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Liposome preparation using a hollow fiber membrane contactor—Application to spironolactone encapsulation

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ABSTRACT

In this study, we present a novel liposome preparation technique suitable for the entrapment of pharmaceutical and cosmetic agents. This new method uses a membrane contactor in a hollow fiber configuration. In order to investigate the process, key parameters influence on the liposome characteristics was studied. It has been established that the vesicle size distribution decreased with the organic phase pressure decrease, the phospholipid concentration decreases and the aqueous to organic phase volume ratio increases. Liposomes were filled with a hydrophobic drug model, spironolactone that could be used for a paediatric medication. The mean size of drug-free and drug-loaded liposomes was, respectively, 113 ± 4 nm and 123 ± 3 nm. The zeta potential of drug-free and drug-loaded liposomes was, respectively, -43 ± 0.7 mV and -23 ± 0.6 mV. High entrapment efficiency values were successfully achieved ($93 \pm 1.12\%$). Transmission electron microscopy images revealed nanometric sized and spherical shaped oligo-lamellar vesicles. The release profile showed a rapid and complete release within about 5 h. Additionally, special attention was paid on process reproducibility and long term lipid vesicles stability. Results confirmed the robustness of the hollow fiber module based technique. Moreover, the technique is simple, fast and has a potential for continuous production of nanosized liposome suspensions at large scale.

1. Introduction

In recent years, it has become more and more evident that the development of new drugs alone is not sufficient to ensure progress in drug therapy. A promising strategy involves the development of suitable drug carrier systems. Colloidal systems, ranging in size from 10 to 1000 nm, including emulsions, micro and nanoparticles, micro and nanocapsules and liposomes are widely used as therapeutic systems.

Liposomes are enclosed spherical vesicles that are organized in one or several concentric phospholipidic bilayers with an internal aqueous phase (Israelachvili et al., 1977). Because of their structure, liposomes can entrap hydrophilic pharmaceutical agents in their internal aqueous compartment or lipophilic drugs within the lipid membrane (Torchilin, 2005). Due to their biocompatibility, biodegradability and low toxicity, potential applications of liposomes as pharmaceutical carriers for efficacy enhancement and toxicity reduction are well recognized (Lian and Ho, 2001).

Since the pioneering discovery of Bangham, several techniques have been reported in the literature for liposome preparation: the thin-film hydration (Bangham, 1978), reversed phase evaporation (Szoka and Papahadjopoulos, 1978), solvent-injection techniques (Deamer, 1978; Stano et al., 2004), detergent dialysis (Zumbuehl and Weder, 1981) are the most commonly applied methods. Other techniques are the supercritical fluid reverse phase evaporation (Otake et al., 2006), spray drying (Skalko-Basnet et al., 2000) and freeze drying (Li and Deng, 2004). Some methods allow liposome size homogenization, for instance the high pressure extrusion (Hope et al., 1985), sonication (Saunders et al., 1962) microfluidization (Vemuri et al., 1990; Jahn et al., 2004; Pradhan et al., 2008) and cross flow injection technique (Wagner et al., 2006). Membrane contactors, applied for the preparation of emulsions, precipitates, polymeric and lipidic nanoparticles (Charcosset, 2006) have known increasing interest and were recently reported for the liposomes preparation using SPG membranes (Jaafar-Maalej et al., 2010). In the present study, the preparation method was based on the membrane contactor principle and used for the first time a hollow fiber module. The module configuration increases the membrane area and thus may offer a better efficiency than the SPG membrane. Furthermore, the hollow fiber configuration allows a uniform flow which may make easily the extrapolation of the results for an industrial production.

This new method was applied to spironolactone encapsulation. Spironolactone is a specific aldosterone antagonist, which has long

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been used as a potassium sparing diuretic in the treatment of heart failure in both adults and infants (Simmonds et al., 2006). As it is commercially available only in a solid dosage form and children have difficulty in swallowing whole tablets or capsules, spironolactone liquid formulations are preferable (Standing and Tuleu, 2005). Thus, several extemporaneous formulations have been developed especially suspensions that showed incomplete oral behaviour, slow dissolution rate and a risk of degradation during storage (Allen and Erickson, 1996). Spironolactone encapsulation into liposomes may enhance its availability by improving the dissolution rate, and protect the drug from degradation by confining it within lipid vesicles.

For pharmaceutical and clinical use of liposomes, an appropriate formulation and a suitable production method should be selected (Wagner et al., 2006). Liposomes must fulfill several criteria in terms of size, zeta potential, morphology, and stability. For liposomes without surface modifications, the circulation time in the blood stream is limited by uptake through the reticulo-endothelial-system (RES). Liu et al. (1992a) had showed that liposomes smaller than 70 nm are taken up from the blood stream by liver parenchymal cells, while liposomes larger than 300 nm accumulate in the spleen. An optimum size range of 70–200 nm has been identified to give highest blood concentration of liposomes. In another study, Liu and Huang (1992b) had demonstrated that biodistribution of liposomes depends not only on the mean particle size but also on size distribution. Liposomes must be of unimodal narrow distribution. In addition to size, the surface charge of liposomes is an important determinant of their clearance from the general circulation and their tissue disposition after administration. Among small liposomes, those with a negative surface charge are cleared rapidly but positively charged or uncharged liposomes remain in the circulation for higher periods. The impact of zeta potential in organ distribution and therapeutic efficacy has been demonstrated by many studies (Hawley et al., 1995). On the other hand, the process used for liposome preparation must be reproducible with low costs and economic scale-up production (Wagner et al., 2002).

