Encapsulation of 5-fluorouracil in liposomes for topical administration

Carla Andréia Miranda da Costa and Ângela Maria Moraes*

*Departamento de Processos Biotecnológicos/Faculdade de Engenharia Química. Universidade Estadual de Campinas - Cidade Universitária "Zeferino Vaz" Caixa Postal 6066, 13083-852, Campinas, São Paulo, Brasil. *Author for correspondence. e-mail: ammoraes@feq.unicamp.br*

ABSTRACT. This work aims to compare different methodologies for the preparation of dipalmitoylphosphatidylcholine/cholesterol (DPPC/Chol) liposomes entrapping the anticancer agent 5-fluorouracil (5-FU), a drug usually employed in melanoma therapy, designed for topical administration. The lipid vesicles were produced by dry lipid film hydration followed by extrusion, by ethanol injection and by reverse phase evaporation, while 5-FU was passively or actively incorporated into the liposomes. The results demonstrated that vesicle loading and stability can be controlled by the experimental procedure used to entrap the drug. Different initial drug to lipid molar ratios strongly affect encapsulation efficiency. The reverse-phase evaporation method resulted in the largest 5-FU encapsulation efficiency, around 6%. The preparation of these vesicles did not result in detectable drug degradation. During 4 weeks, no aggregation of liposomes was observed, however, extensive drug leakage from the vesicles was noticed.

Key words: liposomes, lipid vesicles, 5-fluorouracil, encapsulation, topical administration, controlled drug release.

RESUMO: Encapsulamento do 5-fluorouracil em lipossomas para administração tópica. Este trabalho tem o objetivo de comparar diferentes metodologias de preparação de lipossomas de dipalmitoilfosfatidilcolina/colesterol (DPPC/Col) encapsulando o agente antineoplásico 5-fluorouracil (5-FU), uma droga normalmente utilizada na terapia de melaomas, projetados para administração tópica. As vesículas lipídicas foram produzidas através da hidratação do filme lipídico seco seguida de extrusão, por injeção de etanol e por evaporação da fase reversa, e a 5-FU foi incorporada nos lipossomas ativa ou passivamente. Os resultados mostraram que a incorporação da 5-FU e a estabilidade das veiculas podem ser controladas pelo procedimento empregado para encapsular o fármaco. Diferentes razões molares iniciais fármaco/lipídico afetam fortemente a eficiência de incorporação. O método de evaporação em fase reversa resultou na maior eficiência de incorporação, de aproximadamente 6%. A preparação destas veiculas não resultou em degradação detectável do fármaco. Durante 4 semanas, não se observou agregação dos lipossomas, entretanto, apreciável liberação do fármaco das vesículas foi notada.

Palavras-chave: lipossomas, vesículas lipídicas, encapsulamento, administração tópica, liberação controlada da droga.

Introduction

Liposomes are vesicular structures consisting of hydrated lipid bilayers which can incorporate hydrophilic, hydrophobic and amphiphilic compounds. A schematic presentation of an ideal closed phospholipid bilayer (unilamellar liposome) is shown in Figure 1. Many studies have demonstrated the potential benefits of liposomal encapsulated agents for dermatological application, including increase in drug penetration in the skin, reduction in drug-related toxic side effects and enhanced drug cytotoxicity (Korting *et al*., 1992). Liposomes are biodegradable, biocompatible and can act both as a formulation ingredient (encapsulating a compound) and as an active component itself, supplying the lipid needed by skin and enhancing its moisture. In addition, liposomes can be incorporated in different vehicles, such as gels, lotions, creams, aerosols, and powders (Lasic, 1993).

Pharmacological efficacy for various liposomal encapsulated anti-cancer agents has already been

proven (Lasic, 1998). Skin-protecting and light-protecting agents, corticosteroids, antifungals, anesthetics, and retinoids are among the substances of dermatological interest frequently incorporated in liposomes (Korting *et al*., 1992). The method of liposome manufacture and lipid layer composition can directly affect drug encapsulation, release, permeation, absorption, distribution, metabolism, and elimination, as well as its toxicity profile (Lasic, 1993; Sharata and Katz, 1996). For dermal administration, fosfatidylcholines are normally employed for liposome preparation and dry lipid film hydration is one of the most used approaches for vesicle production, in spite of its difficulties to process scale up.

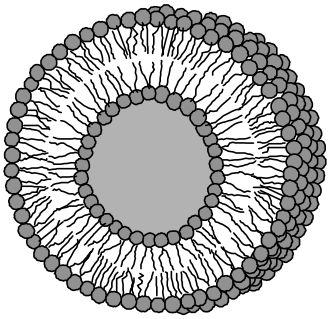


Figure 1. Structure of a typical unilamellar liposome.

