidence of TB is attributable to several factors as HIV epidemics, immigration from third world countries and failure of directly observed treatment short course (DOTS) therapies (Vigorita et al., 1994; Ferrarini et al., 1999; Maher et al., 2002).

In this regard, improper use of antibiotics in chemotherapy easily produces multiple drug-resistance (MDR) strains to conventional antitubercular drugs. Drug-resistant TB is treatable, but it requires extensive chemotherapy, often prohibitively expensive and very toxic to patients. Therefore, less common and generally more toxic drugs have to be employed alone or in combination.

Most antibiotics are relatively ineffective for the treatment of intracellular infections, such as tuberculosis, due to poor penetration into cells or decreased intracellular activity. Improvement of antimicrobial agent efficacy against microorganisms located inside cells has been achieved by drug entrapment within liposomes (Deol and Khuller, 1997; Gursoy, 2000; Pinto-Alphandary et al., 2000). Liposomes may enhance molecule penetration within infected cells and simultaneously reduce the side effects of very toxic antitubercular drugs such as CS (Le Conte et al., 1994).

CS is a highly water-soluble peptide characterized by four co-existing cyclic forms (Fig. 1). It is used, intramuscularly (15–20 mg/kg/day), in combination with other effective drugs, in the treatment of tuberculosis that failed to respond to first-line agents (Martindale, 1997). It is active against *Mycobacterium tuberculosis*, *M. bovis*, *M. kansasii* and *M. avium*. Recently the Italian National Institute of Health (ISS) has shown that only about 10% of the 46 drug-resistant strains of *M. tuberculosis* isolated from Italian patients were resistant to CS (Fattorini et

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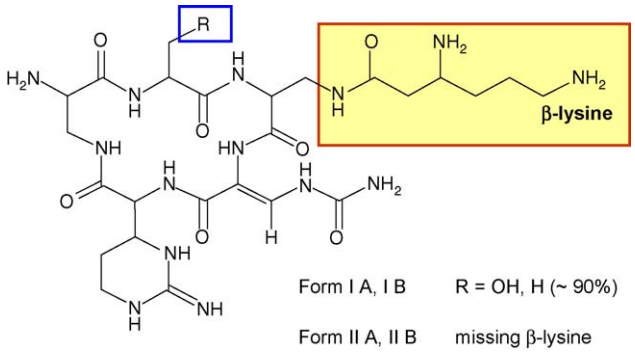

Fig. 1. Capreomycin sulfate structure. The molecule exists as four different forms.

al., 1999). Moreover, it has been demonstrated that entrapment of CS in multilamellar vesicles (MLVs) reduces renal toxicity, enhances peptide penetration into tissues and increases CS activity in Beige mouse model of *Mycobacterium Avium complex* (MAC) infection (Le Conte et al., 1994) and that unilamellar liposomes may potentially represent good candidates for CS delivery (Giovagnoli et al., 2003).

These findings showed that the development of CS liposomal formulation may provide a suitable tool for improving CS performances and make possible its use in antitubercular therapies.

Unfortunately, entrapment of very hydrophilic drugs, such as CS, is not an easy task as, in virtue of their own affinity with the outer aqueous phase, diffusion of molecules to be entrapped is usually increased. For this reason, a freeze–thawing method was chosen for CS encapsulation to achieve successfully high encapsulation efficiencies for hydrophilic molecules (Corvo et al., 2002).

To achieve the goal to improve pharmaceutical formulation, experimental design protocols were employed. In particular, 2^k designs represent time and material saving tools used to assess the effects of variables on a particular property of a pharmaceutical system (Avinash and Ambikanandan, 2002). Moreover, the use of a full factorial design avoids possible aliasing of main effects and interactions.

In light of these considerations, the aim of this paper was to develop a new CS liposomal respirable formulation for peptide delivery to the lungs in the treatment of tuberculosis. This target was pursued by applying a 2^3 full factorial design to assess the effects of % loading, number of cycles and duration of each cycle, on % peptide content. For this purpose, CS loaded large unilamellar vesicles (LUVs) were made of dipalmitoylphosphatidylcholine (DPPC), hydrogenated phosphatidylcholine (HPC) and distearoylphosphatidylcholine (DSPC) mixed together with dipalmitoylphosphatidylglycerol (DPPG) in a 90 to 10 ratio. LUVs were characterized in terms of peptide content, morphology and size, and the effect on size distribution of the peptide loading process was evaluated as well.

First order models were employed and their adequacy was investigated by adding central points to the design dataset to evaluate a possible response curvature. Moreover, contour plots describing the variable effects on the response were built to define the proper conditions for an encapsulation improvement. In addition, model prediction efficiency was assessed by check point analysis in the central region and in the maximum drug content region as reported from the contour plots.

2. Materials and methods

2.1. Materials

CS from *Streptomyces Capreolus* and DPPC, HPC, DSPC phospholipids were purchased from Sigma–Aldrich Chemical. Sodium hydrogen orthophosphate was provided by Farmitalia Carlo Erba (Milan, Italy) and chloroform by J.T. Baker (Milan, Italy). Poly L-lysine, $HClO_4$, phosphotungstic acid and Triton X-100 reduced form were purchased from Sigma–Aldrich (Milan, Italy). Ultra pure water was obtained by reverse osmosis through a Milli-Q system (Millipore, Rome, Italy). All other reagents and solvents were of the highest purity available.