The aims of our study are to develop and optimize a novel preparation strategy, based on a membrane contactor and using a hollow fiber module. The influence of process parameters (aqueous to organic phase volume rate, organic phase pressure, type and concentration of phospholipids) on liposome properties was investigated. The optimized method was applied to spironolactone encapsulation and the spironolactone-loaded liposomes were characterized for their size, zeta potential, dissolution rate and stability. Finally, the reproducibility and scale-up of the process, as well as the stability of the liposomal suspensions were studied.

2. Materials and methods

2.1. Materials

2.1.1. Reagents

Phospholipids were purchased from Lipoïd GmbH (Ludwigshafen, Germany): Lipoid® E80 obtained from egg yolk lecithin (contains 82% of phosphatidyl-choline and 9% of phosphatidyl-ethanolamine), EPC-3 obtained from hydrogenated egg lecithin (contains not less than 98% of phosphatidyl-choline), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine DPPC (contains not less than 99% of phosphatidyl-choline).

Spironolactone and cholesterol were supplied by Sigma–Aldrich Chemicals (Saint Quentin Fallavier, France).

All reagents were acquired with their analysis certificate. Organic solvents (ethanol 95% and chlorhydric acid HCl 37%, w/w) were supplied by Carlo Erba Reagenti (Milano, Italy). They were of analytical grade and used such as without further purification.

Ultra-pure water was obtained from Millipore Synergy® system (Ultrapure Water System, Millipore). According to the British standards for water use in the laboratory, the ultra-pure water, otherwise known as type 1 water, has a resistivity of 18.2 MΩ cm at 25 °C (conductivity = 0.055 μS/cm), total organic carbon (TOC) < 10 ppb, sodium < 1 ppb, chlorine < 1 ppb and silica < 3 ppb.

2.1.2. Hollow fiber module

The Liqui-cel® Mini Module X50 was purchased from Alting (Hoerdt, France). This module contains 2300 polypropylene hollow fibers distributed in a uniform way around a central tube, so as to allow the use of the total membrane surface. The hollow fiber module dimensions are as follows: an inner diameter of 220 μm, an outer diameter of 300 μm, a porosity of 40% and a pore size estimated at 40 nm. The fiber length is 0.115 m and the total active membrane surface is 0.18 m^2.

The polypropylene hollow fibers are naturally hydrophobic. Hence, they must be treated before their first use as follows: a water/ethanol (50:50, v/v) solution was maintained in circulation through the module by applying a low pressure (0.2 bar). After 10 min, water droplets appeared on the filtrate side instead of the outlet side of the membrane device. The module lost its hydrophobic character and was then be rinsed with ultra-pure water. This method to treat the membrane module was suggested by the manufacturer.

2.2. Methods

2.2.1. Liposome preparation

A schematic diagram of the experimental set-up used in this study is shown in Fig. 1.

The system included a positive displacement pump (Filtron, France), a pressurized vessel (equipped with a manometer M_3) connected on one side to a nitrogen bottle (Linde Gas, France) and on the other side to the hollow fiber module (with two manometers M_1 and M_2, respectively placed at the inlet and outlet of the device).

For the liposome preparation, the required amounts of phospholipids (DPPC, EPC-3 or Lipoid® E80, 20, 40 or 80 mg/ml depending on the experiments) and cholesterol (20%, w/w) were dissolved in 250 ml ethanol. The organic phase was placed in the pressurized vessel. The connecting valve to the nitrogen bottle was opened and the nitrogen pressure was set at a fixed level. The aqueous phase (500 ml) was then pumped through the membrane contactor module using the positive displacement pump. When the water arrived to the inlet of the hollow fiber module, the valve connecting the pressurized vessel to the filtrate side of the membrane device was opened so that the organic phase permeated through the pores of the hollow fibers into the aqueous phase. Spontaneous liposome formation occurred as soon as the organic solution was in contact with the aqueous phase. The experiment was stopped when air bubbles started to appear in the tube connecting the pressurized vessel to the membrane module, indicating that the pressurized vessel was empty. Then, the liposomal suspension was stabilized for 15 min under magnetic stirring (RW 20, Ika-Werk). The experiments were conducted at 22 ± 2 °C. Finally, the ethanol was removed by rotary evaporation (Rotavapor R-144, Buchi, Flawil, Switzerland) under reduced pressure.

The experiments were carried out in an open loop configuration to avoid the recirculation of the liposomes formed in the experimental set-up. Therefore, the flow rates for the aqueous and organic phases were set to have both phases passed in the same time.

At the end of the experiment, the hollow fiber module was regenerated. The washing was performed by flushing the module twice with 500 ml of water and 250 ml of ethanol in the pressurized vessel. The membrane permeability (the slope of the permeate flow