Efficient liposomal encapsulation of therapeutic agents can be achieved using passive and active liposomal loading procedures, depending on the characteristics of the drug (Barenholz and Crommelin, 1994). In passive loading, the therapeutic compound can be added during liposome formation, while in active loading, the drug can be added to preformed liposomes presenting an established pH gradient.

The passive loading of hydrophilic compounds during hydration of dry lipid films usually results in low trapping efficiencies and its mechanism is based on the capture of the aqueous solution in the liposome core. Therefore, efficient passive hydrophilic drug incorporation is related to drug solubility in aqueous solutions, to its ability to cross the lipid bilayer, as well as to the molecular weight of the therapeutic compound. Amphipatic and hydrophobic drugs, on the other hand, can be associated with the lipid bilayer components and therefore their passive incorporation can be performed during the lipid bilayer formation. However, reducing the vesicle size hinders drug penetration in the lipid bilayer and causes a drop in loading efficiency. The solubility of very hydrophobic molecules in the bilayer can also be limited because of geometrical constraints.

The mechanism of drug uptake in active loading is based on the fact that unprotonated (neutral) lipophilic drugs can cross the liposome membrane and accumulate inside vesicles if there is an internal excess of protons. The accumulated drug, positively charged, will not permeate the liposome membrane but will remain encapsulated within the liposome. The redistribution of drugs with these characteristics across liposomal membranes in response to a pH gradient can result in trapping efficiencies approaching 100% (Mayer *et al*., 1990; Madden *et al*., 1990).

The purpose of this study was to design liposomes suitable for topical delivery of high concentrations of the chemotherapeutic agent 5-fluorouracil (5-FU), used to treat, among other diseases, skin problems such as melanoma or conditions that could become cancerous if not treated. In Figure 2, the molecular structure of this synthetic pyrimidine is shown. For skin diseases, this drug, formulated in cream or in solution, is employed as a therapeutic complement or for primary treatment. Liposomal encapsulation of 5-FU could accelerate tumor regression and reduce adverse effects such as bone-marrow suppression, gastro-intestinal problems, burning feeling (where the medicine is applied), increased sensitivity of skin to sunlight, itching, skin rash, soreness and tenderness. In addition, increased drug stability could be obtained by 5-FU encapsulation in liposomes, since this drug is photosensitive (Jarugula and Boudinot, 1996; Krogsgaard-Larsen *et al*., 1996).

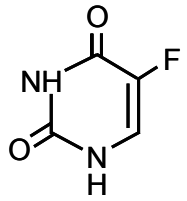


Figure 2. Chemical structure of 5-fluorouracil.

Liposomal encapsulation of 5-FU is described in literature by several research groups (Simons *et al*., 1988; Joondeph *et al*., 1988; Elorza *et al*., 1993; Fresta *et al*., 1993; Dudnitchenko and Krasnopolsky, 1996; Valero *et al*., 1999; El Maghraby *et al*., 2001). The first two groups evaluated the applicability of liposomal 5-FU to improve the treatment against ocular diseases such as glaucoma and vitreoretinopathy, while Valero *et al*. (1999) investigated the efficacy and safety profile of a chemotherapy regimen against metastatic breast

cancer, consisting of liposome encapsulated cyclophosphamide, doxorubicin and 5-FU. Promising results were obtained in these studies, as well as in the development of an industrial technology to produce liposomal forms of 5-FU and other cytostatic agents based on high pressure homogenization (Dudnitchenko and Krasnopolsky, 1996).

The remaining mentioned groups discuss in detail 5-FU incorporation in liposomes. Two different methods for vesicle manufacture were evaluated by Elorza *et al*. (1993), both relatively difficult to scale up, freeze-thawing extrusion and reverse-phase evaporation, employing bovine brain sphingomyelin (SM) or synthetic distearoylphosphatidylcholine (DSPC) in liposome composition. Passive encapsulation efficiencies from 3 to 6% for vesicles with mean diameters varying from 106 to 295nm were achieved. Liposomes consisting of DSPC retained 70% of the encapsulated 5-FU after one hour at 37°C, whereas SM vesicles retained only 15% over the same period. No data are provided concerning to storage stability.

Fresta *et al*. (1993) investigated four different 5-FU-loaded liposome preparation procedures as well as various phospholipids, observing that the lipid mixtures containing dipalmitoylphosphatidylserine seemed to be the most adequate in encapsulation efficiency, fusogenic properties, serum and storage stability. The addition of cholesterol in liposome composition increased vesicle stability.

