Studies on size reduction of liposomes and their stabilization

A thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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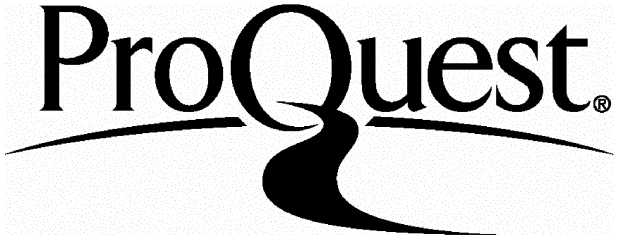

DEDICATION

To my large family.

ACKNOWLEGMENTS

I would like to express my regards and my gratitude to my supervisor, Professor Gregory Gregoriadis, whose help and advice were precious and valuable at all time during my studies and work within his laboratory and team. I am also grateful for the opportunity he gave me to evolve freely and confidently in a scientifically inspired environment gaining at the same time from the benefit of his experience and knowledge. His support, both professional and financial, made it possible to get to the accomplishment of the present thesis work.

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ABSTRACT

The aim of this work was to obtain a better insight into the requirement for size reduction for liposomal formulations, their stability in terms of retention of encapsulated compounds and size conservation upon drying and rehydration.
Chapter three is devoted to the investigation of the microfluidisation of liposomes according to planned experiments. This made possible the quantification of the effect of some specific parameters, the prediction and the optimization of the outcome of microfluidisation (retention of the encapsulated compound upon optimal size reduction). The size reduction was very substantial but always correlated with a loss of the encapsulated compound. Problems associated with liposomal suspensions remained unsolved.
In chapter four the spray-drying of liposomes was investigated aiming at providing some solutions with a view to improve the liposomal stability. The morphology of the dried product (consisting of mixtures of liposomes and carbohydrate) were studied along with any occurring oxidative damage. The nature and the amount of carbohydrate used were varied in order to study their effect on the liposome characteristics on rehydration. Liposomal size reduction prior to spray-drying was found to be a key factor in improving the retention of the encapsulated compound upon rehydration. In chapter five the release of the encapsulated drug from spray-dried liposomes upon incubation with lung surfactant and Triton X-100 is studied. The emphasis was laid on the effect of the temperature of incubation as well as on the liposomal size reduction. Liposomes made out of lipids exhibiting a high Tc exhibited a better stability and a slower release of the marker.The size reduction of liposomes prior to drying appeared to be a decisive factor in minimising drug leakage upon drying and rehydration. Based on these finding, a novel method (Chapter 6) was developed in which a substantial amount of drug could be loaded in relatively small liposomes upon drying and rehydration. Defined amounts of sucrose present with the drug to be encapsulated mixed with the small unilamellar liposomes (SUV) followed by freeze-drying allows the control of the fusion/aggregation processes and the production of small drug-loaded liposomes on subsequent rehydration. The product obtained is dry and it can be stored as such, thus avoiding problems of drug leakage, size growth and lipid degradation. The product can be reconstituted easily, just before administration and does not require any further treatment for size reduction. Parameters such as the molarity of the sucrose solution, the lipid composition and the temperature of rehydration were studied. A comparison of the results obtained by this method and of those obtained when the extrusion technique was employed was carried out. Finally, the overall results obtained in this thesis were discussed in the final chapter (chapter 7). It contains some suggestions and remarks concerning future work.

ABBREVIATIONS IN THIS THESIS

BSA	Bovine serum albumin
CF	Carboxyfluorescein
CHEMS	Cholesterol hemisuccinate
CHOL	Cholesterol
DMPC	Dimyristoyl phosphatidylcholine
DMSO	Dimethyl sulphoxide
DOPE	Dioleoyl phosphatidylethanolamine
DPPC	Dipalmitoyl phosphatidylcholine
DPPG	Dipalmitoyl phosphatidylcholine
DRV	Dehydration-Rehydrarion Vesicles
MDSC	Modulated differential scanning calorimetry
DSPC	Distearoyl phosphatidylcholine
DSPE-PEG	Polyethylene glycol conjugated to disteraoyl phosphatidylethanolamine
DTNB	Dithio nitrobenzoic acid
hr EGF	Human recombinant epidermal growth factor
EPC	Egg phosphatidylcholine
FITC	Fluorescein isothiocyanate
HDL	High density lipoprotein
IgG	Immunoglobulin
LUV	Large unilamellar vesicles
L.S	Lung surfactant

MDSC	Modulated differential scanning calorimetry
MLV	Multilamellar vesicles
mM	Millimolar
MPS	Mononuclear phagocyte system
Nl/h	Normal litre per hour
PBS	Phosphate buffer saline
PCS	Photo correlation spectroscopy
PDI	Polydispersity index
PE	Phosphatidylethanolamine
PEG	Poly ethylene glycol
PSI	lb/in^2
RES	reticuloendothelial system
REV	Reverse phase evaporation
RT	Room temperature
SEM	Scanning electron microscopy
S-PC	Soya phosphatidilcholine
SPD	Spray-drying
SUV	Small unilamellar vesicles
Tc	Bilayer phase transition temperature
TCA	Trichloro-acetic acid
Tg	Glass transition temperature
Tg'	Glass temperature of the cooled suspension

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Chapter one

General introduction

1.1 Liposomes: An overview of structure and applications

Liposomes are microparticulate or colloidal carriers made of organized lipid molecules. They form spontaneously when certain kinds of lipids are hydrated in aqueous medium at a temperature higher than that of their gel/liquid crystalline transition temperature (Tc) (Lasic, 1988). Liposomes are distinguished from other colloidal carriers by the fact that the hydrated lipids forming them are arranged in a lamellar structure (Fig. 1.1). As a rule, the factor governing the behaviour of a molecule when it is dispersed in a medium is its polarity. Being a polar solvent, water will dissolve polar molecules; a non-polar organic solvent will dissolve non-polar and hydrophobic molecules. When polar and non-polar moieties exist on the same molecule, the latter is said to be amphiphilic and its characteristics of dispersion is more complex. The amphiphile molecules are brought into ordered structures where the hydrophobic entities are in contact with one another while the polar groups are organized between themselves in the continuous water environment. The capability of the adjacent amphiphile molecules to align with each other will determine the bilayer or the non-bilayer structure. Polymorphism describes the variety of these structure and phases that can undergo conversions when a particular amphiphile migrates or changes its shape. These changes will be responsible for the transitions from bilayer to non-bilayer structures leading to a decrease of the barrier properties of the membrane.

Liposome formed by the hydration of a dried lipid film with an aqueous phase consist of concentric layers of phospholipid membranes alternating with an aqueous phase in which water soluble molecules are dissolved. The phospholipid bilayer incorporates lipophilic molecules. Other non-polar compounds can also be incorporated at the bilayer interface (Fig.1.1). These lipophilic substances are dissolved with the lipids in an organic solvent. The extent of incorporation of solutes can be expressed as encapsulation efficiency. It is a percentage of the amount of the starting solute, which has been encapsulated.

During their preparation, liposomes can be given, when being prepared, different physical structures and surface properties using different lipid compositions. This enables the tailoring of their properties for different applications. Their versatility and simplicity of preparation has given liposomes superiority over other chemical systems used as drug carriers.

Liposomes resulting from the hydration of a lipid film are known as multilamellar vesicles (MLV) (Fig.1.1). Their size is not homogeneous and on average, it can usually be in the region of 0.5-10 μm. The film hydration method for MLV preparation is simple. One of its major disadvantages is its poor encapsulation efficiency of water soluble compounds, the internal volume being largely occupied by the lamellae. Besides, because of their large size they are rapidly cleared by the reticuloendothelial system (RES) when injected intravenously (Juliano and Stamp, 1975). In certain circumstances, individual lamellae are able to break to form large unilamellar vesicles LUV (Fig.1.1). In the case where the lipids contain a negatively charged amphiphile, repulsive forces between adjacent lamellae induce the formation of LUV liposomes. LUV can also form when the lipid is allowed to hydrate spontaneously (Hope *et al.*, 1986).

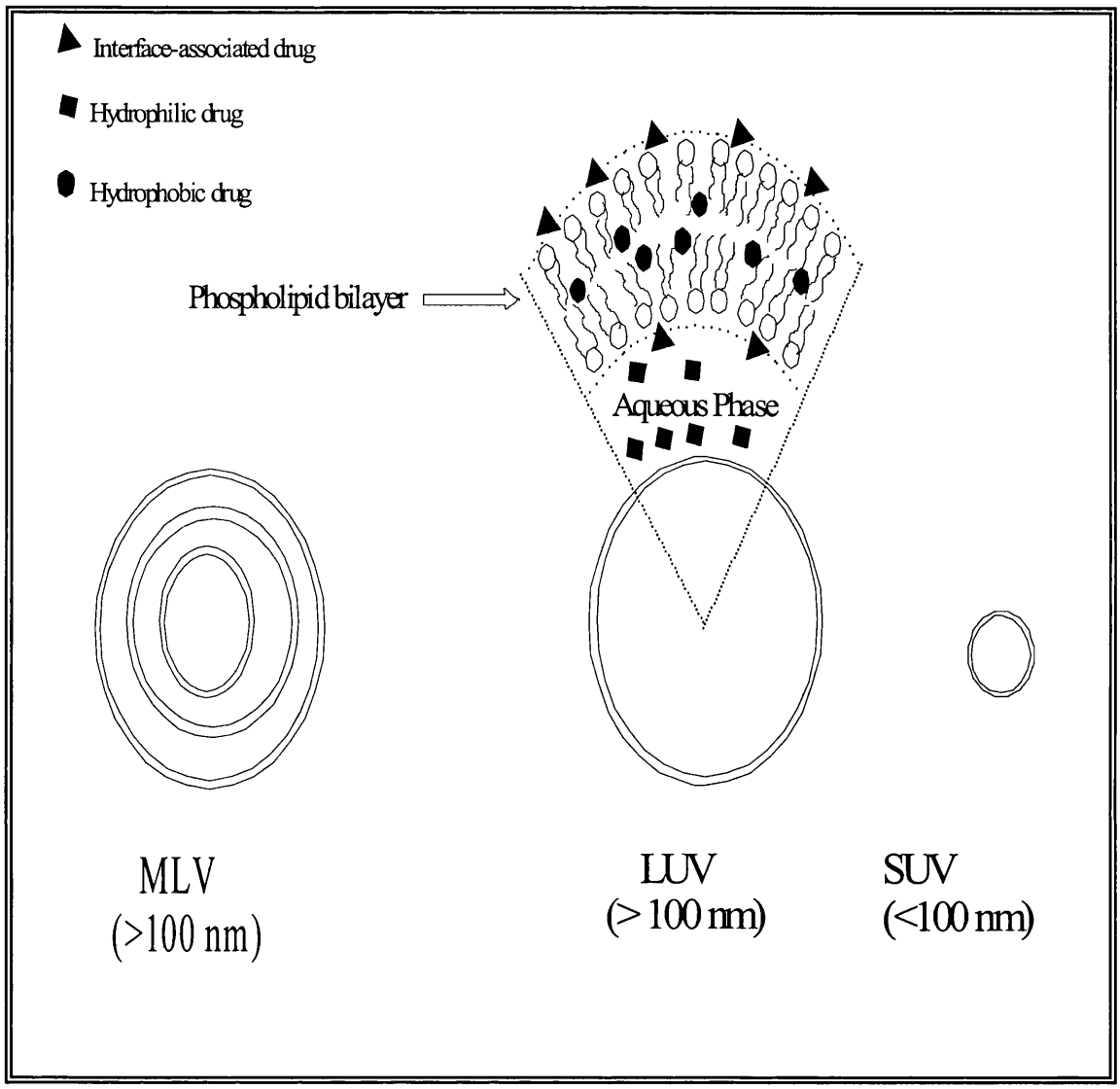

Fig.1.1 Types of liposomes according to the size and number of lamellae

LUVs exhibit an increased permeability to small solutes when compared to MLV s because of their single lamellae. The maximum curvature that the lipid bilayer can achieve fixes the lower limit of the liposome size. These small liposomes are known as small unilamellar vesicles (SUV). Their small size make them circulate in the blood longer when administred (Juliano and Stamp, 1975).

1.2 Methods of liposome preparation

Liposomes of different sizes and characteristics usually require different methods of preparation. In the early stages of liposome development, the interest was to achieve a good entrapment efficiency of solutes. Further developments brought up the need of commercialisation with a new emphasis laid on the formulation stability, reproducibility from batch to batch, scaling up of the process and its validation (Gregoriadis, 1995a). Several methods were developed aiming at fulfilling these requirements. These will be discussed below.

1.2.1 MLV preparation

The simplest method for MLV preparation is the thin film hydration procedure. It concerns liposomes prepared from mixtures of lipids that have to be thoroughly mixed.

The mixtures of lipids are dissolved in an organic solvent or in a mixture of organic solvent (generally chloroform:methanol (2:1 volume)). A rotary evaporator is then used to remove the solvent and to generate the lipid film that is finally hydrated using water or even a solution of the compound to be encapsulated (Hope *et al*, 1986).
One of the major disadvantages of this method is its poor encapsulation yield of water-soluble compounds (Hope *et al*, 1986). The morphology of the MLV s is largely responsible for this, the lamellae occupying a major proportion of the internal volume. One way of overcoming this problem is to include a charged lipid (10 to 20 % molar ratio), causing an electrostatic repulsion and increasing the inter-lamellar

space (Juliano and Stamp, 1975). This increase in the internal volume is used to entrap higher amount of solute. However this approach is only valid when the solute is neutral. When the solute is charged, it may associate with the external surface and depending on its net charge, the encapsulation efficiency will be higher or lower. When the MLVs aimed at are composed of a single phospholipid, this can be directly suspended in an aqueous solution at a temperature higher than the phase transition temperature (Tc) of the lipid. It is preferable that lipids are finely divided and present the highest possible surface area. The hydration of lipids is enhanced with mechanical or magnetic stirring. Lipid oxidation is avoided when the procedure is performed under an inert atmosphere using nitrogen or argon. Liposomes are then formed and the solute is entrapped within the inter-lamellar spaces. The non-entrapped solute can be removed by centrifugation, dialysis or molecular sieve chromatography.

1.2.2 Dehydration/Rehydration Vesicles (DRV)

This is a simple method for high yield drug entrapment in liposomes developed by Kirby and Gregoriadis, (1984). This method, called "Dehydration-Rehydration Vesicles" or DRV, is simple to use, employs mild conditions and shows efficient entrapment of a wide range of materials, and particularly of sensitive biological molecules such as proteins and nucleic acids (Gregoriadis *et al.,* 1998a). In the DRV method, the idea is to bring into contact the solute intended for encapsulation with the lipids so that the lamellae will form around the solute giving liposomes with high encapsulation efficiencies.

An MLV suspension of liposomes is prepared in distilled water. It is then subjected to sonication or any other method of size reduction in order to produce SUVs. This conversion increases the surface area of the lipids allowing a direct contact with the added solute. The water is then removed from the SUV/drug mixture by freeze-drying. A dry product is then obtained where the dry components are in intimate contact (Fig 1.2).

A controlled rehydration step is carried out. A small amount of water is added in order to start the SUV fusion around the solute, forming larger liposomes and allowing a high encapsulation yield. The amount of water added is critical and has to be kept to the minimum possible. For example, a liposome preparation of a total of 37 mg lipids will need about 100 µl of water to be rehydrated.

The preparation is finally diluted in isotonic buffer such as phosphate buffered saline (PBS) and the non-encapsulated material removed either by dialysis or by centrifugation.

Depending on the conditions used to prepare DRV liposomes, they may be oligo- or multilamellar. For some applications, their large size and heterogeneous distribution need further treatment in order to reduce the size, the lamellarity and the dispersity. This will inevitably lead to leakage and to the redistribution of the entrapped material between the liposomes and the external aqueous phase. It can be achieved by using a bath sonication, a high pressure homogenization (fully described later) such as microfluidization or membrane extrusion (Gregoriadis *et al.,* 1990, Brandl *et al.,* 1990). Chapter 3 of this thesis is dedicated to this approach.