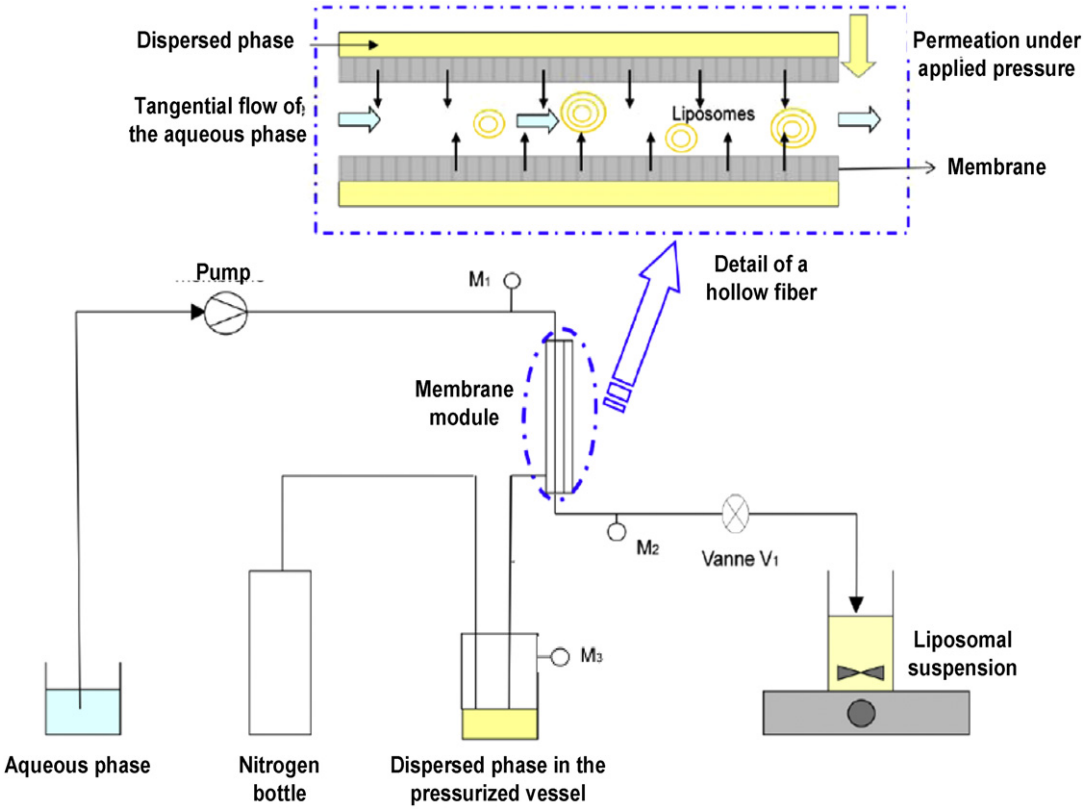

Fig. 1. Schematic diagram of the experimental set-up.

rate versus transmembrane pressure) was measured at the beginning of each experiment and was checked to be around 90% of its initial value.

Drug-loaded liposomes were prepared as described above by dissolving 750 mg of spironolactone in the organic phase.

2.2.2. Liposome characterization

In order to assess the liposome quality and to obtain quantitative measurements that allow comparison between different liposome batches, various parameters were measured. The methods included average mean size and polydispersity index analysis, zeta potential determination, microscopic observation, encapsulation efficiency measurement and drug release study.

2.2.2.1. Size analysis. Dynamic light scattering (DLS), otherwise known as photon correlation spectroscopy (PCS), is extensively used in liposome size distribution analysis. In this study, a Malvern Zetasizer Nano-series (Malvern Instruments Zen 3600, Malvern, UK) was used. Each sample was diluted 100-fold with ultra-pure water and analyzed in triplicate at 25 °C. The data on particle-size distribution were collected using the DTS (nano) software (version 5.0) provided with the instrument. The sizes mentioned correspond to the hydrodynamic diameter of these particles. In addition, the polydispersity index (PI) is calculated in terms of span factor defined as span = ($d90$–$d10$)/$d50$ where $d10$, $d50$ and $d90$ are the particle diameters at 10%, 50% and 90% of the cumulative liposome number, respectively. The PI is an indicator of the width of particle size distribution of a sample. It is ranging from 0 (monodispersed) to 0.5 (relatively broad distribution).

These data (particle size and PI) were expressed as the mean ± standard deviation (S.D). Prior to measuring size and zeta potential, no calibration is needed. The DLS method presents the advantage to be simple and fast (Berger et al., 2001; Kölchens et al., 1993; Provder, 1997).

2.2.2.2. Zeta potential determination. Measurements of zeta potential are commonly used to predict the colloidal system stability. The zeta potential was determined using a Malvern Zetasizer Nano-series (Malvern Instruments Zen 3600, Malvern UK). All the measurements were performed at least three times after dilution in water. The zeta potential was calculated from the electrophoretic mobility by the Helmholtz–Smoluchowski equation (Hunter and Midmore, 2001).

2.2.2.3. Microscopic observation. Transmission electron microscopy (TEM) images were taken using a CM 120 microscope (Philips, Eindhoven, Netherlands) operating at an accelerating voltage of 80 kV. The sample preparation was performed according to our previous study (Jaafar-Maalej et al., 2010). A drop of liposome suspension was placed onto a carbon-coated copper grid; the suspension excess was removed with a filter paper leaving a thin liquid film stretched over the holes. Negative staining using a 2% phosphotungstic acid solution (w/w), pH 7.1, was directly made on the deposit during 1 min. Finally, the excess of phosphotungstic solution was removed with a filter paper and stained samples were observed.

2.2.2.4. Encapsulation efficiency. Liposome preparations are a mixture of encapsulated and un-encapsulated drug fractions. Methods for determining the amount of encapsulated material within liposomes typically rely on destruction of the lipid bilayer and subsequent quantification of the released material. In the present study, the liposome encapsulation efficiency was determined from the amount of entrapped drugs using the ultracentrifugation technique. Briefly, total spironolactone amount (TSA) was

determined after having dissolved and disrupted drug-loaded liposomes in ethanol using an ultrasound bath for 10 min. Then, spironolactone-loaded liposome sample was centrifuged (Optima™ Ultracentrifuge, Beckman Coulter, USA) at 50 000 rpm for 50 min at +4 °C. The free spironolactone amount (FSA) was determined in the supernatant.

Spironolactone concentrations were measured at λ absorbance of 237 nm with a spectrophotometer UV–vis (Shimadzu UV mini-1240 V, Kyoto, Japan). The spectrophotometric analytical method was validated as usually required (data not shown).

The spironolactone encapsulation efficiency (E.E.) was calculated as follows:

$$\text{E.E.} = \frac{\text{TSA} - \text{FSA}}{\text{TSA}} \times 100$$

The encapsulation efficiency was determined in triplicate.