More recently, El Maghraby *et al*. (2001) investigated the potential use of ultradeformable liposomes as skin drug delivery systems employing 5-FU as a drug model entrapped by passive loading of a satured aqueous solution. The ultradeformable vesicles comprised phosphatidylcholine (PC) mixed with sodium cholate and contained 7% (v/v) ethanol in the final formulation, incorporating 5-FU with an entrapment efficiency of 8.8%. The ultradeformable formulation delivered around 3 to 6 times more 5-FU to the skin than four traditional liposomal preparations entrapping 5-FU with efficiencies around 9%. Pure PC increased drug penetration by 2.5 fold when compared to the control, whereas rigid formulations (PC vesicles containing the membrane stabilizer cholesterol or DPPC bilayers presenting or not cholesterol) showed a tendency of doubling the total amount of permeated drug. In spite of the superior behavior of the ultradeformable liposomes concerning to skin drug delivery, more than 80% of the entrapped drug was released in less than 10 minutes at 32°C in distilled water.

Most of the work previously developed on 5-FU encapsulation in liposomes involved passive drug loading procedures, in spite of the low solubility of 5-FU in aqueous solution. This compound should be a good option for encapsulation into liposomes by active loading, since it presents low molecular weight (130.1 Da) and a pKa of 8.04 (Krogsgaard-Larsen *et al*., 1996).

This report details the preparation and characterization of liposomes entrapping 5-FU, by using different approaches to produce the vesicles and entrap the drug, aiming at the treatment of skin melanoma, one of the most rapidly increasing malignancies in man. Dipalmitoylphosphatidylcholine (DPPC) and cholesterol (Chol) at a ratio of 60:40 mol% were employed as components of the lipid vesicles. This composition was not tested by any of the previously mentioned authors and was selected aiming a compromise between adequate vesicle storage stability in aqueous solution and drug permeation into the skin. Passive and pH gradient-mediated drug loading procedures were evaluated.

Material and methods

Material

Dipalmitoylphosphatidilcholine (DPPC), cholesterol (Chol), and 5-Fluorouracil (5-FU), were obtained from Sigma Chemical Co. (St. Louis, MO). All reagents used were of at least analytical grade.

Methods

Vesicle Preparation: DPPC/Chol (60:40 mol%) liposomes were prepared by three different methods: by dry lipid film hydration followed by extrusion (MacDonald *et al*., 1991), by ethanol injection (Batzri and Korn, 1973) and by reverse phase evaporation (New, 1990).

In the first procedure, the lipids were initially dissolved with chloroform/methanol (9:1 v/v) in a 50mL round-bottom flask and dried in a rotary evaporator. Passive loading of 5-FU was performed during lipid bilayer hydration for 15 minutes and at 55°C, with 5-FU at concentrations from 10 to 50mM solubilized in a buffer consisting of 10mM TRIS-HCl and 140mM NaCl at pH 7.4, while total lipid concentration was maintained at 7.5mM. Unilamellar liposomes were obtained extruding the suspension 15 times through two stacked 100nm pore size polycarbonate membranes **(**Osmonics Inc.) using a T 001 stainless steel extruder (Lipex Biomembranes Inc.), at 65°C.

Alternatively, 5-FU was actively loaded into extruded liposomes presenting a transmembrane pH gradient as follows (Madden *et al*., 1990; Mayer *et al*., 1990). The lipid film, after dried, was hydrated for 15 minutes with a 300mM citric acid aqueous solution at pH 4 and 65°C. The same extrusion conditions previously described were employed for these vesicles, which presented final lipid concentration equal to 7.5mM. The vesicles, prepared at pH 4.0, had their external media titrated to pH 7.5 with 2M Na_2CO_3. The liposomal samples, now presenting an internal pH equal to 4 and external pH of 7.5, were diluted to 2mM in lipids and mixed with appropriate amounts of 5-FU dissolved in a 10mM HEPES buffer at pH 7.4. The resulting suspensions were incubated at several temperatures for different periods, according to a 2^3 experimental design.

The ethanol injection method consisted of injecting both lipids (DPPC and Chol) solubilized in ethanol (total lipid concentration equal to 40mM) into a 50mM 5-FU solution in a TRIS-HCl buffer, using a 4μm (in diameter) stainless steel needle coupled to a 1mL glass syringe, while sonicating for 5 minutes at 25°C in a bench sonicator. A drug to lipid molar ratio of 1:25 was employed and the final lipid concentration was 2mM. A variation of this method was also tested, mixing the drug with the lipids in ethanol before the injection of the solution in TRIS-HCl buffer. In this situation, total lipid concentration in ethanol remained equal to 40mM, while drug concentration was 20mM (maximum 5-FU solubility in ethanol at 25°C). In the same conditions previously described, lipid concentration in sample was 2mM, after the injection.