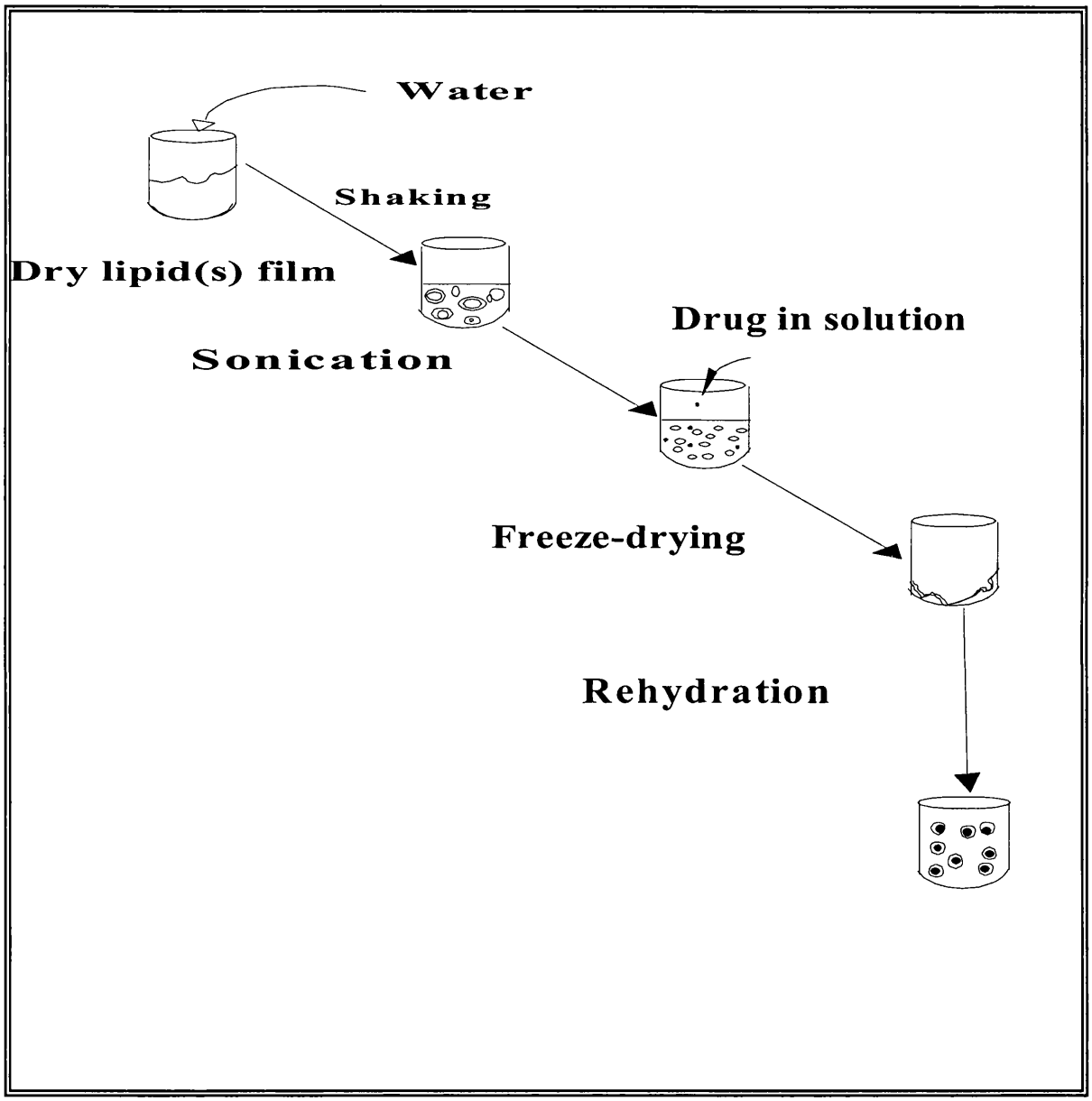

Fig 1.2: Diagram of preparation of dehydration-rehydration liposomes. The material to be entrapped is added to the SUV s suspension and is freeze-dried. A controlled rehydration step will induce the fusion of the SUVs giving liposomes with a high yield entrapment.

1.2.3 Solvent injection methods

1.2.3.1 Hydration of lipids solubilized in organic solvents

Lipids or mixtures of lipids are dissolved in an organic solvent and injected in an aqueous solution to produce liposomes (Kriftner, 1992; Isele *et al.*, 1994). The water-miscible organic solvent can be removed at the end of the procedure by dialysis or filtration.

Water immiscible solvents such as ether, chloroform, dichloromethane can also be used They are removed by evaporation during the process of liposome preparation One of the major drawbacks of this method is the poor encapsulation efficiency obtained.

1.2.3.2 Reverse-phase evaporation vesicles (REV)

An aqueous phase of the solute to be encapsulated is emulsified in a lipid solution (mixture of chloroform: ether 50% v/v) using a bath sonication to form a water in oil emulsion. The solvent is then removed by rotary evaporation. A monolayer of lipid forms around microdroplets of the solute giving inverted micelles. A proportion of these inverted micelles will collapse in a way that their aqueous content will form a continuous phase while the remaining lipids start forming the outer leaflet of this new vesicular structure.

In the case where a single lipid is used, the vesicles will be uni- or oligolamellar with average diameters in the range of 0.05 - 0.5 μm. High encapsulation efficiencies (up to 65%) can be achieved (Szoka and Papahadjopoulos, 1978). One of the limitations is the need to use volatile organic solvents and exposing labile molecules to these solvents and to sonication.

Some derivations of this method will be discussed below.

1.2.3.2a First variant of the REV to produce stable plurilamellar vesicles

The same strategy as for the DRV procedure is used aiming at achieving high level of encapsulation of drugs by including them before fixing the final lamellar configuration (Gruner *et al.*, 1985). The aqueous phase containing the drug is emulsified in a chloroform: ether (50% v/v) solution of lipids using a bath sonication. The solvent is then evaporated from the oil-in-water emulsion by a stream of nitrogen. The differences from the original method are the continuous use of sonication and the high amount of lipids involved. However, the use of organic solvents and sonication are two serious disadvantages, especially when the sealing of the vials containing the final product is intended.

1.2.3.2.b Second variant of the REV to produce multilamellar vesicles (MLVs)

In this variant of the REV method, a higher amount of lipids and a lower volume of aqueous phase are used in order to encourage the formation of multilamellar structures (Pidgeon *et al.,* 1986). The mixture of chloroform: ether (1:10% v/v) solubilising the lipids is removed by rotary evaporation. The gel obtained is removed, vortexed and then re-treated by rotary evaporation to remove any trace of the remaining solvent. These MLV liposomes show typical values of 80-90% of encapsulation. The use of organic solvent still remains a major disadvantage.

1.2.4 Double-emulsion evaporation

Here, a multiple water-in-oil in water (w/o/w) type emulsion is needed.

The lipids are dissolved in a solution of chloroform/ether in which an aqueous solution of the drug is dispersed. The water-in-oil (w/o/) emulsion is dispersed again into a second aqueous phase to form a multiple emulsion.

Each droplet of the organic phase will contain a single microdroplet of the drug solution. The preparation obtained is in the form of an aqueous suspension of LUV (Kim and Martin, 1981). Two of the limitations of the method are the use of specialised lipid components and the complexity of the procedure itself.

1.2.4.1 Variant of the double emulsion evaporation to produce multivesicular liposomes

The method also involves the use of multiple w/o/w emulsions. The same procedure as in the mother method is carried out except that the compositions and the conditions are such that multiple droplets of the initial drug solution will be dispersed in each droplet of the dispersed organic phase (Kim *et al.,* 1983). The solvents are removed under a stream of nitrogen and multicompartment vesicles are obtained. Encapsulation efficiencies of up to 90 % can be achieved when using this method.
The limitations are the requirements of specialized lipids and the use of complex conditions and of volatile organic solvents.

1.2.5 Solvent infusion

This method consists of slowly infusing an organic solution of lipids into an aqueous phase maintained at a temperature above the boiling point of the solvent that then forms bubbles. The solvent can be diethyl ether, petroleum ether, ethyl methyl ether or dichlorofluoromethane. The vapor/water interface is the site of the deposition of the multilayered lipids while the solvent is evaporating (Deamer and Bangham, 1976). The morphology of the formed liposomes is uni- and oligolamellar. Entrapments of up to 46% can be achieved using concentrated solution of lipids. The average diameter of the obtained vesicle population is in the range of 100-400 nm. The use of

organic solvent still constitutes a major limitation because of the possible damage to labile compounds destined for encapsulation.

1.2.6 Method based on the removal of lipid solubilising agents

This method is based on the solubilisation of lipids by the aqueous solution of a detergent to form mixed micelles of lipid and detergent (Allen, 1984).

Uni-or oligolamellar vesicles ranging in diameter from 80 nm up to several microns are obtained upon removal of the detergent by diafiltration, dialysis or by gel chromatography. The Lipoprep is an apparatus that relies on a dialysis cell to remove the detergent (Weder and Zumbuehl, 1984) and produce liposomes at a laboratory scale. This technique allows the control of the liposomal size and size homogeneity. The encapsulation efficiency achieved with this method is low compared to other methods of LUV preparation. Another disadvantage is the residual detergent that increases liposomes permeability. Damage can also occur on the labile material being encapsulated.

1.2.7 Preparation of small unilamellar vesicles (SUV)

Probe sonication is a non gentle method by which liposome dispersions with an average size as low as 70 nm can be produced. Generally, MLV s prepared by the film hydration method are maintained in an inert atmosphere at a low temperature by using a cooling bath to evacuate the heat produced.

Sonication using a titanium probe of a fixed diameter is carried out for a certain time of bursts at a peak amplitude. The time and the peak amplitude are fixed according to the amount of lipid used in the liposome preparation This procedure results in a wide liposomes size distribution (bimodal), the diameter ranging from 25-150 nm.

Prolonged sonication will not improve size reduction (Talsma *et al*, 1989). There is an optimum time and amplitude for a fixed amount and composition of lipids. A disadvantage of this method is the oxidative damage that can occur to the lipids, especially when the heat produced is not evacuated with efficacy.

Another method to produce SUV is the use of high pressure homogenisation devices which include the French Pressure Cell (Hamilton and Guo, 1984), the microfluidizer (Microfluidics, Newton, MA, USA), (Talsma *et al*, 1989), the Gaulin Micron ® lab. 40 (Brandl *et al.*, 1990), the Nanojet (Nanojet Engineering, Dortmund, Germany), (Purman *et al.*, 1993).

The Microfluidizer is a special homogeniser that uses cavitational phenomena responsible for very high shear forces resulting in a pronounced size reduction. Liposomes are recirculated through an interaction chamber via a reservoir for a number of cycles. A full chapter of this thesis **(Chapter 3)** is dedicated to size reduction of preformed DRV liposomes using the microfluidization technique.

A last method to prepare SUV liposomes is extrusion through polycarbonate membranes with a defined pore size (Olson *et al.,* 1979). Polycarbonate filters with pore sizes ranging from 5 to 0.05 μm are available in the market. The obtained vesicle size and lamellarity are correlated with the pore size of the membranes.

1.3 Large scale preparation of liposomes

Numerous techniques have been developed for the preparation of liposomes on a laboratory scale. It is established that the large scale production of liposomes presents

specific problems (Martin, 1990; Kriftner, 1992; Weder and Zumbuehl, 1984; Schwenderer, 1986; Amselem *et al.,* 1990).

In the process of liposome preparation the following steps are encountered: hydration of the lipids for vesicle formation, solute entrapment, and sizing of the resulting vesicles. In some situations some of these steps are interdependent.

1.3.1 Lipids hydration starting from organic solvent solution of lipids

An approach for the preparation of liposomes is the injection of a lipid organic solution, miscible with water (e.g.: methanol, ethanol and DMSO). The dilution of the organic solvent induces vesicle formation. Operational parameters such as the stirring rate, the lipid charge and the ionic strength will affect the size and the size distribution of the vesicles obtained. This procedure was successfully scaled up and used for the production of liposomes encapsulating ecanozol, which is a lipid soluble antifungal drug (Kriftner, 1992).

Residual traces of the ethanol used remain in the liposomes without influencing the bilayer stability. Furthermore ethanol is a pharmaceutically acceptable solvent which can help to maintain the sterility of a formulation.

1.3.2 Detergent removal methods

Liposome preparations can also be achieved by dispersing the lipids and the detergents in an aqueous solution of the drug. The detergent is then removed by ultracentifugation, dialysis or gel filtration.

This method has been successfully scaled up, particularly for drugs that associate with the bilayer. Batches of liposome suspension incorporating cytostatic drugs of up to 5

liters in volume were prepared for use in clinical trials (Schwenderer, 1986). The incorporation efficiencies of these lipophilic drugs were in the range of 85 to 100 %. Concern is expressed about the safety of the residual detergent in liposomes formulations prepared by this method (Allen, 1984).

1.3.3 Solute entrapment

The high encapsulation efficiency of a drug into liposomes and the stability of the liposomes during storage are a prerequisite for a successful commercialisation.

This is easily achievable for drugs that associate with the bilayers. Water soluble drugs present more difficulties and a successful preparation will depend on the properties of the drug being encapsulated. Mechanical methods described earlier are adequate for liposome preparation when low encapsulation efficiencies are acceptable.

Interaction of water-soluble drugs with the bilayer enhances the encapsulation efficiency. Strategies to improve the latter for water-soluble drugs have been developed. Lipophilic chains attached to the drug molecule increases its lipophilicity enhancing the interaction between the drug and the bilayer (Rahman, 1986). Altering the bilayer properties such as the surface charge can enhance the interaction between the drug and the bilayer. (Yagi *et al.,* 1991; Kurono *et al.,* 1992; Kikuchi *et al.,* 1992). Otherwise, liposome production procedures allowing high encapsulation efficiencies must be used.

Liposome preparation methods using volatile organic solvents such as the reverse phase evaporation or one of its derivatives might be considered when the use of organic solvent is acceptable.

Another approach is to use the DRV method sizing the liposomes afterwards, by using extrusion or microfluidization techniques (Gregoriadis and Florence, 1993).

In a two-chamber aqueous system separated by a membrane, solute displacement will occur towards the low pH side under dynamic equilibrium conditions. This can be exploited for the loading of solutes into preformed liposomes. (Deamer *et al,* 1972). This method is classified as an active loading method (Madden, 1990) by which high encapsulation efficiencies can be achieved. For example the transmembrane pH-gradient causes accumulation of doxorubicin at the low pH side. In the unprotonated form, doxorubicin can diffuse through the bilayer membrane of the liposome. Doxorubicin is then protonated at low pH lowering in this way the concentration of the unprotonated form and promoting its diffusion to the low-pH side of the bilayer.

Another analogous method consists of creating such a pH-gradient using ammonium sulfate.

The diffusion of NH_3 on the outside of the liposomes, e.g. upon dilution, creates a pH-gradient by which compounds can be entrapped (Bally *et al*, 1988; Barenholz, 1989; Haran *et al*, 1993). Furthermore, sulfate anions present a low bilayer permeability which stabilises the ammonium sulfate gradient, enhancing the accumulation of the encapsulated drug and improving its retention due to the formation of gel-like doxorubicin sulfate aggregates (Lasic *et al,* 1995). The antibiotic ciprofloxacin when loaded into liposomes did not present the same gel-like structure but it rather crystallised within the liposomes. These liposomes exhibited more leakage of the encapsulated ciprofloxacin. This method has the advantage of high encapsulation efficiency and reduced leakage of the encapsulated compound when it precipitates as a gel within the liposome. A third advantage is that the loading of the drug can be performed at the bed side optimising the retention of the drug and limiting its

chemical degradation during storage. One of the limitations is that the method is not applicable to many drugs.

1.3.4 Size reduction of liposomes

High pressure homogenisation techniques are widely applied in the food and the cosmetic industry at a large scale.

The reproducibility is achievable even when large different batches are used. The Gaulin Micron range homogeniser is claimed to be able to transform highly concentrated lipid suspension prepared out of powder lipids, directly into small unilamellar vesicles when operating continuously (Schneider *et al,* 1994). This suggests that the equipment is well adapted to scale up (Amselem *et al*, 1990)

Generally, the average size obtained depends on the instrument design and on the setting of the operational parameters such as the number of cycles through the interaction zone, the temperature of the homogenization medium and its concentration. The pressure of the propelling gas and the lipid concentration are also determinant factors.

A particular problem that may arise when processing liposomes using these techniques is the clogging of the interaction area. By bypassing it with an evacuation system activated when the working pressure goes over the maximum set pressure (set for safety reasons) the processed sample is then evacuated by the bypass. Another problem is the erosion of metallic particles from the components of these instruments. The use of appropriate filters may help to solve the problem.

1.4 Liposomal stability

Liposomes as drug carrier systems require chemical and physical stability. The

phospholipids constituting the liposomes in suspension are subjected to hydrolysis (Zuidam and Crommelin, 1995). Some leakage of the encapsulated drug can occur during storage as a result of the difference in concentration between the inside and the outside of the bilayer membrane.

The fusion and aggregation of liposomes induce changes in vesicle size. Freeze-drying and spray-drying were suggested as alternative methods to produce a stabled-up dry liposome product (Hauser *et al*, 1987; Kikuchi *et al*, 1991; Crowe and Crowe, 1993). The cake or the powder obtained can be rehydrated and reconstituted. However the drying/rehydration cycle can be damaging in itself. The original properties of the formulation will be affected as changes in vesicle size and leakage of the encapsulated drug occur. A liposome suspension subjected to a drying and rehydration step without additives will undergo a process of fusion and aggregation leading to a large liposome size as well as some leakage of the encapsulated water-soluble compound. Adding carbohydrates can diminish this damage (Crowe and Crowe, 1988a).

The carbohydrates will act as spacers between the vesicles preventing fusion and will interact with the phospholipid head group: the hydroxyl groups of the sugars form hydrogen bonds with the phosphate group of the phospholipid in the dry state and thus replace water (water substitution theory) (Crowe and Crowe, 1993). Drying bilayers will result in the removal of the water molecules between the phospholipid head groups. The interaction between the acyl chains will then be stronger and the energy needed to bring the chains from the ordered gel phase to the liquid crystalline phase will be higher. These phenomena can be inhibited by the use of carbohydrates in sufficient amounts (Crowe and Crowe, 1988a; Crowe *et al*, 1988) allowing the stabilization of liposomes in terms of size conservation and retention of the water-soluble compounds.

1.4.1 Role of the bilayer fluidity

The liposome formulation of various drugs can be optimised in terms of drug content, stability, desirable biodistribution patterns and cellular uptake by altering their physicochemical parameters such as the lipid fluidity.

The phase transition temperature (Tc) characterises the bilayer fluidity. Lipids exist in different physical states above and below Tc. They are in a rigid, well-ordered arrangement (solid, gel-like phase) below the Tc, and in a liquid–crystalline (fluid) phase above the Tc (Marsh, 1991).

The fluidity of liposome bilayers can be altered by using phospholipids with a different Tc which can vary from $-10\ ^0$C to $60\ ^0$C depending upon the length and the nature (saturated or unsaturated) of the fatty acid chains.