2.2.2.5. Drug release study. Spironolactone release was evaluated using the dialysis tube technique. The dialysis membrane Spectra/Por 7 (Spectrum Labs, Breda, Netherlands) was selected according to drug permeability so that no spironolactone adsorption occurred on the membrane (molecular weight cut off of 50 kDa). A 4 ml aliquot of liposomal suspension was placed in the dialysis bag, hermetically tied and dropped into 1.5 l of an aqueous receptor medium (chlorhydric acid HCl 0.1 N mentioned on the 31st edition of the American Pharmacopeia). Perfect sink conditions prevailed during the drug release studies and the entire system was kept at 37 ± 2 °C under continuous magnetic stirring at 70 rpm. The receptor compartment was closed to avoid evaporation of the dissolution medium. Samples (3 ml) of the dialysate were taken at various time intervals and assayed for spironolactone concentration by spectrophotometric method. The same volume was replaced with fresh dissolution medium so that the volume of the receptor compartment remained constant. All kinetic experiences were conducted in triplicate and the mean values were taken.

2.2.3. Reproducibility test

Once all the process parameters were assessed, the experiment under the optimum conditions was repeated three times in order to study the technique reproducibility.

2.2.4. Stability study

The drug-free liposome suspensions were stored at 5 ± 3 °C during a period of 3 months. The stability was evaluated by comparing the initial particle size, zeta potential and morphological investigation with those obtained every month during the storage period.

In addition, the spironolactone-loaded liposome suspensions were stored during a period of 2 months under conditions required by the 2008 guidelines of the ICH (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use): 5 ± 3 °C for normal stability study and 25 ± 2 °C, 60 ± 5 RH (relative humidity) for accelerated stability study. The stability was evaluated in terms of encapsulation efficiency and particle size, using the methods described above.

2.2.5. Scale-up

A 4-fold factor increase of the liposome suspension volume prepared under the optimum conditions was realized by increasing all the amounts and the volumes involved in the preparation. This experiment was aimed to assess the scale-up ability of the preparation process.

Table 1
Influence of formulation factors and process parameters on drug-free liposome mean size.

Aqueous to organic phase volume ratio	Organic phase pressure (bar)	Phospholipid concentration (mg/ml)	Mean size ± S.D.[a] (nm)
0.4	1.8	20	189 ± 6
1.2	1.8	20	151 ± 7
2	1.8	20	114 ± 5
2	1.8	20	114 ± 5
2	2.6	20	129 ± 6
2	3.8	20	126 ± 9
2	1.8	20	114 ± 5
2	1.8	40	114 ± 8
2	1.8	80	228 ± 10

[a] standard deviation ($n = 3$).

3. Results and discussion

3.1. Influence of formulation factors and process parameters on drug-free liposome characteristics

3.1.1. Aqueous to organic phase volume ratio effect on liposome characteristics

The influence of the aqueous phase volume was determined by comparing the mean size of liposomes prepared using 250 ml of organic phase and respectively 100, 300 and 500 ml of water; corresponding to aqueous to organic phase volume ratio of 0.4, 1.2, and 2. The influence of the aqueous to organic phase volume ratio on the liposome mean size and standard deviation of the mean size is shown in Table 1.

The mean size decreased as the aqueous phase volume increased. Our results suggested that an aqueous to organic phase volume ratio of 2 was the optimal ratio, since it produced liposomes with the smallest mean size and the narrowest size distribution (the effect on size distribution is not shown here) under this set of conditions.

In Jaafar-Maalej et al. (2010), liposome preparation using a SPG tubular membrane with 0.9 μm pore size led to a similar result; increasing the aqueous to organic phase volume rate from 1.6 to 2 decreased the mean size from 203 to 61 nm. Our results are also in agreement with those of Limayem-Blouza et al. (2006) who prepared spironolactone-loaded nanocapsules using a membrane contactor method.

Thus, in all following experiments, the aqueous phase volume was set to 500 ml and the organic phase volume was set to 250 ml (ratio of 2).

3.1.2. Organic phase pressure effect on liposome characteristics

The pressure can control the dispersed phase flow rate across the membrane pores. During preliminary studies, the organic phase pressure has been increased progressively to 5 bar (the pressure should not exceed 5 bar for security reasons). It has been observed that below 1.8 bar no organic phase flow was obtained (the pressure was not sufficient to inject the organic phase through the membrane) and beyond 3.8 bar the organic phase flow was too high for an appropriate liposome formation. Thus, the effect of the organic phase pressure over the range of 1.8–3.8 bar on the mean size was investigated. Table 1 shows liposome mean size of samples prepared at three different organic phase pressures. It can be observed that as the pressure increased from 1.8 to 2.6 bar, the liposome mean size slightly increased (+13%), whereas higher pressures (from 2.6 to 3.8 bar) did not affect mean size values.

In the literature, the effects of organic phase pressure have not yet been clarified. The influence of the pressure differs in several studies. For instance, in contrast to our results, Charcosset et al. (2005) observed a small decrease of solid lipid nanoparti-

cles size with the increasing pressure, from 190 to 175 nm for lipid phase pressure from 3 to 6 bar. On the other hand, it has been also reported in the study of Sheibat-Othman et al. (2008) that increasing the lipid phase pressure when preparing pH-sensitive particles by membrane contactor, led to a slight displacement of the particle size distribution to bigger sizes. As well, Jaafar-Maalej et al. (2010) observed that as the pressure increased the liposome size distribution increased. At a pressure of 3 bar, a narrow peak was observed in the size range around 45 nm, whereas at 5 bar the liposome suspensions showed a peak about 80 nm.

In the present study, we considered that 1.8 bar was the optimum pressure, since it produced liposome with the smallest size. In case of industrial applications, a high pressure may be advised as it does not change significantly the liposomes size but decreases considerably the processing time (Charcosset and Fessi, 2005). However the pressure used should not be too high, because it may lead to jets of the dispersed phase at the outlet of the membrane pores, which may give large droplets and large particle size distribution.