The reverse phase evaporation liposomes were prepared with the injection of a 5-FU solution (at 15 or 50mM in drug) in a TRIS-HCl buffer containing or not 140mM NaCl at pH 7.4 in DPPC/Chol solubilized with diethylic ether/chloroform (1:1 v/v) at 2 or 3mM (total lipid). Aqueous to organic phase volume ratios varied from 1:3 to 1:10, while initial drug to lipid molar ratios varied from 0.75 to 8.30. The mixture was sonicated in a bench sonicator for 2 minutes and afterwards, the organic solvents were removed in a rotary evaporator at 37°. Due to the organic solvents evaporation, a gel was formed. The gel, after intense agitation in vortex, collapsed and turned into a viscous fluid consisting of suspended liposomes. The remaining organic solvents were removed in a rotary evaporator and when desired, the vesicles were extruded at 55°C as described above.

After the encapsulation of 5-FU, unentrapped drug was removed by gel permeation chromatography or by dialysis. For gel permeation chromatography, a 2.5x10cm^2 Sephadex G-50 column equilibrated with 10mM HEPES buffer at pH 7.4 containing NaCl 120mM (for the actively loaded extruded liposomes) or with 10mM TRIS-HCl buffer at pH 7.4 including 140mM NaCl (for ethanol injection preparations) was used. Dialysis against 200 volumes of 10mM TRIS-HCl buffer at pH 7.4 and 4°C using 10kDa molecular weight cut-off Inlab bags was performed only for reverse phase evaporation liposomes. All prepared samples were stored at 5°C.

Vesicle Characterization: The liposomes were assayed for vesicle size and for lipid and encapsulated drug concentration as described below.

The hydrodynamic radii of the vesicles before and after antibiotic entrapment were determined by quasi-elastic light scattering (QLS), based on the measurement of time-dependent fluctuations of scattered light intensity to determine the particle diffusion coefficient in diluted solutions. Measurements were performed at a 90° scattering angle, using an Autosizer 4700 equipment (Malvern Instruments).

Determination of the phospholipid concentration of liposomal samples using modifications of a total phosphate spectrophotometric assay technique (Chen *et al*., 1956) was performed using a Beckman DU 640 UV-visible spectrophotometer. The molar concentration of total lipid was calculated by dividing the phospholipid concentration determined using the phosphate assay by the mole fraction of phospholipid in the vesicle preparation (equal to 0.6), to account for the presence of cholesterol.

5-FU concentration was determined at 254nm, on a Beckman DU 640 UV-visible spectrophotometer, immediately after dissolving the liposomes with methanol (4:1 v/v organic solvent to sample) or one hour after dissolution with a microemulsion consisting of 0.81% iso-octane, 6.61% 1-butanol and 3.31% sodium dodecyl sulfate in 10mM borate buffer at pH 9.0. Drug determination was always performed within 12 hours after liposome preparation, to avoid 5-FU degradation.

Percentual encapsulation efficiencies were calculated as the drug to lipid molar ratio after removal of unentrapped compound divided by the initial molar ratio, before free compound removal. The 5-FU concentration in liposome aqueous core

was estimated using both the final compound and lipid molar concentrations in the sample as well as the average vesicle diameter. The cross sectional areas of single phosphatidylcholine and cholesterol molecules are 71 $Å^2$ and 19 $Å^2$, respectively (Israelachvilli and Mitchell, 1975), resulting in an average area per lipid molecule for vesicles composed of DPPC/Chol equal to 50.2 $Å^2$. The number of lipid molecules per liposome of a given size, as well as the concentration and the number of drug molecules encapsulated per liposome were calculated considering that approximately 60% of the lipid molecules are on the outside layer of a unilamellar vesicle.

Evaluation of Liposome Storage Stability: Vesicle stability concerning to storage at 5°C was evaluated monitoring changes in vesicle mean diameter and in drug retention during 4 weeks.

Results and discussion

Efficient drug loading and stable drug retention in liposomes are strongly related to lipid bilayer characteristics, such as charge and permeability, to the physico-chemical features of the drug itself, such as molecular weight, charge distribution, solubility and partition between hydrophilic and hydrophobic environments as well as to vesicle production and drug encapsulation methodology.

Several methods are available for liposome preparation, however, few of them are effectively adequate for scaling up. The purpose of this work was, therefore, to compare different procedures for 5-FU containing liposomes production, suggesting methodologies that could be industrially implanted with relatively low costs, such as the ethanol injection technique.

The specific lipid composition (DPPC/Chol 60:40 mol%) was selected to obtain lipid bilayers rigid enough to prevent extensive drug leakage short after its encapsulation, while still allowing suitable transdermal drug release. DPPC has a phase transition temperature of 41°C and the addition of cholesterol at ratios above 33 mol% should result in elimination of phase transition (New, 1990).