2.2. Liposome preparation

Large unilamellar vesicles were made by DPPC–DPPG, DSPC–DSPG or HPC–DPPG (90:10, w/w). Blank liposomes were prepared by a thin layer evaporation (TLE) method. Briefly, lipids were dissolved in chloroform into 250 mL round bottom flasks. Then the organic solvent was evaporated under nitrogen stream and the dry lipid films were maintained under reduced pressure to remove traces of the solvent.

Films were hydrated by adding an appropriate amount of water to yield 10 mg/mL phospholipid concentration, while shaking in a Gallenkanp orbital incubator (Fisons Instruments, Crawley, UK) at a temperature of 10 °C above the phospholipid gel–liquid crystalline phase transition temperature (T_m), until homogeneous milky suspensions were obtained. The MLV suspensions were extruded through a polycarbonate filter (pore size 1 μm) using an Emulsiflex C5 (Avestin, Faucitano s.r.l. Milan, Italy) and the LUVs obtained were stored overnight at 4 °C.

CS containing liposomes were prepared by modification of the freeze–thawing method elsewhere proposed (Ramaldes et al., 1996). Three milliliters of blank LUVs, prepared as described previously, were mixed with an equal volume of CS aqueous solution and shaken with a vortex mixer for 5 min. Then, each suspension was frozen using a dry ice-ethanol bath for different periods of time and thawed in a water bath at 50 °C. Five to 10 cycles of freeze and thawing were performed. Free CS was separated, from liposome-encapsulated CS, by ultracentrifugation (70,000 rpm, 2 h, 4 °C) using an Optima™ TL Ultracentrifuge with a TLA 100.4 rotor (Beckman, Palo Alto, CA, USA). Supernatant volumes were filtered through a syringe filter (pore size 0.22 μm) and the final volume was adjusted to 5 mL.

2.3. Evaluation of CS content

According to a previously validated method (Rossi et al., 2004), the amount of peptide encapsulated was evaluated by UV spectrophotometry using a UV–vis Agilent 8453 spectrophotometer (Agilent, Germany). Liposome suspensions were prop-

erly ultracentrifuged (70,000 rpm, 2 h, 4 °C) by an Optima™ TL Ultracentrifuge equipped with a TLA-100.4 rotor (Beckman). Supernatant aliquots were directly analyzed by measuring CS absorbance at 268 nm. The entrapped CS within the unilamellar vesicles was determined after resuspension of the pellets in PBS buffer pH 7.4 and addition of 10% reduced Triton X-100 solution according to the aforementioned method (Rossi et al., 2004). Similarly, total CS concentrations of the three LUV formulations were determined on freshly prepared suspensions and error was expressed as standard deviation (±S.D.). All measurements were performed in triplicate.

The entrapped CS amount was reported as % drug content according to Eq. (1):

$$\%\ \text{drug content} = \frac{\text{amount of entrapped drug}}{\text{amount of entrapped drug} + \text{amount of phospholipids}} \quad (1)$$

The amount of phospholipids was determined by using a phosphate analysis spectrophotometric method (Ramirez-Munoz, 1975). Briefly, 7 μL of pellet vesicles were dried up by a rotavapor and 0.65 mL of a concentrated $HClO_4$ water solution were added and incubated at 180 °C for 30 min. After cooling, 3.3 mL of HPLC grade water, 0.5 mL of a 25 mg/mL ammonium molibdate solution and 0.5 mL of a 0.1 g/mL ascorbic acid solution were added upon vortexing. The solution obtained was heated at 55 °C for 5 min. The analyses were performed by reading the absorbance at 818 nm. All measurements were carried out in triplicate and the error expressed as S.D.

2.4. Size distribution and morphology

Liposomes were morphologically characterized by means of transmission electron microscopy (TEM) using a Philips EM 400T microscope (Eindhoven, NL). Samples were prepared according to a method elsewhere reported (Moscho et al., 1996). In particular, a drop of liposome suspension was floated on the surface of a 200 mesh formvar coated copper grid earlier treated with a poly-l-lysine solution. After 3 min, the liposome suspension was drawn off and replaced with a drop of negative stain (phosphotungstic acid 2%, w/v, pH 6.5 in distilled water).

Table 1
Factors and levels employed for the 2^3 full factorial designs

	Factor	Minimum	Maximum	Minimum coded value	Maximum coded value
X_A	% Loading	16	32	−1	+1
X_B	No of cycles	5	10	−1	+1
X_C	Time of each cycle (min)	5	7	−1	+1

Dimensional distribution analyses were performed using a Nicomp 380 autocorrelator (PSS Inc., Santa Barbara, USA) equipped with a Coherent Innova 70-3 (Laser Innovation, Moorpark, CA, USA) argon ion laser. The effect of the freeze–thawing cycles was investigated on size distributions of blank and loaded vesicles. Polydispersity (PD) of liposome size distribution was also calculated before and after the freeze–thawing process on triplicate samples. PD was reported as Span = $[D(v,90) - D(v,10)]/D(v,50)$, where $D(v,90)$, $D(v,10)$ and $D(v,50)$ are the equivalent volume diameters at 90, 10 and 50% cumulative volume, respectively.