3.1.3. Phospholipid nature effect on liposome characteristics

In the present study, three phospholipids were tested: lipoid® E80, DPPC and EPC-3. Only the lipoid® E80 allowed the formation of liposomes with a convenient size. DPPC and EPC-3 led to aggregates with very high mean size, which had not been measured (exceeding the limits of the Nanosizer: 800 nm).

Aggregates formation could be explained by the high phase transition temperature of both DPPC and EPC-3. The DPPC phase transition temperature is about 41 °C as reported by Leonenko et al. (2004). Hence, to work at room temperature, lipoid® E80 was selected. Liposome preparation using DPPC or EPC-3 required working at a higher temperature. Another alternative would consist in putting the experimental set-up in a warm bath.

3.1.4. Phospholipid concentration effect on liposomes characteristics

The phospholipid concentration effect on the liposome size distribution was investigated. As shown in Table 1 the vesicle size did not change when the phospholipid concentration was varied between 20 and 40 mg/ml, but it increased when the phospholipid concentration was about 80 mg/ml.

Our results are in agreement with those reported in the literature. Charcosset et al. (2005) observed that the size of solid lipid nanoparticles, prepared by a membrane contactor method, increased from 150 to 195 nm when the lipid amount in the organic phase increased from 63 to 500 g. As well, Jaafar-Maalej et al. (2010) observed that the liposome size distribution was around 50 nm for the 20 mg/l phospholipid concentration and around 95 nm for the 60 mg/ml phospholipid concentration.

The larger vesicle size obtained with the higher lipid contents may be explained by the fouling of membrane pores by lipid molecules, which leads to a decreasing lipid phase flow rate with time. This result is also described for other preparations methods such as the ethanol injection. Kremer et al. (1977) reported that liposome mean size was proportional to phospholipid concentration. Thus, in a general rule, when lipid molecules are present in a high amount, they induce the formation of large size vesicles (Pradhan et al., 2008).

20 mg/ml was selected as the phospholipid concentration in subsequent parts of the study, since it gave vesicles with a narrow size distribution.

3.2. Drug-loaded liposome characterization

Based on the previous results, the formulation composed of 500 ml of water, 250 ml of organic phase (lipoid® E80 20 mg/ml in ethanol) was selected to produce an optimal liposome suspension using a hollow fiber module at a feed pressure of 1.8 bar. These selected conditions allowed the formation of liposomes with convenient characteristics (mean size of 113 ± 4 nm and polydispersity index of 0.18 ± 0.01).

Table 2
Drug-free and drug-loaded mean size and zeta potential.

Batch	Mean size ± S.D.[a] (nm)	Zeta potential ± S.D.[a] (mV)
Without drug[b]	113 ± 4	−43 ± 0.7
With drug[b]	123 ± 3	−23 ± 0.6

Experimental conditions of liposome suspension preparation: 500 ml of water, 250 ml of ethanol containing 5 g of lipoid® E80 and 750 mg of spironolactone, feed pressure 1.8 bar.
[a] standard deviation ($n = 3$).
[b] The mean of 3 batches.

To prepare drug-loaded liposomes, 750 mg of spironolactone was added in the organic phase, since it is poorly soluble in water and very soluble in ethanol. The preparation process was the same as drug-free liposomes.

3.2.1. Drug-loaded liposome mean size and zeta potential

The effect of the drug entrapment on the vesicle size was investigated. Table 2 presents the mean size and the zeta potential of the liposome suspension with and without spironolactone.

The addition of the drug increased slightly the vesicle size (respectively, 113 and 123 nm without and with spironolactone). This increase of the mean size could be explained by the entrapment of the spironolactone in the vesicles bilayers.

The spironolactone-loaded liposome suspension had an upper zeta potential compared to drug-free formulation. Indeed, the zeta potential was −43 mV for the drug-free liposome suspension and became −23 mV for the drug-loaded liposome suspensions. Zeta potential measurements give information about the surface properties of the carrier and therefore can be useful to determine the type of the association between the active substance and the carrier (whether the drug is encapsulated in the body or simply adsorbed on the surface) (Barratt, 2003). In our study the negative surface charge was further shielded in the presence of the drug, suggesting that at least a part of the association was surface-adsorption and the rest was incorporated within the lipidic matrix. These zeta potential data allowed predicting a very good stability of the preparations, since it was previously reported by Lyklema and Fleer (1987) and Wiacek and Chibowski (1999) that a negative zeta potential higher than 20 mV was sufficient to prevent vesicle coalescence.

3.2.2. Microscopic observation

As shown in Fig. 2, the morphological investigation using transmission electron microscopy revealed nanometric sized and quasi-spherical shaped liposomes. According to TEM micrographs, liposomes ranged in size from 60 to 180 nm correlating well with measurement obtained by PCS. Vesicle membranes were composed of several phospholipids bilayers resulting in oligo-lamellar vesicles.