The presence of high Tc lipids (Tc > 37^0 C) makes the liposome bilayer membrane less fluid and subsequently less leaky at the physiological temperature. Liposomes composed of low Tc lipids (TC <37^0 C) are more susceptible to leakage of drugs encapsulated in the aqueous phase at physiological temperatures. There is a correlation between liposomal stability and clearance from blood circulation. Indeed, the facility by which the HDL will remove the phospholipid molecules from the bilayer is dependant on the bilayer packing and rigidity (Gregoriadis and Senior, 1980). The HDL insert more in loose bilayers then packed ones. These HDL will create gaps on the bilayer and therefore allowing the opsonins to associate with the vesicles. Extensive opsonins adsorption on the vesicles lead to important uptake by the reticulo-endothelial system (RES) (Gregoriadis, 1994).

Liposomes without or with low amount of cholesterol exhibit a less condensed bilayer membrane (Kirby and Gregoriadis, 1981). Upon contact with plasma these liposomes

lose some of their phospholipid to high density lipoproteins (HDL). This is associated with the liberation of the entrapped agent. (Kirby et al, 1980). Upon incorporation of increasing amount of cholesterol, leakage of solute is reduced. This is presumably the result of the condensing effect of the sterol of the acyl chain of the phospholipids, which reduces their loss to HDL and consequently the size of formed pore (Kirby and Gregoriadis, 1981).

1.5 Role of amphiphiles with hydrophilic head groups included in the bilayer

A rapid clearance of the liposomes from the blood circulation by the RES was a major obstacle to the wider use of liposomes as drug delivery system by the parenteral route. The inclusion of a small fraction (5-10 % molar) of material containing hydrophilic groups can overcome this obstacle. Materials such as monosialoganglioside (GM1) when included in the liposome bilayer substantially reduced the clearance rate (Gregoriadis, 1998; Allen *et al.,* 1985; Allen and Chonn 1987; Chonn and Cullis, 1992).

The same effect could be achieved by incorporating lipids conjugated to polyethylene glycol in the bilayer (Kilbanov *et al,* 1991; Lasic *et al*, 1991; Papahadjopoulos *et al*, 1991; Senior *et al*, 1991). The liposomes obtained are said to be sterically–stabilised and exhibit an extended circulation half–life compared to the conventional ones (liposomes without hydrophilic surface coating).

By reducing their rate of clearance, liposomes could be used as circulating drug reservoirs improving the pharmacokinetic control of agents and increasing the stability of sensitive substances in the blood. This is accomplished by the dual control of the rate of drug release from the liposomes acting reservoirs and their clearance

rate. This improvement in circulation time still requires liposomal size reduction down to around 100nm

1.6 Targeted liposomes

The control of biodistribution of the administered carrier will enable to direct the effects of the associated drug on a targeted site maximizing the therapeutic index. Liposomes by their structural versatility play an important role in the targeting of drugs.

With unmodified liposomes we can achieve what is known as passive targeting. Liposomes exhibiting large size (generally MLVs) are cleared by the RES. This was exploited to deliver therapeutic compounds to the RES cells such as enzymes (Belchetz *et al.,* 1977) and antimicrobial agents.

Small liposomes are taken up less rapidly than large ones (Nagayasu *et al*, 1995). They are able to pass through the fenestrae of the sinusoids into the parenchymal cells of the liver allowing a similar exploitation for passive targeting.

This approach appears to be limited and a higher level of control can be achieved by attaching or associating recognizable molecules to the liposome bilayer.

Important requirements for this active targeting have been subject to many studies (Crommelin and Storm, 1998; Gordon, 1998; Zalipsky *et al*, 1998).

In summary:

i. The characteristics of the target cell have to be clearly identified so that other cells will not interfere with the liposomes on their way to the target site,
ii. Targeting ligands must exhibit some exclusive interaction with the targeted polyvalent ligand ,

iii. Ligand attachement to the liposome must generate a stable system able to interact with a polyvalent ligand in the vicinity of the cell surface,

iv. The ligand-bearing vesicle must be stable when administered and circulate for enough time optimizing the interaction with the targeted cell population,

v. Ligand attachement or association must increase the probability of the drug entering the cell interior by membrane disruption or cell-mediated endocytosis.

Different strategies have been adopted to associate the targeting entity and the liposomes. In an early study (Gregoriadis and Neerrunjuin, 1975), a relatively simple approach was used. It consisted of sonicating the liposomes mixed with the ligand allowing the insertion of the hydrophobic end of the protein molecule. Another group of workers used the same approach to prepare a ligand-bearing vesicle and successfully tested their targeting properties (Huang *et al,* 1979). Chemical coupling methods are widely used to attach a targeting moiety to an amphiphilic molecule that is then incorporated into the liposomes bilayers or to an amphiphilic molecule already present in the liposome membrane (Torchilin, *et al,* 1979; Heath *et al*, 1980; Huang *et al,* 1980). Development of the ligand-bearing liposome for targeting encountered many problems and obstacles such as damage to the ligand during the conjugation process which affected its activity. For example, obtaining IgG fragments during the conjugation step will probably result in reduced affinity. Even when the antibody is not damaged, a rapid clearance of the system was observed.

Upon injection, the interaction with blood constituents may result in the detachment of the ligand or in some drug leakage. In some cases the improved stability of the

system might delay the release of the encapsulated drug when the liposome reaches the vicinity of the targeted cell.

1.7 Polymerised liposomes

The principle consists of preparing vesicles out of polymerisable amphiphiles that are then polymerized using conventional methods. The liposomes obtained exhibit high stability and at the same time allow efficient drug release (Bonte *et al*, 1995).

These polymerized liposomes were conferred good stability in the presence of blood constituents and are seen as an alternative to the unstable liposomes made out of natural phospholipids (Freeman *et al*, 1987).

By using mixtures of polymerisable and non-polymerisable lipids, gaps can be obtained during the polymerization process allowing the required drug release.

A potential application is their use as an artificial blood substitute. Synthetic haemoglobin was incorporated by copolymerisation into the bilayer of imadazole dienoylphospholipid liposomes (Rudolph *et al*, 1991). The system was found to be stable for several months and exhibited the same transport and binding properties of oxygen as natural heamoglobin.

However many problems remain unsolved when developing polymerized liposomes such as remaining toxic residues and inert fragments of polymers which accumulate in the body.

1.8 Temperature-sensitive liposomes

At the phase transition temperature of the membrane lipids, liposomes exhibit more leakage. This leakage is enhanced by the presence of plasma proteins which bind to

the membrane and destabilize it. These liposomes are designed to be stable at the biological temperature (37°C), but will be destabilised as they cross an area of the body where the temperature is raised for example externally (Rensen *et al*, 1997).

The release of a proportion of the drug around the phase transition temperature from liposomes in the absence of proteins is due to the increased permeability at the Tc of an intact membrane. Furthermore, in the presence of protein (HDL), a proportion of the vesicles are disturbed and release all their encapsulated drug while another proportion remains intact.

This is exploited to design liposomes containing cytotoxic drugs (Ishida *et al*, 2000) that are then injected in the systemic circulation. By applying localised heat externally to the target area, the liposomes are forced to release their content in the heated area.

1.9 pH-sensitive liposomes

A similar approach to the one previously described is adopted for the construction of liposomes sensitive to pH.

In this approach the non lamellar amphiphile PE is used with another measurable amphiphile that will stabilise the bilayer as a function of pH. Such amphiphiles include fatty acids, palmitoyl homocysteine, cholesterol hemisuccinate (CHEMS) and N-succinyl PE. The mixing of these compounds with PE in a proportion of 10-30 % generates liposomes which are stable at pH 7 but that undergo fusion when the pH is lowered to 5 (Yatvin *et al*, 1980).

This type of liposomes can be applied in the delivery of bioactive materials to cells (Kono *et al*, 1997). During the intracellular processing, liposomes enter an endosome and become fusogenic upon exposure to the intravesicular pH of 5. They may be able

to fuse with the wall of the endosome and release their content into the cytoplasm of the cell.

1.10 Cationic liposomes

The positive charge of the cationic lipid is used to neutralize the polyanionic nucleic acid chain to produce a dense microstructure when cationic SUVs are mixed with DNA (Gregoriadis *et al,* 1998b). The particles obtained have a positive charge allowing the interaction with the negatively charged cell membranes.

A variety of cationic lipids for transfection have been synthesised aiming at improving the efficiency of transfection, increasing their biodegradability and stability and decreasing their toxicity (Harries *et al*, 1998).

The majority of these cationic lipids have been used with a neutral lipid which has frequently been dioleyl phosphatidyl ethanolamine (DOPE). The incorporation of cholesterol also proved to be useful.

The neutral lipid entering in the liposome formulation was able to promote the formation of the fusogenic hexagonal H_{II} phase allowing endosomal disruption and release (as for pH-sensitive liposomes) into the cell cytoplasm.

1.11 Outline of work in thesis

Water-soluble drugs can diffuse out of the liposomes. Liposome dispersions are subject to fusion and aggregation, especially in the case of formulations with neutral lipid bilayers. Moreover, phospholipids can undergo oxidation and hydrolysis depending on the pH of the suspension and the storage temperature.

Because of the importance of the vesicle size on the stability and on the in vivo clearance of liposomes form the blood, we investigated in **Chapter 3** the microfluidization (high-pressure homogenization using circulation of fluids in microchannels) of liposomes. This included the entrapment of ^{14}C labelled sucrose in dehydration rehydration vesicles. Microfluidisation was carried out according to a factorial design in order to quantify the effect of the following operational parameters: the pressure of air in the intensifier pump, the number of cycles through the interaction chamber and the presence or absence of non-entrapped material. The establishment of mathematical models enabling the prediction of the vesicle characteristics was studied. Yet, the stability problems associated with liposomal suspensions remained unsolved. Therefore, investigations concerning the production of stabled-up liposomes using the spray-drying technique were carried out in **Chapter 4.** The stability upon rehydration of liposomal formulations was investigated as a function of the lipid composition, the vesicle size and the amount of carbohydrate used for protection and stabilisation.

The release of the encapsulated marker from the spray-dried liposomes upon incubation with lung surfactant and Triton X-100 was studied in **Chapter 5.**

Because of the many steps involved and the problems encountered in maintaining adequate liposomal characteristics (vesicle size and entrapment efficiencies), **Chapter 6** was devoted to the development of a novel method of high yield entrapment of solutes into small liposomes. The spray drying or the freeze-drying techniques were used to produce dry liposomal formulations exhibiting the desired characteristics of vesicle size and retention of solute upon rehydration.

Conclusions and suggestions for future work are presented in **Chapter 7.**

Chapter Two

Materials and methods

2.1 Sources and grades of materials

2.1.1 Phospholipids

Egg and soya phosphatidylcholine (EPC and SPC), distearoyl phosphatydilcholine (DSPC), dimyristoyl phosphatidylcholine (DMPC), dipalmitoyl phosphatydilcholine (DPPC), dipalmitoylphosphatidylglycerol (DPPG), (grade I, 99% pure) were purchased from Lipoid GmbH, Ludwigshafen (Germany). Cholesterol was obtained from Sigma (London). DSPE-PEG (2000) was as well purchased from Lipoid

2.1.2 Glutathione

Reduced glutathione (γ-Glu-Cys-Gly; GSH), 98-100% pure, was purchased from Sigma (Sigma chemical Co. Dorset, UK).

2.1.3 Radioactive sucrose

(U - ^{14}C) sucrose (9.25 MBq) was purchased from Amersham International (Slough. UK). It consists of a sterilised aqueous solution containing 3% ethanol.

2.1.4 Radioactive penicillin

Benzyl (^{14}C) penicillin potassium (1.85 MBq) was purchased from Amersham International.

2.1.5 Fluorescein isothiocyanate (FITC) -albumin and epidermal growth factor

FITC-albumin (98% pure) was purchased from Sigma.

Human recombinant epidermal growth factor hrEGF was a present from Dr Maria Lanio (Centre for Genetic Engineering and Biotechnology, Havana).

2.1.6 Carboxyfluorescein, doxorubicin, daunorubicin and penicillin G

Carboxyfluorescein (CF) (5-Carboxyfluorescein, 99% pure by HPLC), doxorubicin (doxorubicin HCl, 99% pure by HPLC), daunorubicin (HCl 99% pure by HPLC) as well as penicillin G (99 % pure by HPLC) were purchased from Sigma.

2.1.7 Riboflavin

Riboflavin-5'-phosphate sodium salt dihydrate 96% pure was purchased from Lancaster (Morecambe, U.K).

2.1.8 Sucrose, lactose and trehalose

Sucrose, lactose and trehalose (96 % pure) were purchased from Sigma.

2.1.9 Molecular sieve chromatography

Sepharose 4B-CL, Sephadex G25 (fine grade), were purchased from Pharmacia Biotech, Uppsala, Sweden.

2.1.10 Lung surfactant

Lung surfactant Survanta was purchased from Abbot laboratories (Lancaster, UK).

2.1.11 Dithio-bis (2 nitrobenzoic acid) reagent

5.5' Dithio-bis (2 nitrobenzoic acid) (DTNB or Ellmans reagent) was obtained from Sigma.

2.1.12 Reagents for glutathione assay

See methods.

2.1.13 Reagents for lipid extraction and quantification.

See methods.

2.1.14 Reagents for protein iodination using the chloramine-T method

See methods.

2.2 Equipment

2.2.1 Microfluidizer S110

A microfluidizer 110S equiped with a Y (F112) designed interaction chamber was from Microfluidics Corp (Newton, M.A., USA.). It can take up to 15 ml of sample to optimise the product recovery.

The working pressure can vary from 135 to 965 bar. The microfluidizer can be connected to the main supply of air or to an air or nitrogen cylinder. The preparations can be recirculated through the interaction chamber for the desired number of cycles at the desired pressure, (at the inlet of the intensifier pump), ranging from 20 to 80 psi.

2.2.2 Continuously operated extruder (Maximator® HPE 10-250)

An Extruder maximator ® (Model HPE-10-250) was puchased from Dispex, GmbH, Berlin, Germany. The design of this instrument is very similar to that of the

microfluidizer. Instead of a ceramic interaction chamber, the extruder is equiped with a filter holder through which the sample is recirculated. This extruder requires a propellant pressure (compressed air or nitrogen) of about 2 bars. The maximum hydraulic pressure achievable is about 140 bars. Since high working pressures could damage the screen in the high-pressure filter holder due to its design, a safety valve set for 120 bars has been integrated in the holder. The high-pressure filter holder with screw cap serves to accommodate two polycarbonate filter discs (47 mm in diameter) one on top of the other.

2.2.3 Filter holder (operated discontinuously)

A filter holder for discontinuous extrusion was purchased from Fisher Scientific (Loughborough, UK). It is equiped with a 142 mm filter holder base working at a maximum of 6.9 bars and requiring a 47 mm diameter polycarbonate membrane. It is working on the same principle as the continuous extruder and can hold up to 25 ml of preparation.

2.2.4 Polycarbonate membranes

Polycarbonate membranes of pore diameters ranging from 1μm to 0.050μm were purchased from Millipore, (London, UK). At each extrusion step two stacked membranes are used at a time.

2.2.5 Spray dryer

A mini spray-dryer Buchi 191 equipped with a 0.7 mm nozzle and fully automated with digital hardware was purchased from Buchi Laboratories (Flawil, Switzerland). The pressure required for the atomisation air is in the range of 3-8 bars. The optimal

flow rate of the drying air is around 35 m^3 /h. The temperature of the drying air can be set at a maximum of 220 ^{0}C.

2.2.6 Freeze-dryer

A micro moduylo freeze dryerequippedwith avacuumpump Edwards (Crawley, UK) was used to perform the freeze-drying procedures described in this thesis.

2.2.7 Scintillation counter

A scintillation counter Wallac 1409 (Milton Keynes UK) is used to measure the radioactivity of radiolabelled markers. The scintillation fluid "optiphase safe" was purchased from the same company. It is used for aqueous and non-aqueous samples.

2.2.8 Photon correlation spectroscopy

Samples of the liposome dispersions were diluted in doubly distilled water and the Z-average vesicle size and polydispersity index were determined at 25 ^{0}C by photon correlation spectroscopy with a Malvern Autosizer 2C. Larger vesicles (μm range) were sized using a Mastersizer. Both sizers are from Malvern Ltd (Malvern. U.K.)

2.3 Methodology

2.3.1 Buffers

Phosphate-buffered saline (PBS) pH 7.4 :

The phosphate–buffered saline consisted of: 140 mMNaCl, 0.18 mM Na_2HPO_4, 3.2 mM $NaH_2PO_4.2H_2O$, 2.7 mM KCL, the pH was adjusted using a 1M NaOH.

Phosphate-buffer pH 8.0

Di-sodium hydrogen orthophosphate (Na_2 $HP0_4$ $2H_20$).(35.8 g) was dissolved in 1 litre of distilled water (solution A). 3.12 g of sodium dihydrogen orthophosphate (Na $H_2PO_42H_2O$) were dissolved in 200ml of distilled water (solution B). A volume of 870 ml of the solution A was mixed with a volume of 130 ml. of solution B. The pH was then adjusted with an aliquot of solution B.

Carbonate buffer pH 9

A. 10.6 g of disodium carbonate (Na_2CO_3) was dissolved in 100ml of doubly distilled water.
B. 8.4 g of sodium hydrogen carbonate ($NaHCO_3$) was dissolved in 100ml of doubly distilled water.
C. 58 ml of solution obtained in A was mixed with 10 ml of the solution obtained in B. The pH was then adjusted to 9.

2.3.2 Handling of lipids and storage

Lipids were stored as powders at -30°C. The correct amounts were weighed and dissolved in chloroform just before being used in liposome preparation.