2.5. Factorial design study

A 2^3 full factorial design was built to evaluate main effects and interactions of the three factors chosen on % peptide content. The factors were selected according to the parameters characterizing the preparation method, namely % peptide loading (X_A), number of freezing and thawing cycles (X_B) and time of each cycle (X_C) (Table 1). Sixteen batches, one preparation and one replicate, of CS loaded LUVs were prepared for each liposome system.

The complete first order regression model chosen is described by Eq. (2):

$$Y = \beta_0 + \beta_1 X_A + \beta_2 X_B + \beta_3 X_C + \beta_4 X_A X_B + \beta_5 X_A X_C + \beta_6 X_B X_C + \beta_7 X_A X_B X_C + \varepsilon \quad (2)$$

where Y is the dependent variable, namely % drug content, and β_i are the multiple regression coefficients representing estimates of main effects and interactions. In particular, β_1, β_2, β_3 represent main effects, β_4, β_5, β_6 two-factor interactions and β_7 the three-

Table 2
Complete 2^3 full factorial disegn set up for the three liposomal formulations

HPC	X_A	X_B	X_C	X_{AB}	X_{AC}	X_{BC}	X_{ABC}	I	HPC response		DPPC response		DSPC response	
									% Content	Replicate	% Content	Replicate	% Content	Replicate
1	−1	−1	−1	+1	+1	+1	−1	+1	2.1617	2.1596	4.5302	4.6954	2.9411	2.9444
a	+1	−1	−1	−1	−1	+1	+1	+1	4.4390	4.5522	4.0903	4.1643	6.5617	6.5637
b	−1	+1	−1	−1	+1	−1	+1	+1	5.3792	6.3886	4.5159	4.5629	3.4161	3.4523
ab	+1	+1	−1	+1	−1	−1	−1	+1	4.7994	5.6632	4.1706	4.1660	4.7835	4.7898
c	−1	−1	+1	+1	−1	−1	+1	+1	6.7538	6.8092	6.9556	7.8951	3.3730	3.7636
ac	+1	−1	+1	−1	+1	−1	−1	+1	4.8103	4.4220	2.2741	2.1774	4.1606	4.1151
bc	−1	+1	+1	−1	−1	+1	−1	+1	5.2704	5.2329	2.0028	2.0455	3.3300	3.3341
abc	+1	+1	+1	+1	+1	+1	+1	+1	1.1798	1.2398	4.3623	4.3714	5.0681	5.1109

factor interaction. X_A, X_B and X_C represent the three factors as reported in Table 1 and ε is the residual error of the model. The model therein presented was applied to the HPC, DPPC and DSPC vesicles and evaluated in term of statistical significance by using ANOVA. The complete setup of the 2^3 factorial designs is reported in Table 2.

Three central points were added to the design to evaluate possible response curvature. In this regard, a lack of fit test was performed together with the ANOVA to quantify possible response deviation from linearity and diagnostics was performed to highlight the possible presence of multicollinearity and outliers (data not shown). Eventually, contour plots were obtained at the minimum (16%) and maximum (32%) level of the X_A variable to determine the influence of the preparation parameters on peptide encapsulation and to define the proper conditions to increase peptide content in liposomes. The statistical and factorial analyses were performed by using Design-Expert® v. 6.0.11 (Stat-Ease, Inc., Minneapolis, MN, USA).

2.6. Check point analysis

Check point analyses were carried out to establish model reliability in describing liposome system behavior. Central points were chosen according to the contour plots obtained and all experiments were performed in triplicate. Bias was estimated to evaluate agreement between actual values and predicted values.

Further experiments were carried out in the region around the maximum response achieved for each liposome system. Predicted and actual values were compared to determine the correlation extent between the experimental peptide content increase and the behavior described by the model.

3. Results

3.1. Characterization of CS loaded liposomes

CS content in LUVs was evaluated on the original suspension and after centrifugation on the pellets and supernatants as well. Drug content ranged from 1 to 8% (w/w) per unit weight of liposomes. The obtained data were employed to generate the factorial design to assess the effects due to preparation variables (Table 2).

Morphology and size analysis revealed a round shape of loaded LUVs (Fig. 2) having mean diameters around 1 μm. DLS studies were performed to highlight possible size and morphological modifications due to the freeze and thawing cycles employed for peptide loading. Blank and loaded LUV preparations were compared by reporting freeze–thawing times and number of cycles variations as well. In general, whether HPC, DPPC or DSPC phospholipids were employed, sizes were moderately increased with mean diameters above between 1 and 1.8 μm (Fig. 3, Panel A). These findings were correlated with a loading process effect on size, perhaps owing to formation of oligolamellar vesicles. Conversely, the effect on size due to number of cycles and time produced slight differences in the distribution profiles (Fig. 3, Panel B). Surprisingly, PD was not worsened by the freeze–thawing process. In fact, it ranged between 1.47 and 2.00 after freeze–thawing, while varied constantly around