3.2.3. Encapsulation efficiency

The high encapsulation efficiency (93 ± 1.12%) was believed to be due to the high lipophilicity of spironolactone and therefore its good solubility in phospholipids. This result is in agreement with that reported by Limayem-Blouza et al. (2006); the encapsulation efficiency of spironolactone in nanocapsules was about 90.5% when prepared by a membrane contactor method using a SPG membrane and about 96.2% when prepared by nanoprecipitation. This high encapsulation efficiency was due to the high solubility of the drug in the lipid phase. Many studies (Barenholz, 2003; Fresta et al., 1996; Xu et al., 2007) demonstrated that the encapsulation efficiency was

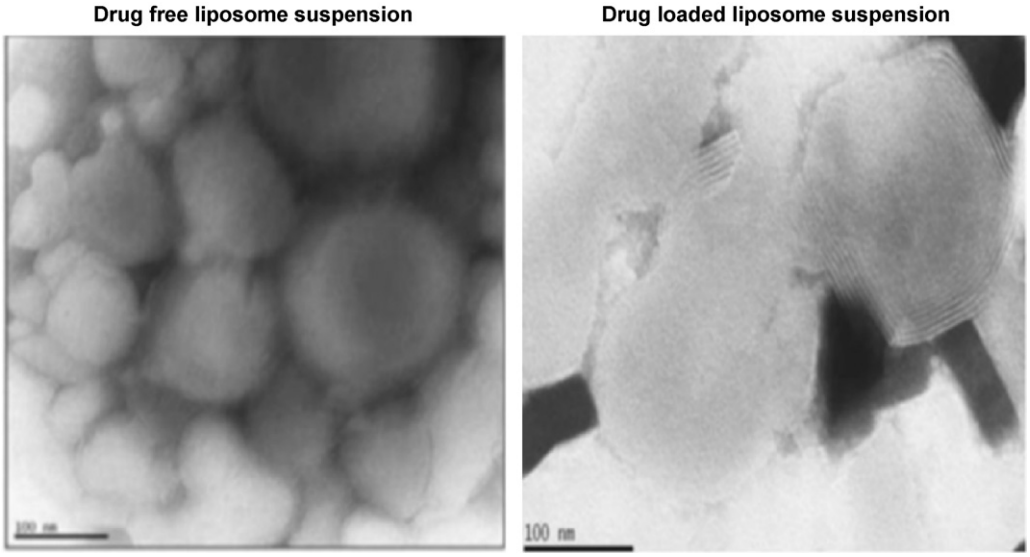

Fig. 2. TEM micrographs of drug free and drug-loaded liposome suspension. Experimental conditions of liposome suspension preparation: 500 ml of water, 250 ml of ethanol containing 5 g of lipoid® E80 and 750 mg of spironolactone, feed pressure 1.8 bar.

proportional to the drug lipophilicity. For instance, the vitamin E loading capacity of solid lipid nanoparticles prepared by Charcosset et al. (2005) was equal to 100%, as well the encapsulation efficiency of beclomethasone in liposomes prepared by Jaafar-Maalej et al. (2010) was 98%.

3.2.4. Drug release study

In sink conditions, the release of spironolactone from liposomes was rapid and complete, as 100% of the drug was released after about 4 h and 30 min (Fig. 3).

As can be seen in our study, 39.31% of spironolactone was released in 60 min. In a recent study (Dong et al., 2009), 24.6% of spironolactone was released from nanoparticles of 300 nm, while 9.2% of the raw spironolactone (several microns) was dissolved when the drug was free in the dissolution medium. According to the Noyes–Whitney equation (Mosharraf and Nystrom, 1995), the drug dissolution rate is directly proportional to its surface area exposed to the dissolution medium. The accelerated dissolution of spironolactone-loaded liposomes could thus be ascribed to their greater surface area in comparison with free drug. In many papers, it has been confirmed that retention time of encapsulated drug within liposomes increased with particle's size.

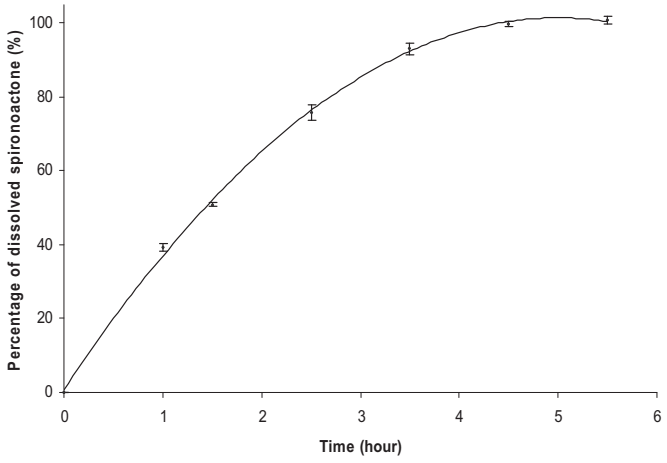

Fig. 3. Percentage of released spironolactone from spironolactone-loaded liposomes. Each value represents the mean ± S.D. ($n = 3$). Experimental conditions of liposome suspension preparation: 500 ml of water, 250 ml of ethanol containing 5 g of lipoid® E80 and 750 mg of spironolactone, feed pressure 1.8 bar.

On the other hand, in vitro release studies revealed that liposomal formulations with higher drug to lipid (D/L) ratios exhibit reduced release rates. Johnston et al. (2006) have showed that for a liposomal vincristine formulation with 60% D/L ratio, the half life time of drug release was 117 h (extrapolated). Whereas in our study (D/L ratio of 12.5%), 50% of the drug was released within about one 1.5 h. The increase of drug retention at the higher D/L ratios appears to be related to the presence of drug precipitates within liposomes.

Recent works have also shown that incorporation of cholesterol into liposomal formulations increased the rigidity of the bilayer and lowered the drug permeability. Thus the fluidity of liposome bilayers is considered as an important influencing factor on drug release rate. In our study, the cholesterol was used at a ratio of 20% (w/w) and the drug release was complete after 4 h and 30 min. In a recent study, the fluconazole released amount from liposomes after 10 h, was about 62.44% for 60% cholesterol ratio. The same study showed also that increasing the cholesterol ratio to 80%, decreased the drug release rate from vesicles (El-Nesr et al., 2010).

Furthermore, phospholipids having a high phase transition temperature (above 37 °C) will be re-organized in a well-ordered manner yielding to a rigid and less leaky liposome bilayer membrane at physiological temperature. In contrast, liposomes composed of phospholipids with low phase transition temperature (below 37 °C), allows quick drug leakage in aqueous phase (Sharma and Sharma, 1997). Thus in our study, the low phase transition temperature of the lipoid E80 may explain the rapid release of the drug.