2.3.3 Preparation of marker and drug solutions

2.3.3.1 Preparation of marker solutions

Carboxyfluorescein:

Carboxyfluorescein was prepared at 1 mg/ml in distilled water. Drops of sodium hydroxide (NaOH) were added to the weighed powder until a dark orange-brown solution was obtained The final volume was made up with distilled water and the pH

adjusted to 7.4. The carboxyfluorescein in solution was assessed by measuring the fluorescence at λ_{ex} =486 nm and λ_{em} = 514 nm.

Riboflavin:

Riboflavin was prepared at 1mg/ml solution in distilled water. The salt form received from the manufacturer is readily soluble in water.

For large batch preparations the riboflavin was weighed and added as a powder. The riboflavin in solution was assessed by measuring the fluorescence at λ_{ex} =445 nm and λ_{em} = 520 nm.

FITC -Albumin:

FITC-Albumin was prepared at 1mg/ml solution in distilled water. The labelled protein in solution was assessed by measuring the fluorescence at λ_{ex} =495 nm and λ_{em} = 520 nm.

Glutathione:

Glutathione (reduced form) was prepared at 10 mg/ml solution in distilled water. Identically to riboflavin, when large batch preparation were needed, the weighed glutathione was added as a powder to the preparations.Glutathione in solution was measured using DTNB and the procedure in 2.3.4.

2.3.3.2 Preparation of drug solutions

Doxorubicin and daunorubicin:

Doxorubicin and daunorubicin were prepared at 1 mg/ml solution in distilled water. The fluorescence in solution for doxorubicin was measured at λ_{ex} = 493 nm and λ_{em}

=560 nm. The fluorescence in solution for daunorubicin was measured at λ_{ex} =490 nm and λ_{em} =560 nm.

Penicillin G:

Penicillin G was prepared at 5 mg/ml solution in distilled water. Trace amount of ^{14}C penicillin was added during liposomes preparation and the drug was assessed by liquid scintillation counting of the ^{14}C isotope.

2.3.4 DTNB or Ellman's reagent for the determination of the glutathione concentration

The Ellman's reagent 5,5' dithiobis (2-nitrobenzoic acid) (DTNB) was introduced in 1959 (Riddles et al, 1979).

It has found extensive use in the estimation of free thiol groups in native and denatured proteins.

The procedure was based on the reaction of the thiol with DTNB to give the mixed disulfide and 2-nitro-5-thiobenzoic acid (TNB) which is quantified by measuring the absorbance of the dianion (TNB^{2-}) at 412 nm.

The reagents used were :

A Phosphate-buffered saline pH 8.0

B 0.1mM solution of Dithiobis (2 nitrobenzoic acid): 39.6 mg of DTNB in 1000 ml of phosphate buffer pH 8.0.

C Glutathione solution (100 μg/ml)

Standards of 20, 40, 60, 80 and 100 μg/ml of glutathione were prepared from the stock solution.

4.5 ml of solution B was added to 0.5 ml of glutathione standard. After mixing, the sample was left at room temperature for 10 min. The absorbance was then measured

at 412 nm. The blank consisted of 4.5 ml of B completed to 5ml. with 0.5 ml of water. A standard curve was established.

2.3.5 Bligh-Dyer two-phase extraction method

Preparation of reagents (New, 1990)

Upper phase

Methanol (20 ml.) was mixed with an equal volume (20 ml.) of chloroform and 20 ml of saline. This mixture was allowed to stand until the two layers separated completely from each other. The top layer was removed and retained for further use.

Method:

1. 100 µl of the liposomes sample was added to 900 µl of saline in a 10 ml centrifuge tube followed by 1 ml of methanol and 1 ml of chloroform, the resulting solution was vortexed for 20 seconds. The sample was then centrifuged at 1000×g for 7 minutes to separate the two phases.
2. The lower layer was removed using a Pasteur pipette and transferred to a clean tube.

1 ml of the upper phase was added to the sample, vortexed and spinned as before.

3. This was repeated three times retaining the lower phase each time. The final lower phase contained the total lipids of the sample separated from the water-soluble liposomal contents and was to be used as starting material in the Stewart assay.

2.3.6 Stewart assay

In the Stewart assay for phospholipids (New, 1990) the property of complexation of phospholipids with amonium ferrothiocyanate in organic solution is used to determine the phospholipid content. The advantage of this method is that there is no interference due to the presence of inorganic phosphate. This method is not applicable to samples where unknown lipids are present.

2.3.7 Preparation of ammonium ferrothiocyanate

0.1 M ferric chloride, hexahydrate (2.7g) and ammonium thiocyanate (3g) were dissolved in doubly distilled water and made up to 100ml. The solution was stable at room temperature.

2.3.8 Standard curve for egg phosphatidylcholine and equimolar cholesterol mixture

The phosphatidylcholine and equimolar cholesterol solution was diluted to have a concentration of 100 μg /ml.

Standards were prepared in the lipid concentration range of 60μg - 600μg /ml. 2 ml of ferrothiocyanate was added to each standard and vortexed vigorously for 20 sec. Each tube was then spinned for 5 min at 300 g in a bench centrifuge, the lower layer was removed and the absorbance read at 485 nm. A standard curve was established.

2.3.9 Protein radioiodination using the chloramine-T method

The chloramine-T method for the iodination of proteins (Redshaw and Lynch, 1974) is as follows:

1. Solutions of 2.5 mg/ml chloramine-T, 1 mg/ml potassium iodide (KI), 5 mg/ml

of metabisulphite and 1mg/ml of protein in 0.3M phosphate buffer pH=7.5, were prepared.

2. Preparation of 0.5% bovine serum albumin (B.S.A.) in glycine buffer (0.2 M, pH=8.8.).
3. 50 µg of protein (20 µl) was mixed with 0.05 mCi (10µl) of ^{125} I (specific activity 3.7 GBq/ml. in NAOH solution, Amersham International, Slough, UK). Chloramine-T (10 µl) was added while mixing. The mixture was incubated for up to 5 min in ice.
4. The reaction was stopped by the addition of 10 µl of sodium metabisulphite and the final volume adjusted to 0.5 ml using KI (1mg/ml).
5. The labelled protein was applied onto Sephadex G-100 column (1 x 50 cm) equilibrated with glycine buffer containing 0.5% BSA.
6. The column was eluted with 0.5 % BSA.
7. 1ml fractions were collected.
8. 5 µl of each fraction was assessed for radioactivity using the minigamma counter.
9. Collection of the main radioactive fractions (peak fractions) which were frozen until further use.

The determination of the percentage radioactivity associated to the protein was carried out by precipitation of 5-10 µl of the labelled main fraction to which 100 µl of glycine buffer, BSA 0.5 % and 1.25ml of TCA (20%) was added. The mixture was incubated for 1 hour at 4 °C and centrifuged at 3000×g. The radioactivity was assessed in the pellet and the supernatant. The efficiency of labelling waa greater than 80%.

2.3.10 Preparation of multilamellar vesicles (MLV)

Phospholipids and equimolar cholestrol were dissolved in chloroform into a 100 ml quickfit round-bottomed-flask. The solvent was removed under vacuum in a rotary evaporator and the film formed was flushed with nitrogen to remove traces of solvent. The lipids were hydrated either with the solution of drug, fluorescent marker, PBS or sucrose, or only with distilled water. The rehydration of the lipid film was done by heating if necessary to achieve a temperature above the Tc of the major phospholipids in the composition. On formation of the vesicles (around 30min), unentrapped component (drug, marker, sugar) was not removed if the preparation were to be microfluidized or extruded. If the preparation was used to prepare MLV, the free material was removed by ultracentrifugation for 30 min at 10000×g for the recovery of large liposomes 0.2-2 μm in diameter (Gregoriadis *et al*,1998a)

2.3.11 Preparation of small unilamellar vesicles (SUV)

The hydrated lipid suspension, as in 2.3.10, was kept at a temperature above the (Tc) of the phospholipid.

Size reduction was achieved using one of the following techniques:

Sonication:

Probe sonication with a titanium probe for discontinuous bursts was carried out at a peak amplitude of 8 microns. The sonication was continued for long enough to achieve a liposome population of an average diameter of 70-90 nm. The sonication time was dependent on the amount of lipids used. Prolonged sonication over this optimum time did not achieve further reduction in size. In order to remove the

titanium particles shed from the probe, the preparation was centrifuged at 5000×g for 10-12 min prior to use.

Extrusion

The MLV preparation was extruded using the high-pressure extrusion apparatus Maximator®. This technique allows the fast continuous extrusion of liposomal suspensions at a hydraulic pressure of up to 10.5 Mpa . MLVs diluted in PBS were forced five times through two stacked polycarbonate membrane filters. Filters with decreasing pore size of 1.2, 0.4 and 0.2 μm were used. The preparation was then centrifuged at 5000g for 10-12 min to remove any particles coming from the extrusion-circuit. The liposome population showed a diameter of around 120 nm after the last extrusion step using the 0.1 μm pore size filter (Schneider *et al*, 1994).

2.3.12 Preparation of dehydration-rehydration vesicles (DRVs)

Small unilamellar vesicles were prepared as described in 2.2.11 with distilled water. The material to be entrapped was dissolved in a volume of buffer or distilled water or it was added as a powder into the SUV sample (typically 1-10 ml depending on the amount of lipid used). After freezing in a deep-freezer for long enough (>1 hour), the samples were lyophilized in a Micro Modulyo freeze-drier unit for 24-48 hours. The controlled rehydration step was carried out at a temperature above the phase transition temperature (Tc) of the main phospholipid present in the formulation. Distilled water (typically 100μl for a preparation containing 37 mg of lipids) was slowly added to the lyophilized cake followed by vigorous vortexing until transformation into a homogenous gel occured. Full rehydration was obtained after 20 min. Dilution to the desired volume was achieved using PBS pH 7.4. The preparation was then diluted in PBS and centrifuged at 25,000 rpm for 30 min. The pellet and supernatant were then

assessed for the drug content and for the estimation of the percentage encapsulation (Kirby and Gregoriadis, 1984). In some extrusion or microfluidization experiments the DRV preparation was processed without removing the unencapsulated material.

2.3.13 Preparation of small liposomes by a novel method

In this case SUV prepared by probe sonication as described in 2.3.11 are mixed with the material to be entrapped as well as sucrose. An amount of sucrose is added to achieve the desired mass ratio of sucrose to lipid (g/g). The suspension volume is then adjusted to a predetermined molarity of the sucrose and the suspension was frozen and freeze-dried. The cake obtained was rehydrated at a temperature above the temperature of phase transition of the main phospholipid present in the formulation in a manner similar to that applied in the classical method. The preparation was then diluted in PBS and centrifuged at 45,000 rpm for 45 min. The pellet and the supernatant were finally assessed for the estimation of the percentage encapsulation.

2.3.14 Preparation of MLV by a variant of the reverse phase evaporation method

The reverse-phase evaporation (REV) procedure is a method for preparing large unilamellar vesicles entrapping a considerable amount of solute (Szoka and Papahadjopoulos, 1978). The idea is to use a water-in-oil emulsion (w/o) where the lipids undergo a process of vesicularisation when the organic phase is removed. Typically, a low ratio of lipid (in organic phase) to solute (in aqueous phase) is used. An example of a typical preparation will consist of dissolving 25mg of PC and equimolar cholesterol in a total of 1 ml of chloroform/ether (50% v/v). One ml of PBS

containing the solute to be encapsulated is emulsified in the organic phase. One or two min of sonication under nitrogen in a bath sonicator achieves the emulsification. The mixture of ether/chloroform is removed using a rotary evaporator. A gel of large unilamellar vesicles is then obtained.

A modified REV method for obtaining MLV-REV liposomes is similar to the REV method except that the ratio of organic phase (ether/chloroform 50% v/v) containing high amounts of lipids (up to 450 mg) to the aqueous phase containing the solute to be encapsulated is very high(Pidgeon *et al.*, 1986).

An example of preparation will be 450 mg total amount of lipid (E-PC: CHOL equimolar) dissolved in 5 ml of chloroform mixed with 5 ml of ether in a round-bottomed flask, to which a maximum of 1.5 to 1.6 ml of PBS containing 12 mg of riboflavin is added. The emulsification is achieved with a bath sonication for 2-3 min. Upon removal of the mixture of chloroform and ether a gel is obtained. This preparation consists of MLV liposomes entrapping high amount of solute. When a small liposome size is desired, the preparation can be extruded without removing the unentrapped material. In some cases it was spray-dried after removal of the free material and the percentage of encapsulation achieved was estimated by measuring the drug in the pellet and the free drug in the supernatant.

2.3.15 Liposome extrusion

When size reduction of preformed DRV and MLV-REV liposomes was needed an extrusion step was carried out and the preparations were processed unwashed, using an extruder Maximator® model HPE-10-250 (Schneider *et al*, 1995). The preparation was diluted in PBS pH 7.4 to achieve a minimum volume of 12 ml. Two stacked polycarbonate membranes were set up on the filter holder. The liposomes were then

passed through for 5 cycles. The filter holder was then opened and the membranes replaced with two other stacked membranes of a smaller pore size. This procedure was repeated until processing the sample through the membrane of a pore size of 100nm. The non-entrapped solute was removed by ultracentrifugation and the percentage of encapsulation assessed.

2.3.16 Experimental strategy of liposomes microfluidization

The study was organized as series of experiments that involved setting up a battery of carefully selected tests. Mathematical models were obtained on the influence of the parameters investigated and their interactions with the selected responses as well as their measured responses (Dussert *et al*, 1995, Valero *et al*, 1996).

2.3.16.1 The fixed parameters:

a) Equipment

A microfluidizer S-110 (Microfluidics Corp, MA. USA) equipped with a Y-designed interaction chamber was used. Its intensifier pump was fed with compressed air from a cylinder. The interaction chamber was extensively cleaned every second test and the flow rate of liquid (water) was monitored and compared to the calibration curve in order to check the total decloging of the chamber. All the samples was processed at room temperature.

b) Formulation composition

All samples were constituted of PC and equimolar CHOL liposomes prepared as described earlier. The amount of sucrose used for encapsulation consisted of 3ml of a 50mM solution of sucrose to which trace of ^{14}C-sucrose were added. The final volume was adjusted to 10 ml.

2.3.16.2 The variable parameters

It was decided to investigate the influence of the following parameters:

a) The pressure of air: the pressure of air at the intensifier pump could be adjusted using the air gauge installed with the pump for this effect. The lowest pressure limit was fixed at 20 PSI and the highest at 60 PSI.

b) Washing or not washing: (presence or absence of free ^{14}C sucrose). The liposomes were either fully separated from the unentrapped ^{14}C sucrose (washed) or used as they were (unwashed) for microfluidization.

c) Number of passes (cycles):

The flow rate of water through the interaction chamber was estimated at each inlet air pressure value and the time corresponding to one cycle was deduced. The samples were processed for 1 or 10 cycles.

2.3.17 Design of the experiments

For the first part of the work the design is that of a complete factorial design on two levels with interactions. It consists of eight tests constituting the experiment grid shown in Table 2.1

Inlet air pressure, X_1	Level +1: 60 PSI
	Level –1: 20 PSI
Washed or unwashed liposomes, X_2	Level +1: unwashed
	Level –1: washed
Number of cycles, X_3	Level +1: 10 cycles
	Level –1: 1 cycle

Table 2.1 Grid of experiments for the evaluation of the effect of the coded parameters.

Experiment Number	X_1	X_2	X_3
1	-	-	-
2	+	-	-
3	-	+	-
4	+	+	-
5	-	-	+
6	+	-	+
7	-	+	+
8	+	+	+

Calculations were performed in order to estimate the parameters associated with each variable or combination of variables in a postulated linear model. The intervention of a parameter or an interaction between parameters is considered to be significant only when the non-zero probability of the associated coefficients in the Student's test performed exceeds 98 %. The second part of the work aimed at quantifying the effect of the real variables P and N using a multiple regression analysis with the liposomes processed unwashed. For this purpose, the data corresponding to the set of experiments in which the liposomes were processed unwashed was retained. Complementary experiments were conducted at 40 psi and 5 cycles (Table 2.2). The validity of the established models was checked by comparing the predicted values (from the model) to those measured after processing fresh samples in the same experimental condition.

Table 2.2 Grid of complementary experiments for the evaluation of the effect of P and N.

Experiment Number	P (p.s.i)	N (passes)
9	20	5
10	60	5
11	40	5
12	40	1
13	40	10

Finally, a set of experiment was conducted to check the validity of the models by comparing the values predicted by the models to those measured after processing the samples in the same conditions.

2.3.18 Liposomes microfluidization responses

The measurements taken made it possible to assess the microfluidization process and to express the final properties of the liposomes such as:

2.3.18.1 Percentage of initial entrapment values

The final supernatant and the pellet were assessed for the ^{14}C sucrose content and the final entrapment was estimated.

% of initial entrapment values = (% final entrapment / % initial entrapment)×100

2.3.18.2 Final size and polydispersity index (PDI)

The size as well as the polydispersity index (PDI) after microfluidization and washing of the pellet were measured by the photon correlation spectroscopy technique (PCS) using the Autosizer 2-C.

2.3.19 Spray-dried liposomes

Most solutions and collodial suspensions, as well as emulsions and dispersions can be spray-dried provided that the dried product has the characteristics of solid matter.

The most frequently used method for drying aqueous extracts or solutions is freeze-drying (lyophilization) which is rather time-consuming. However, the result is then, most of the time, a "soft cake" which must subsequently be milled or ground to give a

dry but very hygroscopic powder. This has been observed with freeze-dried liposomes.

When spray-drying in the mini spray-drier Buchi B190, the dried powder is obtained within a few minutes already in the form of a uniform powder. Ingredients which are sensitive to heat, such as enzymes, antibiotics, lipids retain their activity (Adler and Lee, 1998; Hauser and Strauss 1987; Kikuchi *et al.*, 1992).