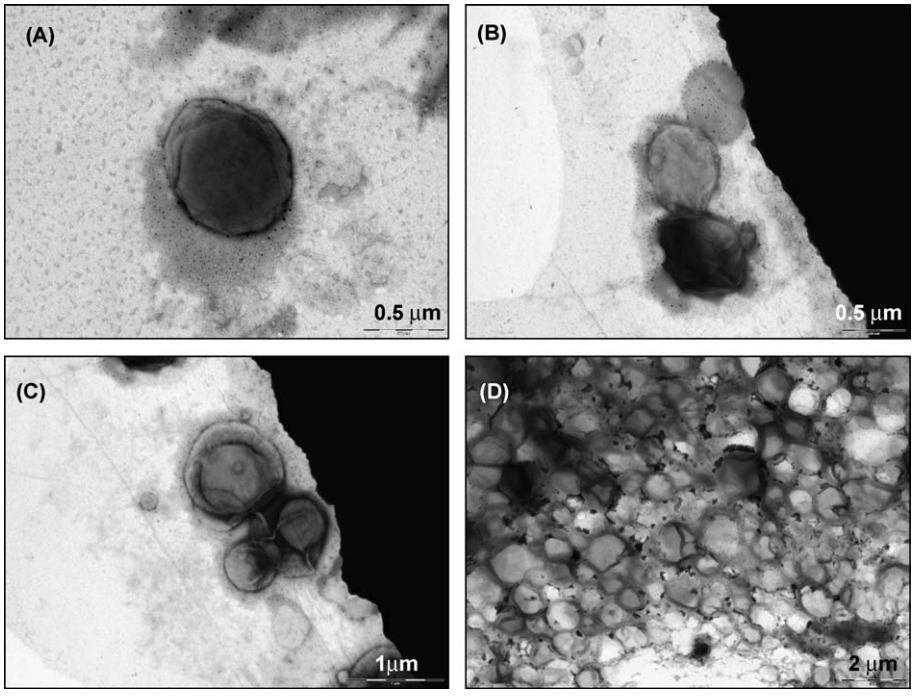

Fig. 2. TEM pictures of CS loaded LUVs: (Panel A) HPC liposomes, (Panel B) DPPC liposomes and (Panel C) DSPC liposomes. Outlook of liposome population distribution is reported (Panel D). Magnification was ×32,000 in Panels A–C and ×24,000 in Panel D. Dimensional bars are reported as well.

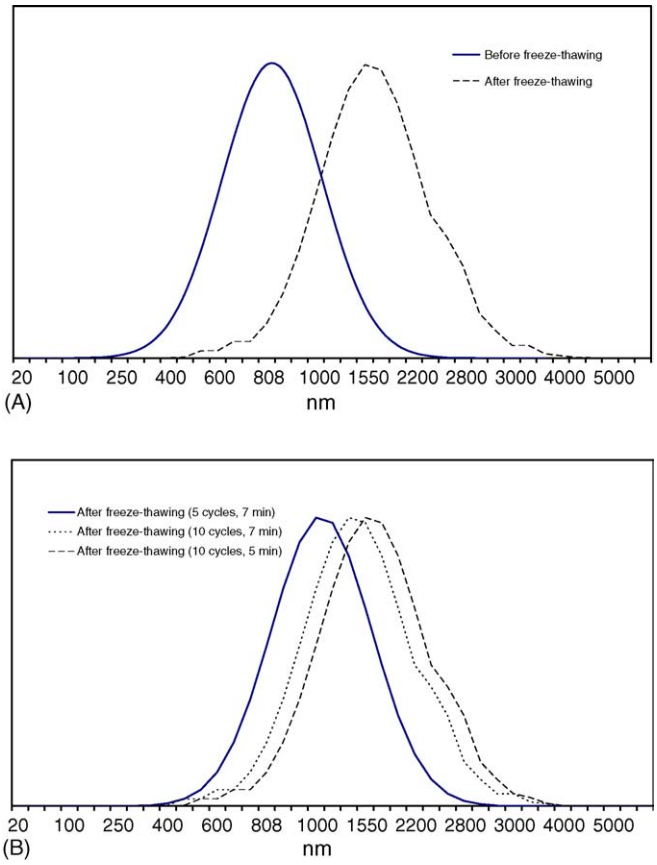

Fig. 3. DLS analysis of blank and loaded liposomes. The effect of the loading process was investigated comparing size distributions before and after the freeze–thawing cycles (Panel A). The effects of the variables number of cycles and time of each cycle were evaluated as well (Panel B). Comparable results were found for all the three liposome systems.

1.80 for the blank LUVs. These data were reproducible for all liposome systems.

3.2. Variable effects and model adequacy

Evaluation of main effects and interactions by the experimental design study showed that all the liposome systems were mainly affected by X_A (% loading) variations. In fact, the estimated contribution of each term of the model showed a major influence of the X_A variable with respect to X_B (time of each cycle) and X_C (number of cycles) as reported in Table 3a–c. Moreover, interaction factors played an important role for HPC vesicles and, partially, for DPPC vesicles, as it can be inferred from the large contribution values calculated for the X_{AB}, X_{AC}, and X_{BC} coefficients for HPC (Table 3a) and X_{AC} and X_{ABC} for DPPC (Table 3b). On the contrary, DSPC liposomes showed relatively lower contributions of the interaction terms but an extremely large X_A effect (68%) (Table 3c).