In conclusion, the optimized liposome formulation increased spironolactone solubility and contained a high drug concentration (1.5 mg/ml) that may allow minimizing the administered volume for children.

3.3. Reproducibility test

3.3.1. Drug-free liposome suspension reproducibility

The experiment was repeated three times in order to study the technique reproducibility (Batches R1, R2 and R3). Resulting data (Table 3 and Fig. 4.) revealed the perfect accordance, in terms of mean size, vesicle size distribution, zeta potential and processing time between the 3 batches prepared under identical conditions.

Table 3
Reproducibility data of drug-free and drug-loaded liposome suspensions.

Batch N°	Mean size ± S.D.[a] (nm)	Zeta potential ± S.D.[a] (mV)	Processing time (s)	Encapsulation efficiency ± S.D.[a] (%)
R1	114 ± 5	−44 ± 1.0	85	
R2	110 ± 3	−44 ± 0.6	84	
R3	115 ± 3	−41 ± 0.6	84	
S1	123 ± 2	−23 ± 1.8	88	94.2 ± 1.51
S2	120 ± 4	−21 ± 0.8	86	92.7 ± 1.62
S3	126 ± 5	−24 ± 1.0	84	92.0 ± 1.60

Experimental conditions of liposome suspension preparation: 500 ml of water, 250 ml of ethanol containing 5 g of lipoid® E80 and 750 mg of spironolactone, feed pressure 1.8 bar.

[a] standard deviation ($n = 3$).

3.3.2. Drug-loaded liposome suspension reproducibility

The reproducibility of the preparation technique of the spironolactone-loaded liposomes was investigated. Three batches were produced under the same conditions (Batches S1, S2 and S3). Data shown in Table 3 and Fig. 4 revealed the perfect reproducibility of the preparation process.

3.4. Stability study

3.4.1. Drug-free liposome stability

Stability studies were carried out over 3 months. The three batches R1, R2 and R3 were selected in order to follow the zeta potential, the vesicle mean size and size distribution variations during storage at 5 ± 3 °C. Data are shown in Fig. 5.

Liposome size remained nearly unchanged during 3 months as no significant differences were observed over this period. In addition, the zeta potential was maintained to its initial value and no particle aggregation was observed during storage. There were also no significant changes in the vesicle size distribution (data not shown).

These results demonstrate the good stability of the liposome suspensions and thus indicate an adequate formulation of the preparation and optimum process conditions.

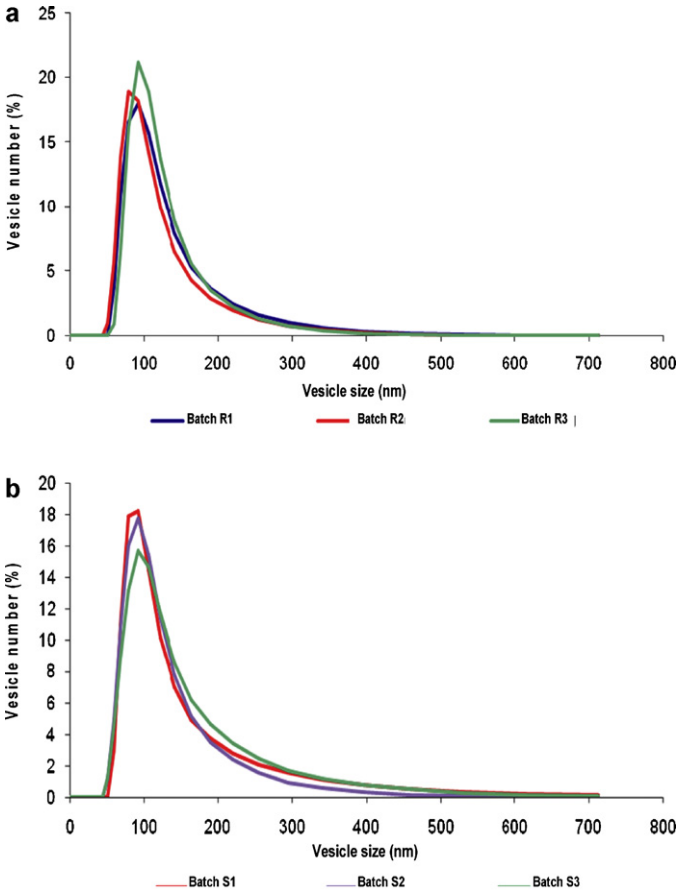

Fig. 4. Reproducibility of liposome size distribution (a) drug-free liposome suspension and (b) drug-loaded liposome suspension. Experimental conditions of liposome suspension preparation: 500 ml of water, 250 ml of ethanol containing 5 g of lipoid® E80 and 750 mg of spironolactone, feed pressure 1.8 bar.