Spray-dryied liposomes, were obtained as follows:

The spray-air flow was set at 600 Nl/hr and the aspirator capacity at 90%.

The inlet temperature was set at 110°C. As soon as the inlet temperature stabilized, the outlet temperature was adjusted using distilled water. The outlet temperature could be controlled by increasing or decreasing the pump output. When the outlet temperature reached 70°C, the suspension of liposomes was sprayed. The concentration of the feed was 10% w/w. We did not need to readjust the outlet temperature. As soon as the liposomal suspension spray-dried, the resulting powder was collected from the collector and from the cyclone.

The powder obtained was then assessed for the size distribution by dispersing an amount of the obtained powder (liposomes and carbohydrate mixture) in a saturated solution of the same carbohydrate. The dispersion was then assessed for size distribution using aMalvern Mastersizer. The shape of the particles was checked with scanning electronic microscopy. An amount of the obtained powder was rehydrated and the size distribution as well as the encapsulation efficiency of the entrapped marker were assessed in order to be compared with the original characteristics of the liposomes (size and % encapsulation before spray-drying).

2.3.20 Incubation of surfactant with spray-dried liposomes

The procedure adopted to assess the effects of surfactants on the release of liposomal contents consisted of treating the liposomal sucrose powders with an amount of detergent to achieve 2:1 w/w of lipids to surfactants. An amount of spray-dried powder was weighed in a dialysis bag. The correct amount of detergent was added in order to achieve the ratio of 1 mg surfactant to 2 mg lipids. The dialysis bag containing the mixtures is then immediately submerged in 25ml of PBS. The release of riboflavin was assessed by taking aliquots from the dialysis media at time intervals and by measuring the fluorescence of the released riboflavin. A total lysis of the same amount of rehydrated powder was performed to determine the total fluorescence of riboflavin in the sample.

Chapter Three

The use of factorial design and regression analysis for the prediction of vesicle characteristics in microfluidized liposomes

3.1 Introduction

The effect of the size of the vesicles on the in-vivo release of drugs and the stability of liposomes in blood was established in earlier studies (Nagayasu. *et al*, 1995; Gregoriadis, 1995b). It appears to be directly related to vesicle plasma retention in the circulatory system. The problem of bilayer destabilization by the HDL has been studied extensively (Gregoriadis and Davis, 1979; Gregoriadis, 1994; Scherphof *et al*, 1979 and 1983).

Changing the liposome composition by adding a high amount of cholesterol or by using lipids which exhibit a high gel-liquid crystalline transition temperature (Tc) reduces the HDL action considerably.Such liposomes are designed so that they retain a considerable amount of entrapped drugs while in contact with the blood components. Delaying and suppressing the intervention of the RES appears to be important and requires other approaches. Successful attempts aiming at increasing the circulation time of liposomes consisted of using stable SUV with a diameter of <100 nm. (Gregoriadis and Senior, 1980) or of attaching hydrophilic substances (covalently attached PEG) to the liposomal surface and also of making the liposomes small in size (around 100 nm) (Kilbanov *et al*, 1990; Papahadjopoulos *et al*, 1991). The polymers at the liposomes surfaces repel the destabilizing opsonins, allowing a longer circulation time.

Intravenous injection of large liposomes show short half-lives in the blood circulation (Senior *et al*, 1985). This makes them inefficient in maintaining a considerable amount of entrapped drug within the vascular system or other targeted area. Large liposomes injected intramuscularly are unable to reach the regional lymph nodes

(Tumer *et al*, 1983). The liposome formulations of a variety of drugs which have been approved for clinical use in several countries or are under clinical trial are represented in Table 3 1.

We can notice that the average diameter rarely exceeds 100 nm and is generally lower. The need of size reduction of liposomes is a prerequisite for their clinical use when a long circulatory half-life is required.

Table 3.1 Some characteristics of the liposomal products approved for use or in clinical trial (from Gregoriadis, 1995a)

Product	Composition	Drug	Size
AmBisome	HSPC, CHOL, DSPG	Amphotericin B	55-75 nm
Daunoxome	DSPC, CHOL	Daunorubicin	60 nm average
Mikasome	HSPC, CHOL, DSPC	Amikacin	35-45 nm
Vincaxome	DSPC, CHOL	Vincristine	30-50 nm
Doxil (intravenous)	HSPC, CHOL, PEG-DSPE	Doxorubicin	80-120 nm
D99	EPC, CHOL	Doxorubicin	100 nm average
C53	EPC	Prostaglandin E1	100 nm average

Industrial scale production (as discussed in Chapter1) has become a reality and methods which use mixing devices classified as the 'family' of the high-pressure homogenizers are currently used in research laboratories and in the pilot-plant of companies. The main characteristics of homogenized liposomes are their single bilayer membrane and their small and homogenous vesicle size. The process mostly works under mild conditions, it is cost-effective and may be scaled up easily.

Microfluidization (double-jet homogenization) is a relatively recent technique which utilises the forces of two streams of a liposome suspension colliding with one another

under high pressure, producing high-shear forces and reducing the vesicle size.

The uniformly hydrated phospholipid suspension is transferred to the reservoir. The liposome suspension is pumped under high pressure through the interaction chamber. In the interaction chamber, the liposome suspension is divided into two streams and is then recombined at high velocity to produce smaller and more uniformly sized liposome vesicles.

In this chapter there will be an investigation of the microfluidization of liposomes. The work will include:

1 Entrapment of ^{14}C-sucrose into DRV liposomes,

2 Microfluidization according to a factorial design; quantification of the effect of the operational parameters such as the pressure of air in the intensifier pump and the number of cycles through the interaction chamber as well as the effect of the presence or absence of the non-entrapped material,

3 Prediction of the vesicle characteristics such as the size, the percentage of initial entrapment values as well as the polydispersity index and its evaluation,

4 Discussion of the approach, implications and prospects.

3.2 Materials and methods

Described in Chapter Two.

For materials, see; 2.1.1, 2.1.3, 2.1.8

For methods, see: 2.2.1, 2.2.6, 2.2.7, 2.2.8, 2.3, 2.3.10, 2.3.11, 2.3.12, 2.3.16, 2.3.18.

3.2.1 Statistical methods

A statistical method was adopted (see Chapter 2) where three characteristics of the vesicles, after microfluidization, were considered at the same time. Drastic size reduction of empty liposomes does not present a particular problem. It is necessary to consider the entrapment of material and the size reduction because size reduction is always correlated with leakage of the entrapped material.

3.2.2 Microfluidization according to factorial design

Each experiment was carried out according to the plan in Chapter 2, Table 2.1. The first experiment for example is a preparation of washed liposomes (free ^{14}C sucrose removed by ultracentrifugation). This preparation is then microfluidized using a Microfluidizer S-110 equipped with an unchanged Y-designed interaction chamber where the pressure gauge on the intensifier pump is set at 20 PSI and the sample processed for only one cycle (1pass).

All the experiments in the grid were carried out according to this plan avoiding any change in the stated conditions.

Complementary experiments for the multiple regression were conducted in the middle of each interval as described under 2.3.17.

3.3 Results and discussion

3.3.1 Entrapment yield of ^{14}C-sucrose in DRV liposomes

The entrapment of ^{14}C-sucrose in dehydration-rehydration liposomes was efficient (34.73 % ± 4.9 of the starting material was entrapped (Table 3.2). A dilution prior to freeze-drying allows a limitation of the stabilizing effect of sucrose. Indeed the

disaccharide acts as a stabilizer by forming an amorphous glass matrix between the vesicles which prevents any fusion or any bilayer damage by crystal formation and/or by interacting with the phospholipid head groups (see Chapter 1) (Crowe and Crowe, 1993). The ^{14}C labelled sucrose was successfully entrapped and did not allow a total SUV stabilization allowing DRV liposomes to form with a considerable entrapment. This was exploited to establish a novel method based on the classical DRV method subject of the study in Chapter 6.

Table 3.2 Characteristics of liposome encapsulating ^{14}C-sucrose

Samples N°	% entrapment	Size (± SD) nm
1	31.5	501 (12)
2	37.2	412 (31)
3	38.7	386 (9)
4	31.7	486 (11)
5	33.5	412 (21)
6	45.2	396 (18)
7	29	410 (9)
8	31.1	421(14)

3.3.2 Effect of the operational parameters

The values of the responses (after microfluidization) in the eight experiments of the factorial design are shown in Table 3.3.

In order to describe the responses, a linear model with interactions has been postulated on the basis that the variables coded x_i associated with physical parameters:

$$Y= b_o + b_i x_i + b_{ij} x_i x_j + b_{ijk} x_i x_j x_k$$

This general model has been fine-tuned for each response by discrimination using the Student's test. We considered the intervention of a parameter or of an interaction between the parameters to be significant only when the non-zero probabilty of the associated coefficient exceeded 98%.

Table 3.3 Characteristics of microfluidized liposomes encapsulating ^{14}C sucrose

Experiment	% of initial entrapment	Size (nm)	PDI
1	46.0	273.8	0.188
2	30.0	247.9	0.230
3	50.4	199.2	0.179
4	43.8	150.4	0.257
5	30.0	207.0	0.19
6	20.0	202.0	0.18
7	40.5	138.4	0.214
8	34.43	117.5	0.161

The models were established using the following calculations:

Table 3.4 Grid allowing the calculation of the associated coefficients

X_1	X_2	X_3	X_1X_2	X_1X_3	X_2X_3	$X_1X_2X_3$	Y
-	-	-	+	+	+	-	Y_0
+	-	-	-	-	+	+	Y_1
-	+	-	-	+	-	+	Y_2
+	+	-	+	-	-	-	Y_3
-	-	+	+	-	-	+	Y_4
+	-	+	-	+	-	-	Y_5
-	+	+	-	-	+	-	Y_6
+	+	+	+	+	+	+	Y_7

$B_o = \sum Yi /8 \qquad i=0,7$

B_1 coefficient of the coded variable X_1:

$$B_1 = (- Y_0+Y_1-Y_2+Y_3-Y_4+Y_5-Y_6+Y_7)/8$$

B_2 coefficient for X_2:

$$B_2=(-Y_0-Y_1+Y_2+Y_3-Y_4-Y_5+Y_6+Y_7) / 8$$

B_3 coefficient for X_3:

$$B_3 = (- Y_o - Y_1 - Y_2 - Y_3+Y_4+ Y_5+ Y_6+ Y_7)/8$$

B_{12} coefficient for the interaction between X_1 and X_2:

$$B_{12}=(Y_0-Y_1-Y_2+Y_3+Y_4-Y_5-Y_6+Y_7) / 8$$

B_{13} coefficient for X_1 X_3:

$$B_{13}=(Y_0 - Y_1+Y_2 - Y_3 - Y_4+Y_5 -Y_6+ Y_7) / 8$$

B_{23} for X_2 X_3:

$$B_{23} =(Y_o + Y_1 - Y_2 - Y_3-Y_4 - Y_5+ Y_6+ Y_7)/8$$

B_{123} for $X_1X_2X_3$:

$$B_{123} = (- Y_0+Y_1 +Y_2 - Y_3 + Y_4 -Y_5 -Y_6+ Y_7) / 8$$

Where Y is one of the following responses:

- percentage of initial entrapment values,
- size (nm) (Z-average diameter),
- polydispersity index.

The resulting equations are shown in Table 3.5

Y_1, Y_2, Y_3, are the coded variables.

Y_1: % of initial entrapment values.

Y_2: size (Z-average diameter) nm.

Y_3: polydispersity index (PDI).

Table 3.5 Equations set: response models on the coded variables before fine tunning.

Y1: coded variable % of initial entrapment value $Y_1=36.9 - 4.83X_1 + 5.38\ X_2 - 5.66\ X_3 + 1.67X_1X_2 + 0.823\ X_1X_3 + 0.836\ X_2X_3 - 0.676X_1X_2X_3$
Y2: coded variable size $Y_2=192 - 12.57\ X_1 - 40.66\ X_2 - 25.81X_3 - 4.86\ X_1X_2 + 6.1\ X_1X_3 + 2.4\ X_2X_3 + 0.883\ X_1X_2X_3$
Y3: coded variable polydispersity index $Y_3 = 0.199 + 7.12510^{-3}\ X_1 + 2.8710^{-3}\ X_2 - 13.62510^{-3}\ X_3 - 8.7510^{-4}\ X_1X_2 - 22.87510^{-3}\ X_1X_3 - 1.62510^{-3}\ X_2X_3 - 9.87510^{-3}\ X_1X_2X_3$

As it was said earlier, a parameter is considered to be significant only when the non-zero probability of the associated coefficient exceeds 98%. So we performed the Student's Test to discriminate between the parameters as shown in Tables 3.6, 3.7 and 3.8.

Table 3.6 The retained coefficients of the coded variable Y1

Student test	
Response Y1	
Coefficients	**Non zero probability**
4.83	
5.38	
5.66	
1.67	**0.9893**
0.823	
0.836	
0.676	

Table 3.7 The retained coefficients of the coded variable Y2

Response Y2	
Coefficients	**Non zero probability**
40.66	
25.81	
12.57	**0.9891**
6.10	
4.86	
2.40	
0.883	

Table 3.8: The retained coefficients of the coded variable Y3

Response Y3	
Coefficients	**Non zero probability**
0.022875	
0.013625	
0.009875	
0.007125	**0.9816**
0.00287	
0.001625	
0.000875	

After fine-tuning the coefficients and retaining those with the non-zero probability (numbers in bold in **Tables 3.6, 3.7** and **3.8**) exceeding 98 %, the following equations are obtained and retained for further analysis:

Table 3.9 Response models on the coded variables after fine-tuning

Response models	
Response	**Equation of the model**
% of initial entrapment	$Y_1=36.9 - 4.83X_1 + 5.38\ X_2 - 5.66\ X_3 + 1.67X_1X_2$
Size (nm)	$Y_2=192 - 12.57\ X_1 - 40.66\ X_2 - 25.81X_3$
PDI	$= 0.199 + 7.12510^{-3}\ X_1 - 13.62510^{-3}\ X_3 - 22.87510^{-3}\ X_1X_3 - 9.87510^{-3}\ X_1X_2X_3$

In these models the amplitude of the impact of a parameter or of an interaction is proportional to the absolute value of the corresponding coefficient; its sign obviously

indicates whether this action results in an increase or in a reduction in the response (Dussert *et al*, 1995).

Thus a first degree model, such as that used in this study, is able to determine which are the key parameters and whether each of the parameters has to be increased or reduced to obtain an increase or a reduction in the result as appropriate.

3.3.2.1 Effect of the pressure of air in the intensifier pump on the responses.

The air pressure in the intensifier pump determines the energy transmitted to the liquid and thus to the liposomes in suspension. This allows them to cross the microchannels of the interaction chamber. The energy at the collision point between the two streams is higher at high pressure. The cavitation phenomena occuring during the impact is intense at higher pressures. One could predict that smaller sizes are obtained at high pressure but would it be beneficial for the retention of the originally entrapped sucrose?

As we look at the equations of the models, we can assert that the size is decreased if X_1 is at its highest setting i.e. maximum pressure (60 PSI). On the other hand, the percentage of initial entrapment values is dependant on the pressure and is, in the same time, decreased by increasing the pressure.

The polydispersity index is also increased when the pressure is increased (X_1 in the equation).

In summary, on one hand, increasing the pressure is favorable for the productionof small liposomes exhibiting low PDI upon microfluidization. On the other hand this is not very favorable for the retention of the encapsulated ^{14}C-sucrose.

3.3.2.2 Effect of the number of cycles on the responses

The percentage of initial entrapment values is decreased if X_3 (number of cycles) is high. Re-circulating liposomes at high pressure through 100 μm diameter channels of the interaction chamber leads to the loss (by leakage) of the encapsulated ^{14}C sucrose. The size is also decreased by increasing the number of cycles but tends to be drastically reduced for the first few cycles of processing. Similar results were also reported by others (Vemuri *et al*, 1990, Talsma *et al*, 1989). Vemuri and co-authors reported, that after the first pass, the liposome size was reduced from 640 nm down to 240 nm. Further treatment by recirculation (increasing the number of cycles) allows an improvement in size reduction only. The polydispersity index will decrease by re-circulating the liposomes for a higher number of cycles.

3.3.2.3 Effect of the presence of non-entrapped material during microfluidization.

Processing unwashed liposomes (X_2 at its highest level) has a positive effect on size reduction. This could be explained by the fact that the presence of some free sucrose increases the viscosity of the suspension allowing a better energy transfer (higher mass involved in the collision inside the interaction chamber).

The percentage of initial entrapment values increases when the liposomes are left unwashed (X_2 increased).

The presence of an unentrapped solute during microfluidization decreases the leakage of the solute possibly by creating an equilibrium between the inside and the outside of the vesicles and by suppressing the gradient across the membranes.

The presence of the non-entrapped ^{14}C sucrose has no effect on the polydispersity index.

Therefore liposomes should be microfluidized unwashed and at the highest pressure. A compromise has to be found between the desired final size and the retention of entrapped sucrose during liposome processing.

The main conclusion is that it is more beneficial to process unwashed liposomes. By quantifying the outcome of microfluidisation one could decide on the conditions to be used (number of cycles and the setting of the pressure).

An attempt, in the next section, has been made at quantifying the effect of the real variables in terms of simulation and prediction of the vesicle characteristics.