Eqs. (3)–(5) represent the linear regression models for the HPC, DPPC, and DSPC LUVs, respectively, as obtained from the factorial study.

$$Y = 4.45 - 0.57X_A - 0.066X_B + 0.011X_C - 0.61X_AX_B - 0.99X_AX_C - 1.17X_BX_C + 0.14X_AX_BX_C \quad (3)$$

Table 3
Effects and contribution of the model coefficients estimated from the factorial study

Term	Effect	% Contribution
(a) HPC		
X_A	−1.13	10.2
X_B	−0.139	0.154
X_C	0.0224	0.00402
X_{AB}	−1.22	11.8
X_{AC}	−1.97	31.1
X_{BC}	−2.35	44.1
X_{ABC}	0.278	0.619
(b) DPPC		
X_A	−1.05	9.72
X_B	−0.956	7.93
X_C	−0.211	0.390
X_{AB}	2.04	36.4
X_{AC}	−0.624	3.41
X_{BC}	−0.935	7.65
X_{ABC}	1.98	34.4
(c) DSPC		
X_A	1.87	68.0
X_B	−0.0938	0.170
X_C	−0.449	3.90
X_{AB}	−0.319	1.97
X_{AC}	−0.612	7.25
X_{BC}	0.550	5.84
X_{ABC}	0.814	12.8

$$Y = 4.25 - 0.53X_A - 0.48X_B - 0.11X_C + 1.02X_AX_B - 0.31X_AX_C - 0.47X_BX_C + 0.99X_AX_BX_C \quad (4)$$

$$Y = 4.21 + 0.94X_A - 0.047X_B - 0.23X_C - 0.16X_AX_B - 0.31X_AX_C + 0.28X_BX_C + 0.41X_AX_BX_C \quad (5)$$

ANOVA and statistical validation of the mathematical models reported a high significance ($P < 0.0001$) as it was pointed out by large F values (Table 4a–c). Moreover, all the terms of the DPPC and DSPC models were extremely significant ($P < 0.0001$) (Table 4b and c). On the contrary, the factors X_B and X_C ($P = 0.402$ and 0.891) for the HPC model were not significant, but the correspondent interaction terms were highly influential ($P < 0.0001$) (Table 4a). Moreover, also the X_{ABC} contribution to the response ($P = 0.110$) was almost neglectable as it can be seen from the small X_{ABC} coefficient reported in Eq. (3) and the low contribution depicted in Table 3a.

Although the presence of not significant terms, the HPC, DPPC and DSPC models showed good characteristics in terms of prediction R^2 values, which were 0.9281, 0.9968 and 0.9994, respectively. Moreover, diagnostics did not point out any particular problem related to the presence of outliers or multicollinearity and lack of normality (data not shown).

In addition, the investigation of response curvature (Table 4a–c) showed that all the models presented a not significant lack of fit, which correlated well with a neglectable non-linear response. Upon this observation, the first order model chosen was considered suitable for this study.

Table 4
Summary of ANOVA analysis of the models derived from the factorial study

	Sum of squares	d.f.	Mean square	F	P
(a) HPC					
Model	49.1	7	7.01	69.4	<0.0001
β_1 (A)	5.11	1	5.11	50.6	<0.0001
β_2 (B)	0.0773	1	0.0773	0.766	0.402
β_3 (C)	0.00201	1	0.00201	0.0199	0.891
β_4 (AB)	5.92	1	5.92	58.6	<0.0001
β_5 (AC)	15.6	1	15.6	154	<0.0001
β_6 (BC)	22.1	1	22.1	219	<0.0001
β_7 (ABC)	0.310	1	0.310	3.07	0.110
Residual	1.01	10	0.101		
Lack of fit	0.00690	1	0.00690	0.0619	0.809
Pure error	1.00	9	0.111		
(b) DPPC					
Model	45.6	7	6.52	1020	<0.0001
β_1 (A)	4.44	1	4.44	692	<0.0001
β_2 (B)	3.62	1	3.62	564	<0.0001
β_3 (C)	0.178	1	0.178	27.8	0.000363
β_4 (AB)	16.6	1	16.6	2590	<0.0001
β_5 (AC)	1.56	1	1.56	243	<0.0001
β_6 (BC)	3.50	1	3.50	544	<0.0001
β_7 (ABC)	15.7	1	15.7	2440	<0.0001
Residual	0.0642	10	0.00642		
Lack of fit	0.00260	1	0.00260	0.380	0.553
Pure error	0.0616	9	0.00685		
(c) DSPC					
Model	20.7	7	2.95	7920	<0.0001
β_1 (A)	14.1	1	14.1	3770	<0.0001
β_2 (B)	0.0352	1	0.0352	94.5	<0.0001
β_3 (C)	0.807	1	0.807	2170	<0.0001
β_4 (AB)	0.408	1	0.408	1090	<0.0001
β_5 (AC)	1.50	1	1.50	4020	<0.0001
β_6 (BC)	1.21	1	1.21	3240	<0.0001
β_7 (ABC)	2.65	1	2.65	7120	<0.0001
Residual	0.00373	10	0.000373		
Lack of fit	0.000620	1	0.000620	1.80	0.213
Pure error	0.00311	9	0.000345		

3.3. Contour plots interpretation

Contour plots were obtained by fixing the X_A factor at its high and low level and varying number of cycles and time of each cycle over the range used in the factorial study (Figs. 4–6). The profiles showed that, as far as HPC and DPPC liposomes were concerned, an increase in peptide content can be achieved by reducing the number of cycles and increasing time at the lowest X_A level (Figs. 4a and 5a), whereas, at the highest X_A level, time should be decreased and the number of cycles augmented (Figs. 4b and 5b). On the contrary, a different behavior was observed for the DSPC vesicles. In fact, peptide encapsulation in DSPC LUVs was improved by decreasing time and number of cycles at the highest X_A level and by increasing number of cycles and decreasing times at the lowest X_A level (Fig. 6a and b). Furthermore, the observed X_A effect improved encapsulation when it was at its low level for both HPC or DPPC liposomes. On the other hand, in DSPC liposomes encapsulation was strongly augmented at high X_A levels. The highest peptide content was recorded (∼8%) for DPPC LUVs at the lowest X_A level.