Incorporation of 5-FU in liposomes prepared by dry lipid film hydration

One of the evaluated encapsulation methods was the passive loading of 5-FU into liposomes performed during the hydration of the dry lipid film. This method is not easily scaled up; however, it is a classic procedure, usually employed as a reference when comparing different encapsulation methodologies. The use of drug solutions at several concentrations in TRIS-HCl buffer resulted in low drug encapsulation efficiencies, as observed in Table 1. The upper initial drug to lipid ratio (D/L) was limited by the solubility of 5-FU in the employed buffer at room temperature.

Table 1. Passive loading of 5-FU in DPPC/Chol liposomes at different initial drug concentrations. Initial lipid concentration: 7.5mM.

Initial 5-FU concentration (mM)	*Final lipid concentration (mM)*	*Final 5-FU concentration (mMl)*	*Mean vesicle diameter (nm)*	*Encapsulation Efficiency (%)*	*Estimated Internal 5-FU Concentration (mM)*
10	4.32	0.070	155.2	1.22	6.3
20	4.87	0.149	176.2	1.15	10.6
30	4.97	0.246	160.3	1.24	18.8
50	5.94	0.237	151.9	0.60	16.0

Increasing the initial drug concentration resulted in increases of drug incorporation, up to concentrations estimated around 19mM of 5-FU in the vesicle aqueous core, indicating a possible saturation phenomena. However, the performance of this method was not satisfactory, possibly due to mass transfer limitation during the dry lipid film hydration, mostly at the larger D/L evaluated, since the effectively captured drug in the vesicle aqueous core was consistently lower than 5-FU concentrations used to hydrate the lipid film. Vesicle mean diameters were not consistently affected by hydration with 5-FU solutions at the different tested concentrations.

The active drug encapsulation approach itself can be easily scaled up, since it basically involves the contact of preformed vesicles presenting a pH gradient with the drug at suitable operational conditions. In this way, liposomes could be virtually prepared by any technique and used to incorporate the drug. The active loading of 5-FU in extruded DPPC/Chol liposomes prepared by dry lipid film hydration was evaluated through factorial design, where the effect of the following independent variables were studied on drug encapsulation: drug to lipid initial molar ratio (from 0.4 to 2), time of contact of drug and liposomes (from 10 to 60 min) and incubation temperature (from 37 to 60°C).

The results illustrated on Table 2 indicate that the vesicles, with initial mean diameters of 144nm, showed some aggregation after contacting the drug, probably as a result of 5-FU superficial activity (Elorza *et al*., 1993).

Table 2. Experimental design for active loading of 5-FU in DPPC/Chol liposomes with final drug to lipid ratio and mean vesicle diameter as responses. Initial total lipid concentration: 2.0mM. Initial vesicle mean average diameter: 144nm.

Initial D/L	Temperature (°C)	Incubation time (min)	Final D/L	Mean vesicle diameter (nm)
0.4	37	10	0.0	184
0.4	37	60	0.0	129
0.4	60	10	0.0	132
0.4	60	60	0.0	132
2.0	37	10	0.0	130
2.0	37	60	0.0	133
2.0	60	10	0.0	130
2.0	60	60	0.0	495
1.2	48.5	35	0.0	224
1.2	48.5	35	0.0	220

Data statistic analysis by Statistica software (Table 3) shows that all factors affect the final vesicle mean diameter at 95% confidence level. However, the ANOVA variance analysis (Table 4) shows that no statistically significant mathematical correlation can be proposed since the calculated F value (13.38) is lower than the listed value for 95% of confidence, that is equal to 19.35. For practical purposes, a model has statistical significance if the calculated F value is at least 4 to 5 times larger than the listed value (Barros Neto *et al*., 1995).

According to the data, the combination of high temperatures (from 48.5 to 60°C), high drug to lipid ratios (from 1.2 to 2.0) and prolonged exposure of the liposomes to the drug (from 35 to 60 minutes) result in increased vesicle mean diameters.

The data on Table 2 also show that, in the tested conditions, 5-FU cannot be accumulated into liposomes presenting a pH gradient of 3.4 units, in spite of the adequate drug pKa of 8.04 (Krogsgaard-Larsen *et al*., 1996). Doxorubicin, an antineoplastic agent, presents a pKa of 8.2 and can be actively incorporated in liposomes with up to 100% efficiency. However, this drug is a lipophilic amine, presenting an octanol/water partition coefficient around 12.6 (Crommelin and Schreier, 1994). 5-FU, on the other hand, does not have a free amino group and presents a much lower partition coefficient in the same system (around 0.13), what indicates a larger difficulty of the therapeutic compound to cross lipophilic barriers, such as the liposomal membrane.