3.3.3 Prediction of vesicle characteristics

As discussed in the previous part (3.3.2), and as shown by the established models, the outcome of the microfluidization of liposomes is better when the liposome preparations are processed without removing the non-entrapped material. It is also more economical to process unwashed DRV-liposomes. So, we decided for this part of the work to quantify the effect of the two operational variables, pressure and number of cycles, on the characteristics of the vesicles treated without removing the free ^{14}C sucrose. For this purpose, we needed to consider the middle of the interval for both the pressure (20,60 PSI) and the number of cycles (1,10 cycles), (see method 2.2.14).

We conducted the experiment grid shown in Table 2 (see methods). The results obtained are shown in Table 3.10.

Table 3.10 Characteristics of microfluidized liposomes entrapping ^{14}C sucrose in experiments conducted in the middle of the interval (5 cycles or 40 p.s.i, n=2)

P(p.s.i)	N(cycles)	Experiment N°	% initial entrapment	Size(nm)	PDI
20	5	9	43.79	141.1	0.212
60	5	10	32.79	128.2	0.168
40	5	11	29.22	140.6	0.202
40	1	12	53.41	137.6	0.245
40	10	13	25.65	134.8	0.164

The complete set of data (measured responses after microfluidization) was processed using the software SPSS PC+ in order to generate a correlation for the percentage entrapment, the size and the polydispersity index of the two real operational variables: P (pressure) and N (number of cycles).

3.3.3.1 Vesicle size

The mean diameters of the microfluidized DRVs measured by photon correlation spectroscopy at each stated experimental condition and constituting the raw data or in the transformation pass for treatment on SPSS/PC+ are as follows:

Table 3.11 Raw data : size of the microfluidized liposomes

P(p.s.i)	N (cycles)	Size(nm)
20	1	199.2
20	5	141.1
20	10	138.4
60	1	150.4
60	5	128.2
60	10	117.5
40	1	137.6
40	5	140.6
40	10	134.8

MULTIPLE REGRESSION

Dependant variable: SIZE

Variables entered on step number: **NP, P, N**

Multiple R	0.81362
R square	0.66198
Adjusted R square	0.45917
Standard Error	**16.86910**

Analysis of variance	Sum of squares	Mean square
Regression	2786.51	928.83
Residuals	1422.83	284.56
F=3.264	**Signif F=0.117**	

Table3.12 Coefficient of the equation model for the prediction of size

Variable	B	SE B
NP	0.072684	0.093525
P	-1.075984	0.606111
N	-6.387432	4.040741
(constant)	204.688525	26.186994

The model

Size(nm) ± SE = 0.0726 NP - 1.075P - 6.387N + 204.688 ± 16.86 (equation 3.1)

Equation 3.1: equation model for the prediction of size

As shown by the analysis of variance there is 88.23 % of confidence in the correlation between NP, P and N. Equation 3.1 was used in a Q.Basic program in order to generate all the values of the size in the interval of study for the pressure P (20-60 PSI) and for the number of cycles N(1-10 cycles) as follows:

Q.Basic program:

```
1    OPEN "Set dat 1" FOR OUTPUT AS#1
2    FOR p=20 TO 60 STEP5
3    PRINT #1, USING "##";p;
4    NEXT p
5    PRINT #1
6    FOR n = 1 TO 10
```

```
7       PRINT #1, USING "##";n;
8       FOR p=20 TO 60 STEP5
9       Size = 0.0726*n*p-1.075*p-6.387*n+16.86#
10      PRINT #1, USING "###.##";size;
11      NEXT p
12      PRINT #1
13      NEXT n
14      CLOSE
```

Table 3.13 shows all the generated and possible values of the size generated from the established model in the interval of work for the pressure (20-60 psi) and the number of cycles (1-10).

Table3.13 Values of the size (Z-average diameter) generated from the model equation 3.1

P(PSI) / N(cycle)	20	25	30	35	40	45	50	55	60
1	178.2	173.2	168.2	163.2	158.1	153.2	148.1	143.1	138.1
2	173.3	168.6	164.0	159.3	154.7	150.0	145.4	140.7	136.1
3	168.4	164.1	159.8	155.5	151.2	146.9	142.6	138.3	134.1
4	163.4	159.5	155.6	151.7	147.7	143.8	139.9	135.9	132.0
5	158.5	154.9	151.4	147.8	144.2	140.7	137.1	133.6	130.0
6	153.6	150.4	147.2	143.9	140.8	137.6	134.4	131.1	128.0
7	148.6	145.8	142.9	140.1	137.3	134.4	131.6	128.8	125.9
8	143.7	141.2	138.8	136.3	133.8	131.3	128.9	126.4	123.9
9	138.8	136.7	134.5	132.4	130.3	128.2	126.1	124.0	121.9
10	133.8	132.1	130.3	128.6	126.8	125.1	123.4	121.6	119.8

3.3.3.2 Percentage of initial entrapment values

The same approach was adopted to quantify the effect of the pressure and of the number of cycles on the percentage retention of ^{14}C sucrose.

The raw data for treatment on SPSS PC+ software are as follows:

Table 3.14 Raw data: percentage of initial entrapment values for microfluidized liposomes

P (pressure)	N (cycles)	% initial
20	1	50.4
20	5	43.8
20	10	40.4
60	1	49.3
60	5	32.8
60	10	34.4
40	1	53.4
40	5	22.2
40	10	25.6

Multiple regression

Dependant variable: % of initial entrapment values

Variables entered on step number **N^2, P^2, N** and **P**.

Multiple R = 0.92528
R square = 0.85615
Adjusted R Square = 0.71230
Standard Error = 5.34273

Analysis of variance	Sum of squares	Mean square
Regression	679.552	169.888
Residuals	114.178	28.544
F=5.951	**Signif F=0.0561**	

Table 3.15 Coefficient of the model equation for the prediction of percentage solute entrapment

Variable	B	SE B
N^2	0.398833	0.189282
P^2	0.014408	0.009445
N	-6.333833	2.167789
P	-1.303500	0.763406
(constant)	82.209444	14.087666

% of initial entrapment ± SE = 0.3988 N^2 + 0.0144 P^2-6.33N-1.303P + 82.2094

± 5.34

Equation 3.2 equation model for the prediction of percentage solute entrapment

As shown by the analysis of variance there is 94.39 % of confidence in the correlation between N^2, P^2 , N and P.

Equation 3.2 was used in a Q.Basic program in order to generate all the values of the entrapment in the interval of P and N in a similar manner as in the previous paragraph (3.3.3.1) except that in step 9 the model equation number 2 for the percentage of initial entrapment is used.

The generated values are shown in the following Table (3.16)

Table 3.16: Values of percent of initial entrapment generated from the model. (equation 3.2)

P(PSI) / N(cycle)	20	25	30	35	40	45	50	55	60
1	56	52.7	50.1	48.3	47.2	46.8	47.1	48.2	49.9
2	50.8	47.5	45.0	43.1	42.0	41.6	42	43.1	44.8
3	46.5	43.2	40.7	38.8	37.7	37.2	37.6	38.7	40.5
4	42.9	39.7	37.1	35.3	34.2	33.8	34.1	35.1	36.9
5	40.2	36.9	34.4	32.5	31.4	31	31.4	32.4	34.2
6	38.3	35	32.4	30.6	29.5	29.1	29.4	30.5	32.2
7	37.1	33.8	31.3	29.4	28.3	27.9	28.3	29.3	31.1
8	36.8	33.5	30.9	29.1	28.0	27.6	27.9	29	30.7
9	37.2	33.9	31.4	29.5	28.4	28	28.4	29.4	31.2
10	38.4	35.2	32.6	30.8	29.7	29.3	29.6	30.7	32.4

3.3.3.3 Polydispersity index.

PDI= (STD deviation / mean) x 1000

In a similar manner as for the size and for the % initial entrapment the following raw data was used for treatment in order to generate a model for the prediction of the P.D.I

Table 3.17 Raw data: polydispersity index of microfluidized liposomes

P (p.s.i)	N (cycles)	Polydispersity index
20	1	0.179
20	5	0.212
20	10	0.214
60	1	0.257
60	5	0.168
60	10	0.161
40	1	0.245
40	5	0.202
40	10	0.164

Multiple regression

Dependant variable: Polydispersity index

Variables entered on step number: NP, P, N

Multiple R = 0.85955
R square = 0.73882
Adjusted R Square = 0.58212
Standard Error = 0.02274

Analysis of variance	Sum of squares	Mean square
Regression	0.00731	0.00244
Residuals	0.00259	0.00052
F=4.714	**Signif F=0.0640**	

Variables in the Equation

Table 3.18 Coefficient of the model equation for the prediction of PDI

Variable	B	SE B
NP	-3.5082E-04	1.26074E-04
P	0.001713	8.17053E-04
N	0.008872	0.005447
(constant)	0.159240	0.035301

PDI ± SE = 0.001713P + 0.008872N - 0.0003508 NP + 0.159240 ± 0.022

Equation 3.3 equation model for the prediction of PDI

As shown by the analysis of variance there is 93.6% of confidence in the correlation between NP, P and N.

A similar Q.Basic program was used. The third equation 3.3 was added in step 9 of a similar program generating the following values of polydispersity index (table 3.19).

Table 3.19 Polydispersity index values generated from the model

P(p.s.i) / N(cycle)	20	25	30	35	40	45	50	55	60
1	0.1954	0.2022	0.2090	0.2158	0.2226	0.2294	0.2362	0.2430	0.2498
2	0.1972	0.2023	0.2073	0.2124	0.2174	0.2255	0.2276	0.2326	0.2377
3	0.1991	0.2024	0.2057	0.2090	0.2123	0.2156	0.2189	0.2222	0.2255
4	0.2009	0.2025	0.2040	0.2056	0.2071	0.2087	0.2102	0.2118	0.2133
5	0.2028	0.2026	0.2024	0.2022	0.2020	0.2018	0.2015	0.2013	0.2011
6	0.2046	0.2027	0.2007	0.1988	0.1968	0.1948	0.1929	0.1909	0.1890
7	0.2065	0.2028	0.1991	0.1953	0.1916	0.1879	0.1842	0.1805	0.1768
8	0.2083	0.2029	0.1974	0.1919	0.1865	0.1810	0.1755	0.1701	0.1646
9	0.2102	0.2030	0.1958	0.1885	0.1813	0.1741	0.1669	0.1596	0.1524
10	0.2121	0.2031	0.1941	0.1851	0.1762	0.1672	0.1582	0.1492	0.1402

3.3.4 Validation of the models

A set of experiments performed in the same experimental conditions with the variables set at the levels shown in Table 3.14 was conducted to validate and/or evaluate the established models.

PC and equimolar CHOL liposomes were microfluidized unwashed. The percentage of initial entrapment values, the size and the polydispersity index were measured at each experiment and compared to the values generated by the models. The following Tables show this evaluation.

Table 3.20 Observed and predicted values of percent initial entrapment values

Experiment number	Microfluidisation		Observed %	Predicted (%) from model	Residuals
	P	N			
1	40	1	54.73	47.19	- 7.5
2	40	5	28.0	31.42	+ 3.42
3	40	10	26.5	29.67	+3 .17

Table 3.21 Observed and predicted values of vesicle size (nm)

Experiment number	Microfluidisation		Observed size (nm)	Predicted size (nm) from model	Residuals
	P	N			
1	40	1	211.7	158.17	-53.53
2	40	5	172.7	144.25	-28.45
3	40	10	167.2	126.85	-40.35

Table 3.22 Observed and predicted values of polydispersity index

Experiment number	Microfluidisation		Observed PDI	Predicted (PDI) from model	Residuals
	P	N			
1	40	1	0.28	0.222	- 0.058
2	40	5	0.222	0.202	- 0.02
3	40	10	0.190	0.176	- 0.013

Table 3.20 displays the values of % of initial entrapment measured and compared to those generated from the established model. The standard error allowed in the model is equal to 5.3%. In experiments 2 and 3 the deviation from the model is less than 5.3 % (3.42 and 3.17 % respectively). The first experiment showed a deviation of 7.5 % from the predicted value. If we assume that allowing an additional error of 2% on the percentage of initial entrapment values is acceptable, the values predicted by the model are very close to those measured. The established model gives an estimation of the values of the % of initial entrapment with an error which exceeds the stated error on the model with 2%.

Table 3.21 shows the values of the size (Z average diameter) measured and compared to those generated from the established model. The standard error allowed in the mathematical model is equal to 16.86 nm. The deviation from the model in the three experiments exceeded this value (53.5, 28.45 and 40.35 nm respectively). This suggests that the model does not predict the size of microfluidized DRV liposomes with the stated error on the model. On the other hand it allows an estimation of the outcome of microfluidization with an error exceeding the average value of 16.9 nm.

This could originate from the fact that the correlation exhibits only 88.3% of confidence. The decreasing efficiency of the interaction chamber of the microfluidizer could also play a role. Indeed, the wearing of the ceramic material of which the chamber is made make the interaction chamber less efficient. This was persceptible on the size measurements rather than on the % initial entrapment values since a variation of a few tens of nanometer on the microfluidized liposomes size does not affect the percentage of entrapment values.

Table 3.22 displays the values of the PDI measured and compared to those generated from the established mathematical model. The standard error in the model is equal to 0.022. The observations made concerning the prediction of the size are valid since the two variables are linked. In experiments 2 and 3 the deviation from the model is equal or less than 0.022 (0.022 and 0.013 respectively). The first experiment exhibited a deviation of 0.058 from the predicted value. This suggests that the model does not predict the PDI of microfluidized DRV liposomes with the stated error on the model. It rather allows an estimation of the PDI with an error exceeding the value of 0.022.

This study shows that it is beneficial to microfluidize liposomes without removing the non-entrapped material. Models predicting the % entrapment values and sizes can be established and used for simulation. By imposing constraints on the % entrapment,

one could choose from the simulated data the setting of the values of pressure and the number of cycles allowing higher residual entrapment and adequat Z-average diameters values as self-imposed by the constraint for the intended use of the formulation.

3.4 Conclusions

This study showed that microfluidization of preformed DRV liposomes lead to vesicles exhibiting small sizes and relatively low entrapment values. The nature of the phenomena involved in the microfluidization process (generation of high shear forces) make the use of high pressures beneficial for the overall procedure.

The presence of non entrapped material (unwashed liposomes) improved the outcome of microfluidization in terms of final sizes and percentage of entrapment values. The procedure is most effective for the first few cycles of liposomes microfluidization. Processing for further cycles generates minimal size reduction.

The leakage of the ^{14}C sucrose during processing limits the application of this procedure. An alternative solution to this problem is the use of high amounts of lipids and correspondingly high amounts of drugs. The requirement for such an alternative is the use of specific equipment (microfluidizer) which can process efficiently highly concentrated liposomal suspensions.

The mathematical modeling could be used to establish correlations for microfluidized liposomes allowing quality control during the process. Simil ar strategy could be followed in order to optimize and limit the number of cycles conducted on a liposome formulation containing a labile compound (sensitive to shear forces) such as proteins.

Finally, microfluidized liposomes will still be subject to stability problems such as hydrolysis, size growing and leakage of the encapsulated solutes. Drying liposomes could provide solutions to this problem as shown in the following chapters.

Chapter Four:

Spray-drying liposomes as an alternative method of stabilisation on storage.

4.1 Introduction

Spray-drying is a process by which a suspension, a solution or even a paste is transformed into a dry product. The physico-chemical form of the treated material can be drastically changed because of the energetic nature of the process.

All the major industries (such as clays, paints, food industry and pharmaceuticals) are users of the spray-drying operation. In the pharmaceutical field it is known to improve formulation characteristics like, for example, the compressibility of a powder for tablet manufacturing. (Seager *et al*, 1982, Broadhead *et al*, 1992). Furthermore, the spray-drying operation can be easily scaled up.

4.1.1 Spray-drying operation

The spray-drying process can be divided into the following stages:

A. Atomisation of the feed consisting of the preparation to be spray-dried,

B. Contact between the drying air and the spray,

C. Drying of the spray,

D. Separation of the dried product from the air.

4.1.1.1 Atomisation stage

Atomisation is the transformation of a liquid feed into fine droplets (spray). These droplets are generally spherical depending on the nature of the feed (solution, suspension or paste) and on its concentration (Masters, 1991a).

The mechanism of atomisation depends on the design of the nozzle and on its source of energy. There are three types of nozzles :

1. Rotary nozzles: these are of two subtypes; atomizing wheels and atomizing discs using a centrifugal energy for atomisation,
2. Pressure nozzles: the feed is fed into the nozzle via a chamber under pressure, then it is issued from the orifice of the nozzle at a very high speed desintegrating and forming a spray,
3. Pneumatic (two fluids) nozzles. They rely on the simultaneous feed of the pressurised air with the material to be dried in the nozzle (Cedik and Filkova, 1985).

The Buchi B191 used for this work is equiped with a pneumatic nozzle.

The size distribution of the droplets is generally governed (for the pneumatic nozzle) by the following equation (Lefebvre, 1992):

$$\frac{D_{vs}}{L_c} = \left[A\left(\frac{\sigma}{\rho_A V_A^2 D_p}\right)^{0.5} + B\left(\frac{\mu_L^2}{\sigma \rho_L D_p}\right)^{0.5} \right]\left(1 + \frac{1}{ALR}\right)$$

Equation 4.1 equation governing the size distribution of droplets generated by pneumatic nozzles

Where A and B are two experimental parameters depending on the design of the atomiser.

D_{vs}: sauter diameter of the droplet,

L_c: Characteristic dimension of the atomiser,

σ: superficial tension (kg/s^2),

ρ_a: air density (kg/m^3),

ρ_l: liquid density (kg/m3),

V_a: air velocity (m/sec),

D_p: liquid diameter before film formation (m),

μ_L: viscosity (absolute) of the liquid (kg/m3),

AL R: Air to liquid flow rate ratio (flow rate expressed in kg/sec).