3.4. Check point analyses

Check points were evaluated in order to confirm predictivity of the regression models. Checking was performed at high and low X_A levels as resulting from the contour plots drawn for the three liposome systems. A good agreement between actual and predicted responses was observed (Table 5). In fact, the estimated biases were always below 10–12% for all systems.

In addition, points in the region of the highest increase of peptide content were also compared to evaluate the agreement between predicted and experimental conditions in term of encapsulation improvement in the liposome systems. The results showed matching values of % peptide content with reasonably low biases (Table 6).

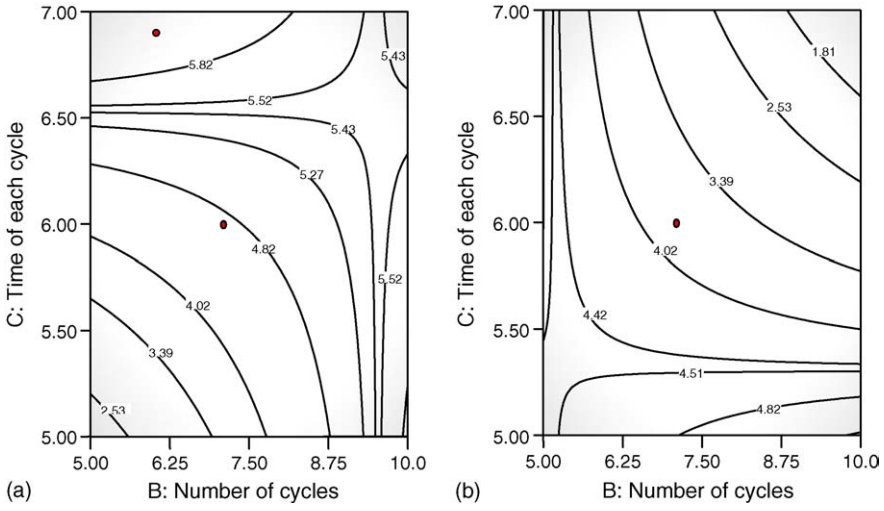

Fig. 4. Contour plots of CS loaded HPC at (a) high (+1) and (b) low (−1) level of factor X_A (% loading). The check points used for model evaluation are reported as solid circles in the central region (Panels a and b) and in the region of maximum response increase (Panel a).

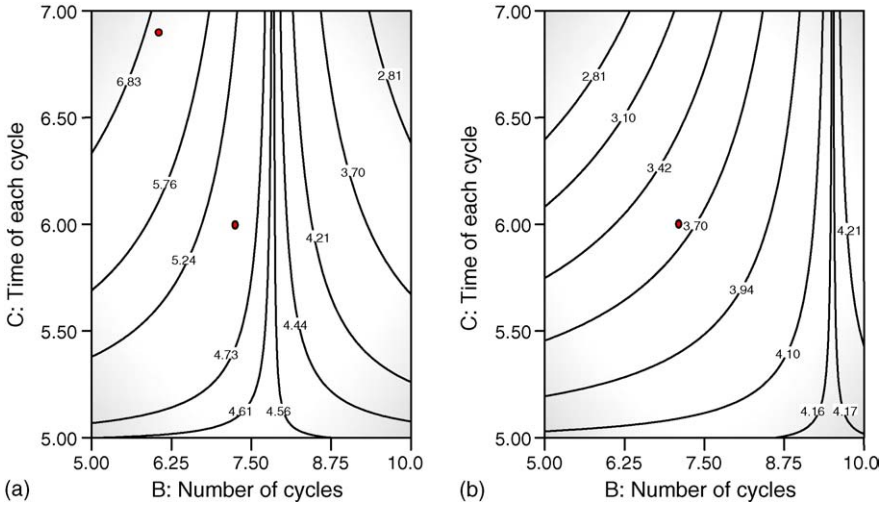

Fig. 5. Contour plots of CS loaded DPPC at (a) high (+1) and (b) low (−1) level of factor X_A (% loading). The check points used for model evaluation are reported as solid circles in the central region (Panels a and b) and in the region of maximum response increase (Panel a).