Another possible reason for the low drug entrapment observed, both in active and passive loading procedures, is that at pH 7.4 the neutral form of 5-FU is surface active, with a critical micelle concentration estimated at 80nM (Elorza *et al*., 1993). Therefore, if some of the drug molecules were organized in the form of micelles, their permeation through the lipid bilayer or their capture in the vesicle aqueous core would be more difficult, due to the larger size of the permeating species, resulting in increased mass transfer limitation. Another drawback of the methodologies above discussed is the low stability of 5-FU aqueous solutions when warmed. Increases around 20% in drug absorbance at 254nm were noticed when the drug was exposed to a temperature of 60°C from 10 to 60 minutes.

These results stimulated testing the ethanol injection procedure, which involves milder operational conditions and could lead to more stable drug encapsulation as well as to higher final drug to lipid ratios.

Incorporation of 5-FU in liposomes prepared by ethanol injection

The lipid hydration step can be a severe constraint on large scale vesicle manufacture. This step can be accelerated and easily scaled up by dissolving the lipids in an organic solvent and injecting the mixture into the desired aqueous phase, which basically comprises the ethanol injection method. Lipophilic compounds can be effectively incorporated by mixing the compound with the lipids, whereas hydrophilic agents can be encapsulated by lipid mixture injection in an aqueous phase containing the agent to be incorporated. However, for water soluble compounds, the encapsulation efficiency is normally poor and the procedure requires a large drug concentration in the aqueous phase.

The mixture of the drug with the lipids in ethanol followed by injection in TRIS-HCl buffer resulted in only 0.4% of 5-FU incorporation in vesicles, corresponding to the extremely low final drug to lipid ratio of 0.002. In this situation, total lipid concentration in ethanol remained equal to 40mM, while drug concentration was 20mM (maximum 5-FU solubility in ethanol at 25°C). After the injection, in the same conditions previously described, lipid concentration in the sample was 2mM. This result is due to the predominant hydrophilic character of the drug, what possibly impels it to migrate to the external aqueous phase, preventing the drug to be incorporated into the vesicles.

Table 3. Effect estimates on mean vesicle diameter resulting from the experimental design for active loading of 5-FU in DPPC/Chol liposomes. Initial total lipid concentration: 2.0mM.

Factor	Effect	Standard Error	t(1)	P	-95%	+95%
Mean	190.900	0.894	213.433	0.003	179.535	202.265
(1) Time	77.750	2.000	38.875	0.016	52.338	103.162
(2) Temperature	78.250	2.000	39.125	0.016	52.838	103.662
(3) Initial drug to lipid ratio	78.250	2.000	39.125	0.016	52.838	103.662
(1) x (2)	102.750	2.000	51.375	0.012	77.338	128.162
(1) x (3)	105.750	2.000	52.875	0.012	80.338	131.162
(2) x (3)	104.250	2.000	52.125	0.012	78.838	129.662
(1) x (2) x (3)	76.750	2.000	38.375	0.016	51.338	102.162

Table 4. Variance analysis of mean vesicle diameter data resulting from the experimental design for active loading of 5-FU in DPPC/Chol liposomes.

Source of variation	Sum of squares	Mean squares	Degrees of freedom	F value
Regression	113589.9	16225.84	7	13.38
Residual	2426	1213	2	
Total	116006.9	12889.66	9	
Correlation coefficient	0.97909	-	-	

The incorporation of 5-FU in liposomes resulted in an encapsulation efficiency of only 0.6% when the lipid/ethanol solution was injected (to a final lipid concentration of 2mM) into a 50mM 5-FU in TRIS buffer solution, corresponding to a final drug to lipid ratio of 0.15. The final vesicle mean diameter in this case was 253nm. These values of drug loading and vesicle size correspond to an internal drug concentration in the aqueous core of the vesicles around 36mM, meaning that 72% of the available drug was effectively incorporated during the injection.

This result in terms of internal drug concentration is more attractive than the ones mentioned above for passive and active loading into extruded vesicles. However, concerning to final drug concentration and to vesicle diameter, the extruded vesicles passively encapsulating 5-FU are more adequate. Higher drug concentration implies in lower therapeutic doses, while smaller vesicles can penetrate more deeply into the skin. It is worth mentioning, however, that the liposomal concentration in systems prepared by ethanol injection can be easily increased by ultrafiltration.

Incorporation of 5-FU in liposomes prepared by reverse phase evaporation

An alternative drug encapsulation technique was used for 5-FU, consisting of the reverse phase evaporation method, which is more difficult to be scaled up but enhances the contact of the drug with the lipid molecules during vesicle formation, improving the incorporation of very hydrophilic compounds. As mentioned before, this method has been previously used to incorporate 5-FU in sphingomyelin or DSPC liposomes with mean diameters from 238 to 295 nm by Elorza *et al*. (1993), which reported encapsulation efficacies up to 6%.