4.1.1.2 Contact between the drying air and the spray

The atomized droplets mix with the drying air, this mixing is dependent upon the air flow pattern into which the spray is projected, as well as upon the way in which it is emitted from the nozzle.

The drying air can enter the drying chamber in three ways: co-current, counter current or mixed flow.

Co-current flow occurs when the atomization and the drying air are located at the top of the drying chamber.

Counter current flow occurs when the atomization and the drying air are localised at the opposite end of the drying chamber.

Mixed flow combines both co-current and counter current flows during the drying process (Masters, 1991b).

The Buchi mini spray-dryer used in our work is a co-current dryer.

4.1.1.3 Drying of the spray

The drying of the droplets is by evaporation of the moisture from the droplets into the surrounding air. The phenomenon is a simultaneous mass and heat transfer between the spray and the surrounding gas (Masters, 1991c).

The heat transfer at the surface surrounding the droplets can be expressed as follows:

$$\frac{dQ}{dt} = \alpha S(T_a - T_h)$$

Equation 4.2 equation describing the heat transfer at the surface surrounding the droplets

α: heat transfer coefficient ($J/m^2s\ °C$),

S: exchange area (total surface area of the droplets),

T_a and T_h: temperatures of the drying air and of the humid air.

The mass transfer (of water) can be expressed as follows:

$$\frac{dm}{dt} = \beta S(P_w - P_a)$$

Equation 4.3 equation describing the mass transfer of water at the surface surrounding the droplets.

β: mass coefficient (sm^{-1}),

P_w and P_a: partial vapour pressures at the surface of the droplet (Pw) and in the surrounding air (Pa).

The rate of drying depends on a number of factors such as the temperature, humidity and velocity of the drying air, the temperature and size distribution of the droplets and the nature of the material dissolved in the droplets.

4.1.1.4 Separation of the drying product from the air

The product separation from the air stream is achieved either by a cyclonic air flow set up in a conical chamber base or by allowing the particles to fall out of the air flow on to a flat chamber base (collector). It is also possible to realise the product separation in a second stage with the help of a bag filter or of an air scrubber positioned after both the cyclone and the drying chamber.

In this chapter the aim was to investigate the spray-drying of liposome preparations. The work includes:

- Entrapment of riboflavin and glutathione into liposomes,
- Spray drying the preparations according to the stated conditions,
- Estimation of the lipid content before and after spray-drying of the liposome dispersions,
- Particle size distribution of the dry product as well as a microscopy study,
- Possible oxidative damage of the phospholipids before and after spray-drying,
- Effect of the lipid composition,
- Effect of the nature and amount of the carbohydrate used ,
- Effect of liposome size reduction,
- Attempt of a thermal study using the MDSC technique.

4.2 Materials and methods

Liposomes were prepared as described in (Material and methods) using the DRV and the modified REV method.

Because of the high amount of lipids used (450mg), we encountered some problems during the rehydration step of the DRV method under the conditions used. At times the lipids collapsed producing quite large lipid aggregates which were very difficult to redisperse. The reverse phase evaporation with modification (see methods) did not show this inconvenience with the amount of lipid used.

The general protocol was as follows:

- Preparation of liposome dispersions encapsulating riboflavin or glutathione by the DRV or the REV methods,

- An extrusion step if desired is performed as described in Methods,
- The preparation is then washed and the non-entrapped material removed,
- The desired amount of sugar is added to the preparation,
- The preparation is then spray-dried in the stated conditions,
- A rehydration step is performed followed by a characterisation in terms of size and percentage of encapsulation and other analytical techniques.

For other details see for materials: 2.1.1, 2.1.2, 2.1.7, 2.1.8, 2.1.11, 2.2.2, 2.2.4, 2.2.5, 2.2.8 and for methods: 2.3.3, 2.3.4, 2.3.5, 2.3.6, 2.3.10, 2.3.11, .2.3.12, .2.3.14, 2.3.19.

4.2.1 Modulated Differential Scanning Calorimetry method (MTDSC)

Experiments were carried out on a TA instruments 2920 MDSC. Matched (± 0.1 mg) Perkin Elmer hermetic aluminium pans were used throughout the study.

The pans were sealed with a dry weld in a Perkin Elmer press. The MTDSC experiments consisted of temperature cycling between – 50 °C and 130 °C with an underlying heating and cooling rate of 1 °C. The linear temperature programme was modulated with a modulation amplitude of 0.159 °C and a modulation frequency of 60 seconds.

4.2.2 Determination of the lipid content of the liposome dispersion after spray-drying

A weighed amount of the spray-dried product was rehydrated and the lipid extraction was preformed using the Stewart assay as described in Chapter 2.

Using the standard curve of the PC and CHOL (equimolar), the amount of lipid present in the rehydrated sample was determined. The amount of lipid was then compared to the original amount of lipid used in the preparation before spray-drying.

4.3 Results and discussion

4.3.1 Effect of the atomization on the liposome suspension integrity

During spray-drying, the liposome suspension is transformed from a whole suspension to a spray consisting of small droplets in which liposomes are suspended. One question may arise: does the spraying process (using air at ambiant temperature) disrupt the vesicle structure?

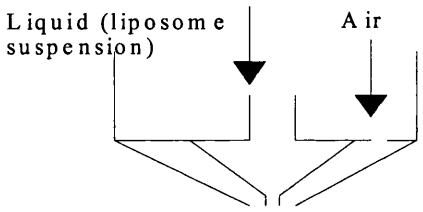

Fig 4.1 Schematic representation of the pneumatic nozzle.

This question can be answered by looking at the design of the spraying nozzle (Fig. 4.1) and by conducting an experiment with empty MLV s which were sprayed with ambiant air and collected just after their passage into the nozzle. The MLVs were sized before and after in order to assess if there were any overall change in vesicle size distribution. The pressure of air used for spraying was set at 3 bar. The liposome suspension met the air flow in a restricted zone producing relatively large droplets below 10 μm up to an average of 25-50 μm (Fig.4.2).

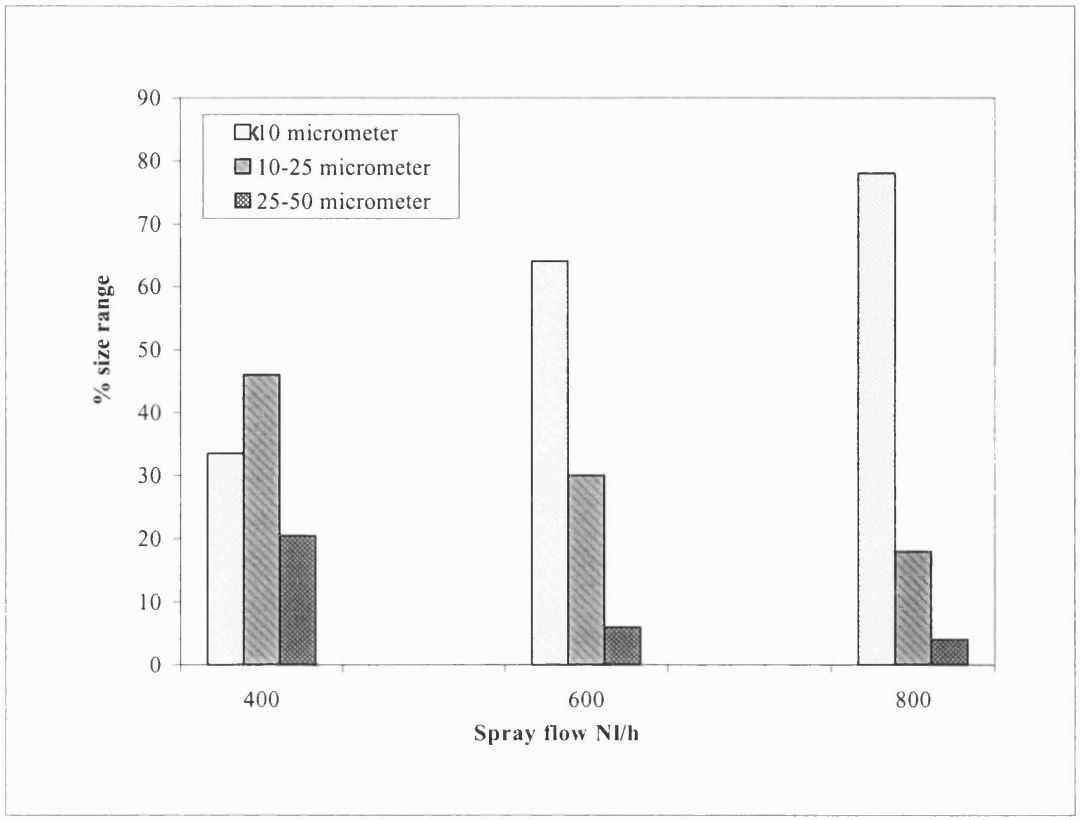

Fig 4.2: Influence of the spray flow on the particle size obtained using a Buchi 190 (from the operational manual of the mini spray-drier 190).

These shear forces produced droplets in which liposomes would presumably be intact. Still, there is a probability of some vesicle disruption situated at the interface of the droplets.

This situation (spraying using the twin nozzle) is not comparable to the high pressure homogenizer such as the microfluidizer and its interaction chamber. The latter consists of microchannels of 100 μm in diameter and of 8 to 10 cm in length. The liposome suspension is pushed inside these channels at a pressure of 40 bars and speeded up to very high velocity. It is divided into two flows, which will meet and collide producing high shear forces and complex cavitation phenomena responsible for the size reduction. As a conclusion, because of the low pressure used and the nature of the phenomena (liquid against liquid for homogenisation, liquid against air

for the spray nozzle) and of the technical design involved, it was not possible to disrupt liposomes significantly when using the spray nozzle.

As shown in Figs 4.3.1 and 4.3.2 the overall liposome size distribution was not affected by the atomization process.

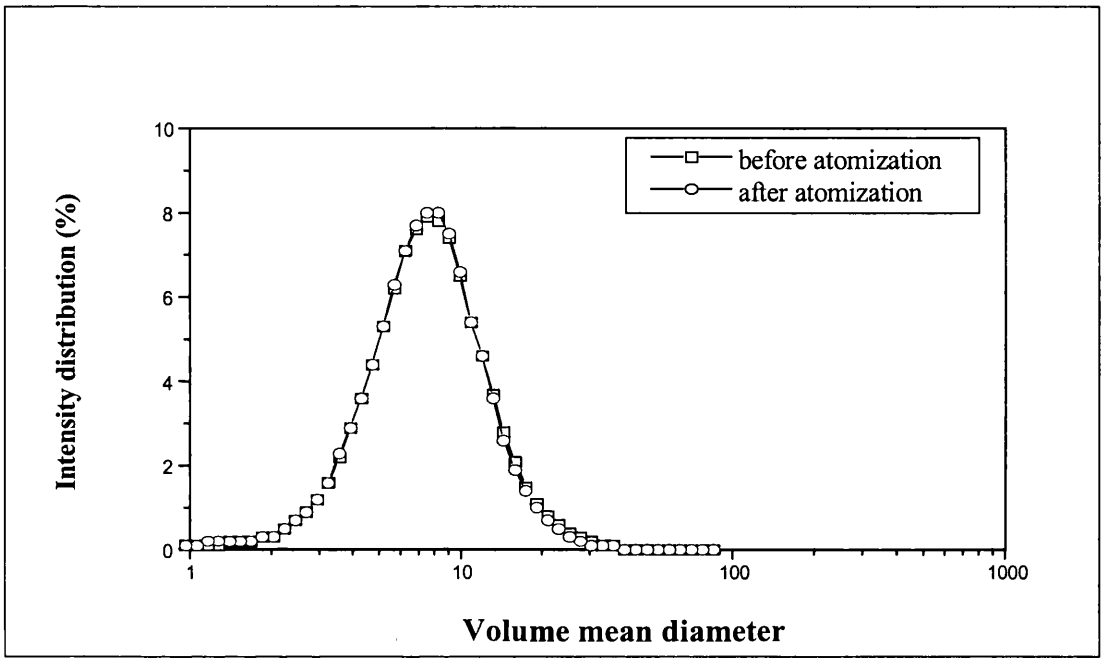

Fig 4.3 Size distribution of empty liposomes made of PC and equimolar CHOL before and after atomization (air at room temperature).

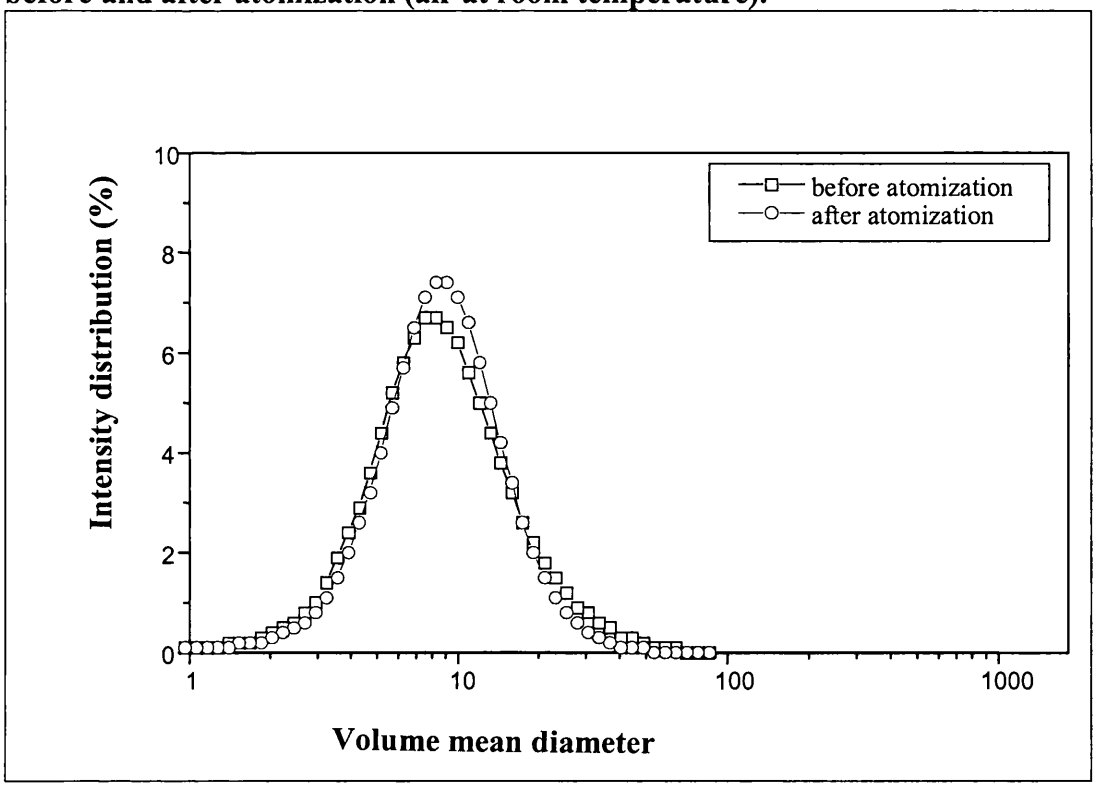

Fig 4.4 Size distribution of empty liposomes made of DSPC and equimolar CHOL before and after atomization (air at room temperature).

4.3.2 Vesicle size distribution and determination of the lipid content

Table 4.1 shows three determinations of the lipid content after spray-drying of the preparations consisting of phosphatidylcholine and equimolar cholesterol.

Table 4.1 Percentage of the lipid recovered (of original amount used) from the spray-dried and reconstituted liposomal suspension

Sugar/lipid mass ratio	Amount of lipid recovered from the dry product (%)
Sucrose at 1/1 (w/w)	93
Sucrose at 3/1 (w/w)	89
Lactose at 3/1 (w/w)	95

The lipid content,expressed as percentage of the originally used amount prior to drying, determined after reconstitution of the spray-dried preparations and lipid extraction was close to the standard liposomes preparations prior to spray-drying suggesting that there was no major loss of lipids.

The vesicle size distribution was determined before and after spray-drying (Table 4.2). The mean diameter of the unextruded liposomes was modified by the spray-drying and by the rehydration cycle, showing that the spray-drying of multilamellar vesicles leads to aggregation and/or fusion of the vesicles. But the mean diameters of the extruded liposomes (Table 4.2) were not significantly modified by dehydration and rehydration

Table 4.2 Z-average diameters of liposomes (PC and equimolar CHOL) before and after spray-drying and rehydration

Carbohydrate	Carbohydrate/Lipid (mg/mg)	Initial Zav diameter(nm) (SD)	Final Zav diameter(nm) (SD)
Lactose	108	283 (16)	281 (10)
Lactose	2.7	248 (15)	295 (7)
Lactose	1	664 (69)	1170 (35)
Sucrose	1	1362 (51)	1967(51)
Sucrose	1	184 (13)	232 (18)

cycle, suggesting that minor aggregation and/or fusion occurs during spray-drying. This last statement is in agreement with the results of Hauser and Strauss (Hauser and Strauss, 1987) and Goldbach and co-workers (Goldbach *et al.*, 1993a). The size of small unilamellar vesicles (SUV) was about the same before and after spray-drying and reconstitution.