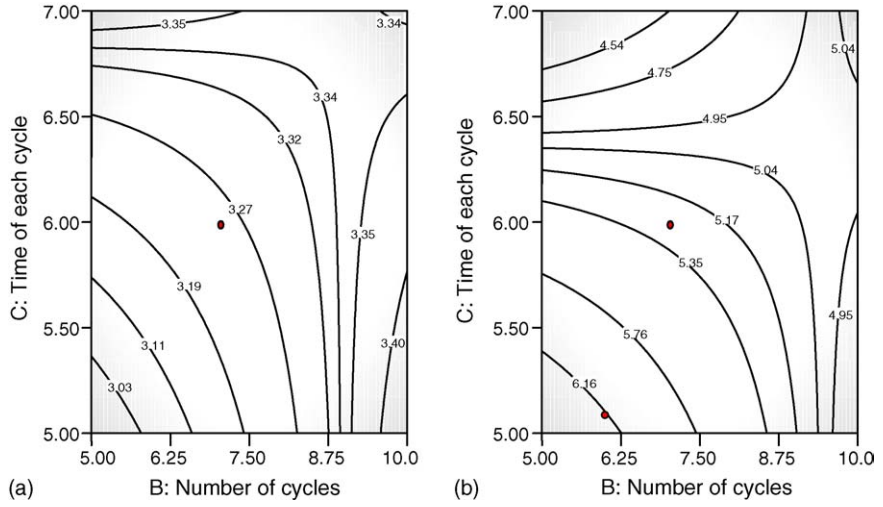

Fig. 6. Contour plots of CS loaded DSPC at (a) high (+1) and (b) low (−1) level of factor X_A (% loading). The check points used for model evaluation are reported as solid circles in the central region (Panels a and b) and in the region of maximum response increase (Panel b).

Table 5
Check point analysis for model adequacy evaluation

Factors			HPC			DPPC			DSPC		
X_A (% loading)	X_B (no. of cycles)	X_C (time of each cycle)	Actual (±S.D.)[a]	Predicted	Bias (%)	Actual (±S.D.)[a]	Predicted	Bias (%)	Actual (±S.D.)[a]	Predicted	Bias (%)
−1 (16)	0[b] (7)	0 (6)	4.82 ± 0.23	4.90	1.66	5.02 ± 0.61	5.08	1.20	3.01 ± 0.22	3.25	7.97
+1 (32)	0[b] (7)	0 (6)	3.62 ± 0.42	4.02	11.02	3.94 ± 0.16	3.62	8.12	5.23 ± 0.34	5.19	0.76

[a] $N = 3$.
[b] Approximate center value.

Table 6
Comparison of predicted and actual values recorded in the region of the highest response increase from contour plots

	% Loading	No of cycles	Time of each cycle (min)	% Actual drug content (±S.D.)[a]	% Predicted drug content	Bias (%)
HPC	16 (−1)	6 (−0.6)	6.9 (+0.9)	6.82 ± 0.97	6.29	7.77
DPPC	16 (−1)	6 (−0.6)	6.9 (+0.9)	6.96 ± 0.94	6.65	4.45
DSPC	32 (+1)	6 (−0.6)	5.1 (−0.9)	6.47 ± 0.19	6.29	2.78

[a] $N = 3$.

4. Discussion

The freeze–thawing process allowed fabrication of loaded liposomes having very homogeneous morphological characteristics (Fig. 2). This method also greatly increased the amount of peptide encapsulated (efficiency > 50%) if compared to other conventional preparation methods, such as TLE, which, on the contrary, was unable to furnish an adequate CS entrapment efficiency (<10%). Furthermore, the possibility of managing vesicle size is one of the advantages of coupling TLE to freeze–thawing so as to obtain respirable LUVs. In fact, the TLE method and extrusion were employed prior to peptide loading in order to prepare blank LUVs having a proper size (around 1–1.8 μm). Vesicle sizes were controlled by extruding LUVs through a 1 μm average pore size filters.

Size distribution analyses showed proper size of the obtained CS loaded liposomes, however, a certain effect of the loading process was highlighted (Fig. 3). In fact, DLS measurements revealed a slight size increase after CS encapsulation due to the number of freeze–thawing cycles. This effect may be correlated with the formation of a population of larger oligolamellar vesicles in place of unilamellar vesicles that, consequently, increased mean diameters. Nevertheless, this underlined effect was not large enough to affect respirability of such formulations as size after peptide loading was still within the limit established by the aerodynamic particle features needed for inhalation. Moreover, as result of their aqueous core, liposomes usually possess low densities, which may favor the reduction of the aerodynamic diameter value. The general expression of the aerodynamic diameter is represented by Eq. (6), in which are reported the main factors affecting particle respirability:

$$d_{ae} \cong d_g \sqrt{\frac{\rho_p}{\rho_0} \frac{1}{\chi}} \tag{6}$$

where d_{ae} is the aerodynamic diameter, d_g the geometric diameter, ρ_p the particle density, ρ_0 the standard density and χ is the shape factor.

Furthermore, an effect of size on vesicle phagocytosis in the alveoli has been widely reported (Suarez and Hickey, 2000; Agu et al., 2001; Aliasgar and Ambikanandan, 2004; Zhou et al., 2005). In particular, particles resulting <1 μm have been reported to persist longer in the lungs as they are preferentially translocated to the connective tissues and cleared by epithelial cells, whereas particles having sizes >1 μm are mainly phagocytosed by alveolar macrophages (Geiser, 2002). In this regard, being the mean diameters of the obtained freeze–thawed liposomes >1 μm, they can be well considered to be potentially prone to phagocytosis by alveolar macrophages when aerosolized to the lungs (Geiser, 2002). Moreover, the size distributions of the freeze–thawed vesicles did not show any PD increase, although the formation of oligolamellar vesicles is plausible during CS loading. Thus, on these basis, the formulations herein described can be considered potentially suitable for CS delivery to the lungs. Therefore, since size and density were not main issues during preparation of CS liposomal formulations, a critical parameter was represented by the amount of encapsulated peptide which is the factor conditioning the possible in vivo applicability of CS loaded LUVs. In fact, the achievement of local therapeutic levels depends directly on the administered dose, and, thus, on the drug content of LUVs formulations. For this reason, drug content was evaluated by an experimental design study. This study allowed the determination of preparation variable effects on CS encapsulation with a relatively low number of experiments as well. This is one of the advantages of such method, which, for this reason, has large application in pharmaceutical system development.