The effects of NaCl presence in the buffer solution and of the aqueous phase/organic phase volume ratio during vesicle preparation were evaluated, and the results are shown in Table 5. With this technique, up to 5.7% of the drug was accumulated into 137nm vesicles (after extrusion). Therefore, this method is more suitable than the others tested in this work concerning to drug incorporation efficiency and also than the results reported by Elorza *et al*. for reverse phase evaporation (1993) when comparing mean vesicle diameters. Also, tests performed following the methodology proposed by Amer *et al*. (1998) shows that the operational conditions involved in the preparation of these vesicles do not affect drug chemical stability, possibly because the remaining amount of organic solvent protects the therapeutic compound from degradation.

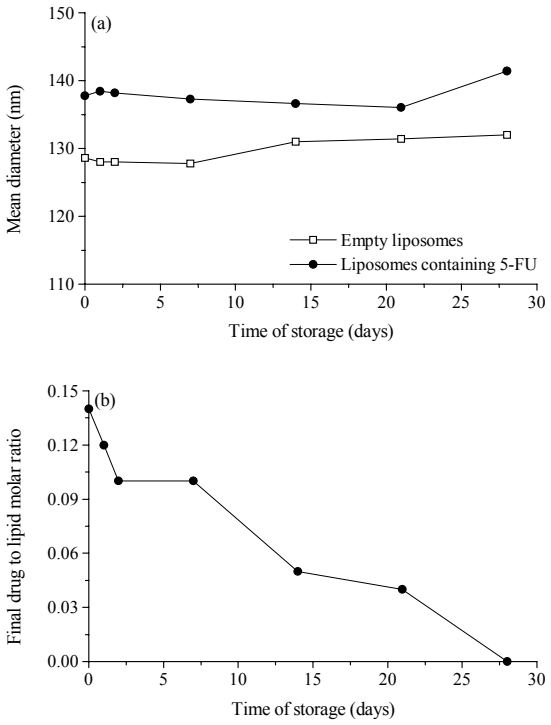
Storage stability of liposomes prepared by reverse phase evaporation encapsulating 5-FU

The storage stability of these vesicles at 5°C concerning to changes in mean vesicle diameter and to drug retention inside the liposomes was monitored for 4 weeks, giving the results presented in Figure 3. The liposomes showed to be stable concerning to vesicle diameters during the entire storage period, with no aggregation or fusion being observed, being able to retain more than 50% of the entrapped drug one week after liposome preparation. At the end of four weeks, however, practically all the encapsulated drug was released. These results suggest that, for adequate pharmacological application, these vesicles should be used immediately after their preparation.

Table 5. Passive loading of 5-FU in DPPC/Chol liposomes prepared by reverse-phase evaporation.

Aqueous Phase	*Initial 5-FU (mM)*	*Initial Lipid (mM)*	*Volume Ratio Aqueous/ Organic Phase*	*Mean vesicle diameter (nm)*	*Encapsulation Efficiency (%)*	*Internal 5-FU (mM)*
TRIS buffer/ 140 mM NaCl	4.54	3.0	1:10	2000[a]	2.7	12.0
TRIS buffer	12.50	1.5	1:3	137	1.8	8.0
TRIS buffer	3.75	1.5	1:3	137	5.7	25.3

a) vesicles not extruded



Fiigure 3. Stability on storage at 4ºC of empty vesicles and of liposomes containing 5-FU. a) Mean vesicle diameter variations; b) 5-FU leakage from liposomes. Empty liposomes: lipidic concentration of 6.0mM and initial mean diameter of 128.6nm. Liposomes containing 5-FU: lipidic concentration of 6.0mM and initial mean diameter of 137.8nm.

Conclusion

The results herein described demonstrate that vesicle loading and stability can be controlled by the experimental procedure used to entrap the active agent. Different initial active agent to lipid molar ratios and distinct preparation methods strongly affect 5-FU encapsulation efficiency. For this drug, the reverse-phase evaporation method was the most adequate.

Acknowledgements

The authors are grateful to the Fundação de Amparo à Pesquisa do Estado de São Paulo (Fapesp) for the financial support to this research.

References

AMER, M.M. *et al*. Spectrophotometric and spectrofluorimetric determination of fluorouracil in the presence of its degradation products. *J. Pharm. Pharmacol.*, London, v.50, p.133-8, 1998.

BARENHOLZ, Y.; CROMMELIN, D.A. Liposomes as pharmaceutical dosage forms. *In:* SWARBRICK, J.; BOYLAN, J.C. *Encyclopedia of Pharmaceutical Technology*, v.9. New York: Marcel Dekker Inc., p.1-39, 1994.