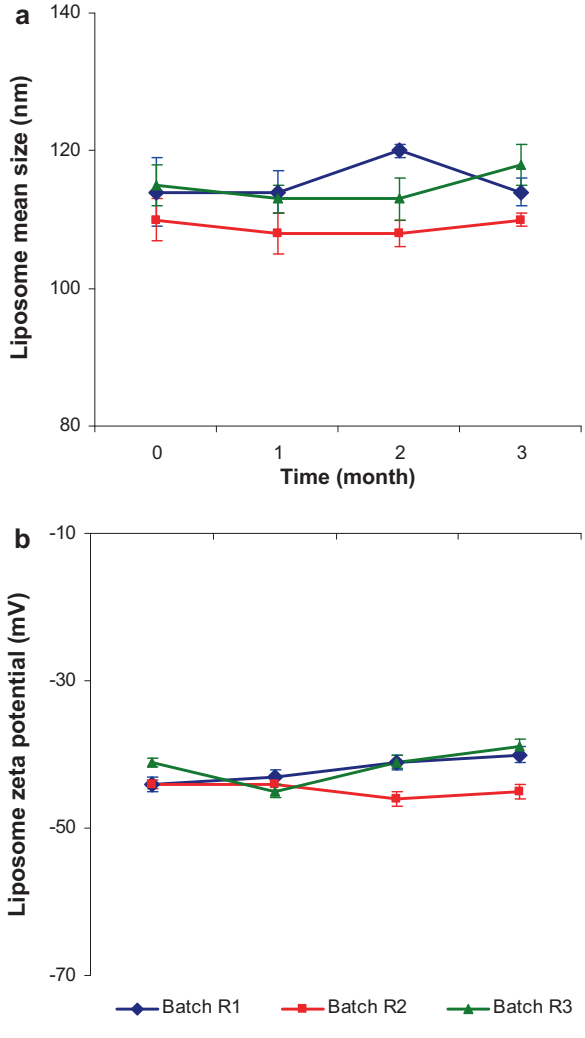

Fig. 5. Stability data of drug-free liposome suspensions stored at 5 ± 3 °C. Each value represents the mean ± S.D. ($n = 3$) (a) mean size stability and (b) zeta potential stability. Experimental conditions of liposome suspension preparation: 500 ml of water, 250 ml of ethanol containing 5 g of lipoid® E80 and 750 mg of spironolactone, feed pressure 1.8 bar.

Table 4
Encapsulation efficiency stability ± S.D.[a] (%) of spironolactone-loaded liposome suspensions stored at +4 °C and +25 °C.

Batch no.	After preparation	Storage at +4 °C		Storage at +25 °C	
		After 1 month	After 2 months	After 1 month	After 2 months
S1	94.2 ± 1.51	94.1 ± 1.33	93.2 ± 0.96	6.76 ± 0.82	0.49 ± 0.35
S2	92.7 ± 1.62	92.4 ± 1.20	90.2 ± 1.10	7.25 ± 0.67	0.67 ± 0.38
S3	92.0 ± 1.60	91.8 ± 1.17	90.6 ± 1.04	7.68 ± 0.66	0.63 ± 0.37

Experimental conditions of liposome suspension preparation: 500 ml of water, 250 ml of ethanol containing 5 g of lipoid® E80 and 750 mg of spironolactone, feed pressure 1.8 bar.
[a] standard deviation (n = 3).

Table 5
Scale-up of liposome suspensions prepared with a hollow fiber module.

Batch	Mean size ± S.D.[a] (nm)	Zeta potential ± S.D.[a] (mV)	Encapsulation efficiency ± S.D.[a] (%)	
Drug-free liposomes	Laboratory scale[b]	113 ± 4	−43 ± 0.7	
	Pilot scale	121 ± 3	−42 ± 0.6	
Drug-loaded liposomes	Laboratory scale[b]	123 ± 4	−23 ± 1.2	93.0 ± 1.58
	Pilot scale	132 ± 4	−31 ± 0.6	93.9 ± 1.39

Experimental conditions of liposome suspension preparation: 500 ml of water, 250 ml of ethanol containing 5 g of lipoid® E80 and 750 mg of spironolactone, feed pressure 1.8 bar.
[a] standard deviation (n = 3).
[b] The mean of the 3 batches.

3.4.2. Drug-loaded liposome stability

Stability data of spironolactone-loaded liposomes upon storage of 2 months are shown in Table 4, for storage temperature of 5 °C and 25 °C. Encapsulation efficiency drastically decreased only at 25 °C (it remained stable at 5 °C). This decrease could be explained by: (i) liposomes are lipidic vesicles which are not stable at 25 °C and the best way to store them is 5 °C, as previously reported in other studies (Du Plessis et al., 1996), (ii) spironolactone stability is influenced by temperature, the drug half time decreased when storage temperature increased (Pramar and Gupta, 1991; Tokumura et al., 2005). Thus, the best way to store the spironolactone-loaded liposomes is at temperature of 5 °C.

3.5. Scale-up

A 4-fold factor increase in the drug-free and drug-loaded liposome suspension volume, prepared under the optimum conditions, was realized. The results shown in Table 5 indicated a similarity for the mean size and zeta potential between the drug-free pilot-scale batch and the three batches R1, R2 and R3. In addition, data showed a similarity for the mean size, zeta potential and encapsulation efficiency between the drug-loaded pilot-scale batch and the three batches S1, S2 and S3.

Thus, it can be concluded that the membrane contactor method using a hollow fiber module allowed the production of liposomes in an easy way, which can be conducted at a large scale.

4. Conclusion

The present study investigated liposome suspension preparation with a membrane contactor in a hollow fiber configuration. Different parameters were tested in order to obtain an optimized formulation and process. Results showed that the hollow fiber module method successfully led to the formation of narrow distributed oligo-lamellar liposomes and satisfying entrapment efficiency of spironolactone. Moreover, obtained liposomes had rapid and complete release and good stability for 2 months.

The advantages of this new process for liposomes preparation are shown to be its facility of use, its reproducibility and its industrial scaling-up abilities. This simple and fast method allowed the production of nanosized liposome suspension at a large scale. Therefore, the hollow fiber module offers new opportunities in the design, rationalization and optimization of industrial processes and appears as a promising way to achieve important benefits in the logic of process intensification strategy leading to several successful applications.

In the near future, this study is intended to be completed by (i) an organoleptic evaluation and a pharmacokinetic study of the spironolactone-loaded liposomes, (ii) an application of this new method to the encapsulation of other pharmaceutical and cosmetic agents, and (iii) an application of the membrane contactor method to the development of new drug carrier systems.

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