Although all the factors showed quite large effects on the response, the evaluation of the equation terms evidenced the presence of not significant coefficients ($P > 0.05$). In particular, the X_B, X_C and X_{ABC} terms in the HPC regression model were found to be neglectable (Table 4a). In spite of the X_B, X_C and X_{ABC} low contribution, these terms were not removed from Eq. (3). In fact, the X_B and X_C correspondent interaction terms were highly significant. It meant that, for HPC, X_B and X_C did not affect directly the % peptide content but had a great effect on the levels of the other factor X_A. Therefore, Eq. (3) cannot be reduced as underestimation of effects may be generated as a consequence of a not hierarchical model. In general, since important information may be lost, it is always preferable to do not remove terms from the full model unless a remarkable improvement in model performances is achieved. In this regard, the HPC model did not show relevant modifications when the other not significant term X_{ABC} was taken out. In fact, prediction R^2 remained almost unchanged, from 0.9281 of the full model to 0.9237 of the reduced model. This implied that no reduction was needed for the HPC model.

Check point analyses were extremely helpful in allowing the assessment of model predictivity and the investigation of response curvature. The correspondence of predicted and experimental values claimed the proper choice of the models employed for data regression analyses. The models obtained were then used to outline contour plots. This approach was particularly useful in order to establish which is the direction the system should move towards to obtain a % drug content increase. It was then possible to determine the conditions, as factor levels, at which CS content reached its highest value, and, furthermore, which direction in space towards CS content increased faster. In this regard, the contour plots were built by fixing the X_A level at its maximum and minimum. X_A was kept constant as result of its major influence on the response. In fact, % loading showed the highest effect and contribution on % CS content either for HPC and DPPC or DSPC liposomes (Tables 3 and 4).

The peculiar differences observed between DSPC vesicles and the other liposome systems resulted important, as reported in the results section. In fact, the different behavior highlighted by DSPC may be ascribed to different attitudes of the phospholipids employed. Bilayer flexibility, capacity of reorganization during the freeze and thawing cycles and affinity with the peptide, possibly contribute to the differences observed for the three formulations. In this regard, HPC, DPPC and DSPC have basically different structures, and thus different properties. In fact, although all three phospholipids possess the same polar head-

groups, they have hydrophobic palmitic (DPPC), stearic (DSPC) and a mixture of palmitic and stearic acyl chains (HPC) that provide extremely different order and rigidity to the bilayer, as it is evident if looking at the different transition temperatures ($T_m \sim 41, 51, 55\,^{\circ}C$ for DPPC, HPC, DSPC, respectively). These features may alter their response to perturbation and to CS loading as it may happen during the freeze and thawing cycles.

Differences between DSPC and HPC and DPPC vesicles were recorded in an our previous work (Giovagnoli et al., 2003) as well. In fact, DSPC vesicles showed interdigitation as result of CS loading increase. This effect, producing a stabilization of the bilayer, may explain the higher sensitivity of the DSPC system to the % loading increase. Moreover, the higher DSPC stability may also cause the lower susceptibility observed with respect to the other factors that, in turn, had a much greater influence on the HPC and DPPC response.

From the interpretation of the contour plots, it was possible to spot the direction in space towards the response increased faster, in the range employed for the design study. In order to confirm model predictivity and the results from the contour plots, correspondence between experimental and predicted responses, recorded around the region of maximum encapsulation increase, was investigated. The experimentally collected triplicate drug content values matched closely with low bias. The values predicted by the model (Table 6) demonstrated that a true response improvement can be achieved according to the conditions suggested by the factorial design study.

Of course there are some issues as far as theoretical and practical aspects of liposome formulation development are concerned. In fact, although, as it was reported for DPPC, drug content can be increased theoretically by reducing number of cycles and loading and augmenting time, in practice it is not possible to reduce number of cycles beyond a certain value as well as it is not recommended to increase time indiscriminately. The same considerations can be made for the HPC and DSPC systems. Furthermore, the center points adopted as check points, although located in the central design region, cannot be considered actual center points, because it was not possible to operate at 7.5 cycles, the X_B true center value, during the freeze–thawing process. In fact, for technical reasons, a half cycle cannot be performed, thus, 7 was considered as the approximate X_B center values. Upon these considerations, it is clear that care has to be taken in designing and interpreting a factorial study.

In conclusion, the results obtained furnished a very useful and helpful input in developing a new CS liposomal formulation. In this regard, the improvement of peptide encapsulation was important to achieve a formulation having a suitable amount of entrapped drug, and dimensional and morphological characteristics appropriate for producing respirable formulations. The innovation of such systems resides in the capacity of improving safety and efficiency of this well known interesting peptide that, in such a way, may find wider and useful applications in tuberculosis treatment, especially when conventional drugs are impaired by resistance and cross resistance occurrences.

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