BARROS NETO, B. *et al*. *Planejamento e otimização de experimentos*. 2. ed. Campinas: Editora da Unicamp, 1995.

BATZRI, S.; KORN, E.D. Single bilayer liposomes prepared without sonication. *Biochim. Biophys. Acta*, Amsterdan, v.298, n.4, p.1015-19, 1973.

CHEN, P.S. *et al*. Microdetermination of phosphorus. *Anal. Chem.*, Washington, DC, v.28, n.11, p.1756-58, 1956.

CROMMELIN, D.J.A.; SCHREIER, H. Liposomes. *In:* KREUTER, J. (Ed.) *Colloidal Drug Delivery Systems*. New York: Marcel Dekker Inc., p.73-191, 1994.

DUDNITCHENKO, A.S.; KRASNOPOLSKY, Y.M. Preparation and pharmacokinetics in vivo of liposome-associated anthracyclines and 5-fluorouracil. *Exp. Oncol., v.*18, n.4, p.392-6, 1996.

EL MAGHRABY, G.M.M. *et al*. Skin delivery of 5-fluorouracil from ultradeformable and standard liposomes in-vitro. *J. Pharm. Pharmacol.*, London, v.53, n.8, p.1069-77, 2001.

ELORZA B. *et al*. Characterization of 5-fluorouracil loaded liposones prepared by reverse-phase evaporation or freeze-thawing extrusion methods: study of drug release. *Biochim. Biophys. Acta*, Amsterdan, v.1153, n.2, p.135-42, 1993.

FRESTA, M. *et al*. 5-Fluorouracil - Various kinds of loaded liposomes - encapsulation efficiency, storage stability and fusogenic properties. *Int. J. Pharm.,* Amsterdam, v.99, n.2-3, p.145-56, 1993.

ISRAELACHVILLI, J.; MITCHELL, D. J. A model for the packing of lipids in bilayer membranes. *Biochim. Biophys. Acta*, Amsterdam, v.389, n.1, p.13-19, 1975.

JARUGULA, V.R.; BOUDINOT, F.D. High-performance liquid chromatographic determination of 5-fluorouracil and its prodrugs tegafur and 4-deoxy-5-fluorouracil, in rat plasma. *J. Chromatogr. B,* v.677, n.1, p.199-203, 1996.

JOONDEPH, B.C. *et al*. Liposome-encapsulated 5-fluorouracil in the treatment of proliferative

vitreoretinopathy. *Ophthalmic Surg.*, Thorofare, v.19, n.4, p.252-6, 1988.

KORTING, H.C.F. *et al*. (Ed.) *Liposome Dermatics*. Muniche, 1992.

KROGSGAARD-LARSEN, P. *et al*. *A textbook of drug design and development*. 2 ed. Copenhagen: Harwood Academic, 1996.

LASIC, D.D. Cosmetic applications of liposomes. *In:* LASIC, D.D. (Ed.) *Liposomes: from Physics to Applications.* Amsterdam: Elsevier Science Publications B.V., p.477-487, 1993.

LASIC, D.D. Novel applications of liposomes. *Trends Biotechnol.,* Cambridge, v.16, n.7, p.307-21, 1998.

MACDONALD, R.C. *et al*. Small-volume extrusion apparatus for preparation of large, unilamellar vesicles. *Biochim. Biophys. Acta*, Amseterdam, v.1061, n.2, p.297-303, 1991.

MADDEN, T.D. *et al*. The accumulation of drugs within large unilamellar vesicles exhibiting a proton gradient: a survey. *Chem. Phys. Li*pids, Charmon, v.53, n.1, p. 37-46, 1990.

MAYER, L.D. *et al*. Characterization of liposomal systems containing doxorubicin entrapped in response to pH gradients. *Biochim. Biophys. Acta*, Amsterdam, v.1025, n.2, p.143-51, 1990.

NEW, R.R.C. - *Liposomes:* a practical approach. Oxford: Oxford University Press, 1990.

SHARATA, H.H.; KATZ, K.H. Liposomes. *Int. J. Dermatol.,* Oxford, v.35, n.11, p.761-769, 1996.

SIMMONS, S.T. *et al*. Pharmacokinetics of a 5-fluorouracil liposomal delivery system. *B. J. Ophthalmol.*, London, v.72, n.9, p.688-91, 1988.

VALERO, V. *et al*. Phase II trial of liposome-encapsulated doxorubicin, cyclophosphamide, and fluorouracil as first-line therapy in patients with metastatic breast cancer. *J. Clin. Oncol.*, Kyoto, v.17, n.5, p.1425-34, 1999.

Received on January 08, 2003.
Accepted on May 05, 2003.