4.3.3 Particle size distribution and morphology of the spray-dried product

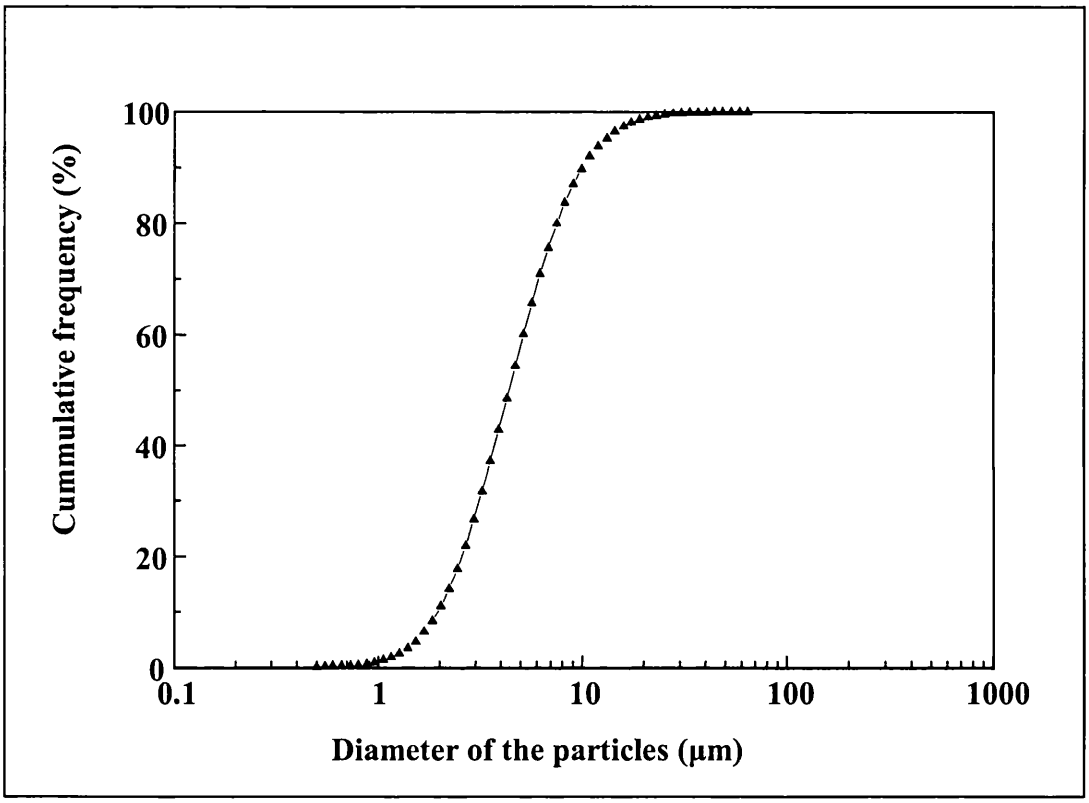

Fig 4.5 Size distribution of the spray-dried product consisting of liposomes and lactose measured in the Mastersizer by dispersion in a saturated solution of lactose.

The particles mean diameter, as determined by the mastersizer, was around 5 μm according to the cumulative frequency curve (Fig 4.5). It is generally accepted that the optimum size for inhaled dry particles ranges between 0.5 and 5 μm (Hindle and Byron, 1995). In such a case, a significant amount of the spray-dried liposome product reaches the lower airways. According to the cumulative frequency distribution, around 90 % of the particles exhibit an average diameter of less than 10

μm. Since the product may contain an amount of absorbed moisture (not determined), no definitive conclusion could be made concerning the inhalability of these powders. Further investigations concerning the stability of the powders at different relative humidities (RH) should be performed

Typical scanning micrographs of the dry product are shown on Figures 4.6-4.7. The particles have a spherical shape and are relatively small-sized. The phosphatidylcholine with equimolar cholesterol based liposomes, when spray-dried in the presence of sucrose, led to melted structures rather than separated spherical particles. The DSPC:CHOL (equimolar) liposomes preparations generated more rigid structures where the particles are separated with a minimum melt observed. The temperature of phase transition (Tc) of the lipids could play an important role in maintaining an adequate mechanical structure, confering to the powder better mechanical properties. Indeed, DSPC presents a higher phase transition temperature Tc (55 °C) than PC (Tc around -10 °C). Therefore, the spray-dried product containing DSPC:CHOL liposomes presents less aggregated particles than the one containing PC and CHOL (equimolar) liposomes.

The examination by SEM of the dry product obtained by spray-drying the PC and equimolar CHOL liposomes in the presence of lactose (Fig 4.8) showed uniform spherical particles of about 5 μm in diameter. For the spray-dried liposomal sucrose the particles were rounded but not totally spherical (Figs 4.6 and 4.7). This has also been reported by Elamin (Elamin *et al*, 1995) This is possibly due to the fact that an amount of absorbed moisture is present within the amorphous structure of sucrose. The combination of the two parameters (residual absorbed moisture and phase transition of lipid) are both determinant in conferring specific properties to the dry product.

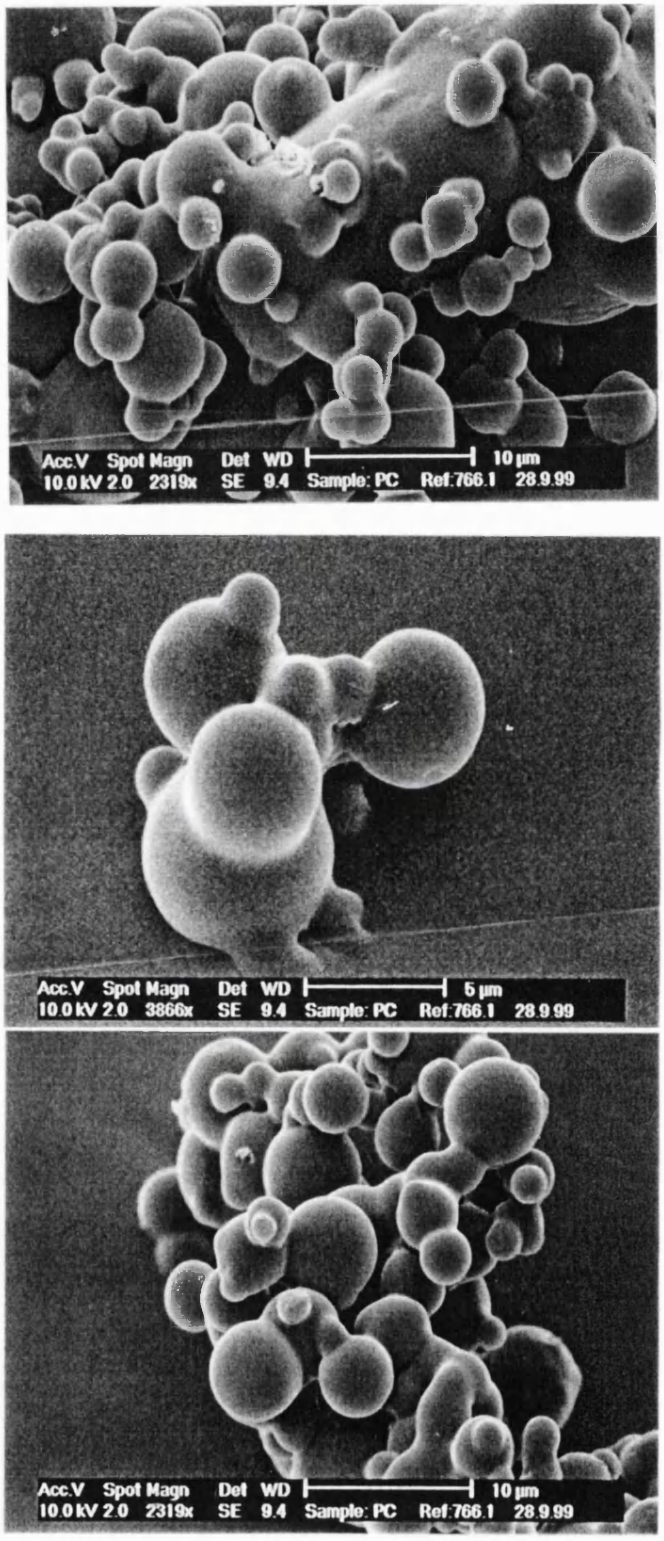

Figure 4.6 Scanning electron microscopy pictures at different magnifications of spray- dried liposomal (PC and CHOL equimolar) powders containing sucrose as a thermoprotectant.

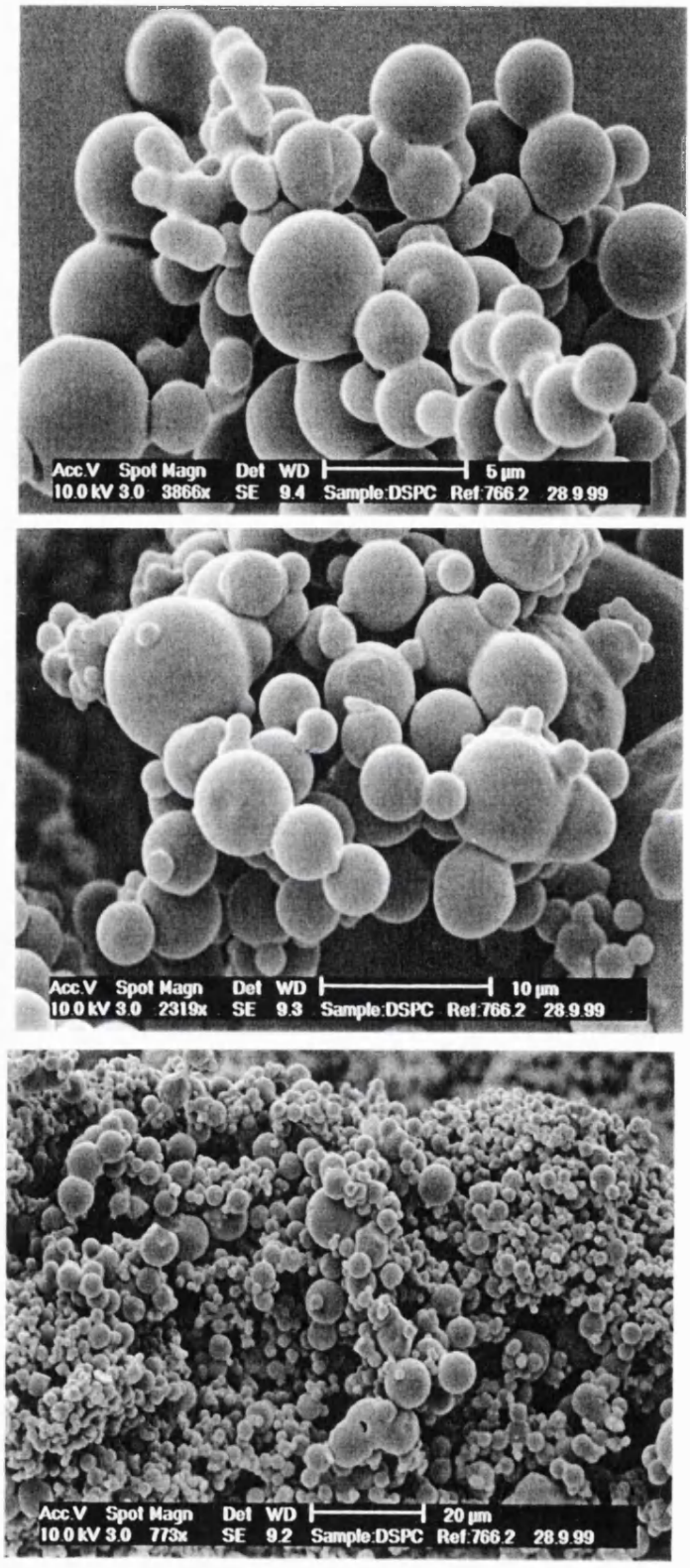

Figure 4.7: Scanning electron microscopy pictures at different magnifications of spray-dried liposomal powders (DSPC and equimolar CHOL) containing sucrose as thermoprotectant.

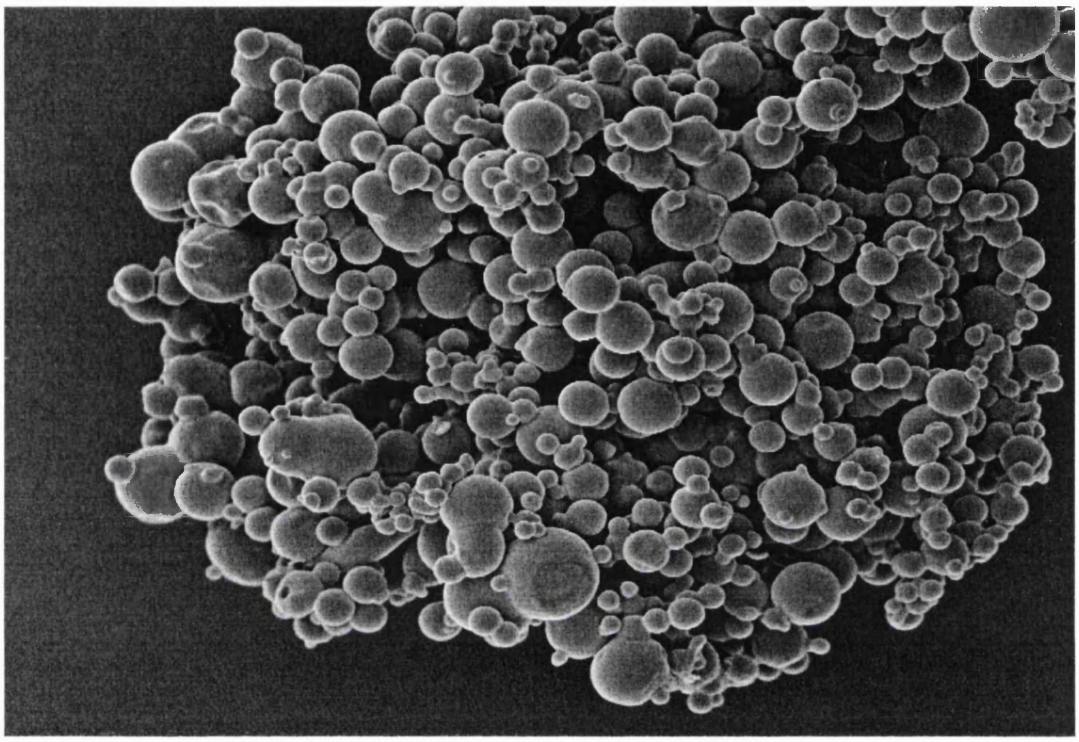

Figure 4.8: Scanning electron microscopy picture of spray-dried liposomal (DSPC and equimolar CHOL) powders containing lactose as thermoprotectant (|——| 20 μm) and exhibiting more spherical shape than liposomal powders containing sucrose as thermoprotectant.

4.3.4 Oxidative damage to the phospholipids during spray-drying

The occurrence of oxidative reactions resulting from the formation of conjugated dienes was examined. Indeed, natural phospholipids only contain double bonds which are non-conjugated and thus have an ultrviolet absorbance peak at a very short wavelength (200-220 nm). The initial step of oxidation of the acyl chain is supposed to involve a free radical chain mechanism which leads to a bond migration and a diene conjugation since conjugated dienes absorb at 233 nm,. The occurrence of oxidative reactions can be monitored by measuring the change in absorbance at this wavelength. The ultra violet spectrum of the lipids extracted from a dispersion of PC and equimolar CHOL shows that the lipids were not oxidised at the time of use. The spectra of the lipids were the same before and after spray-drying(Figs 4.9 and 4.10).

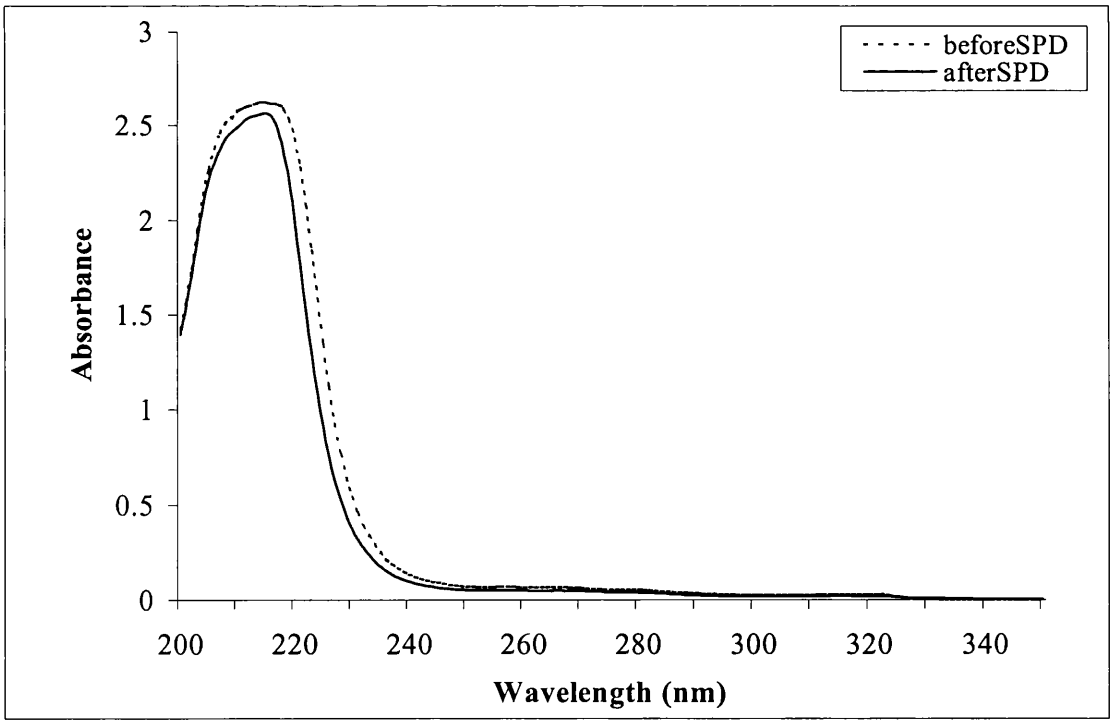

Figure 4.9 Ultra-violet absorption spectra of liposomes made of PC and equimolar CHOL before and after spray-drying (SPD) and rehydration.

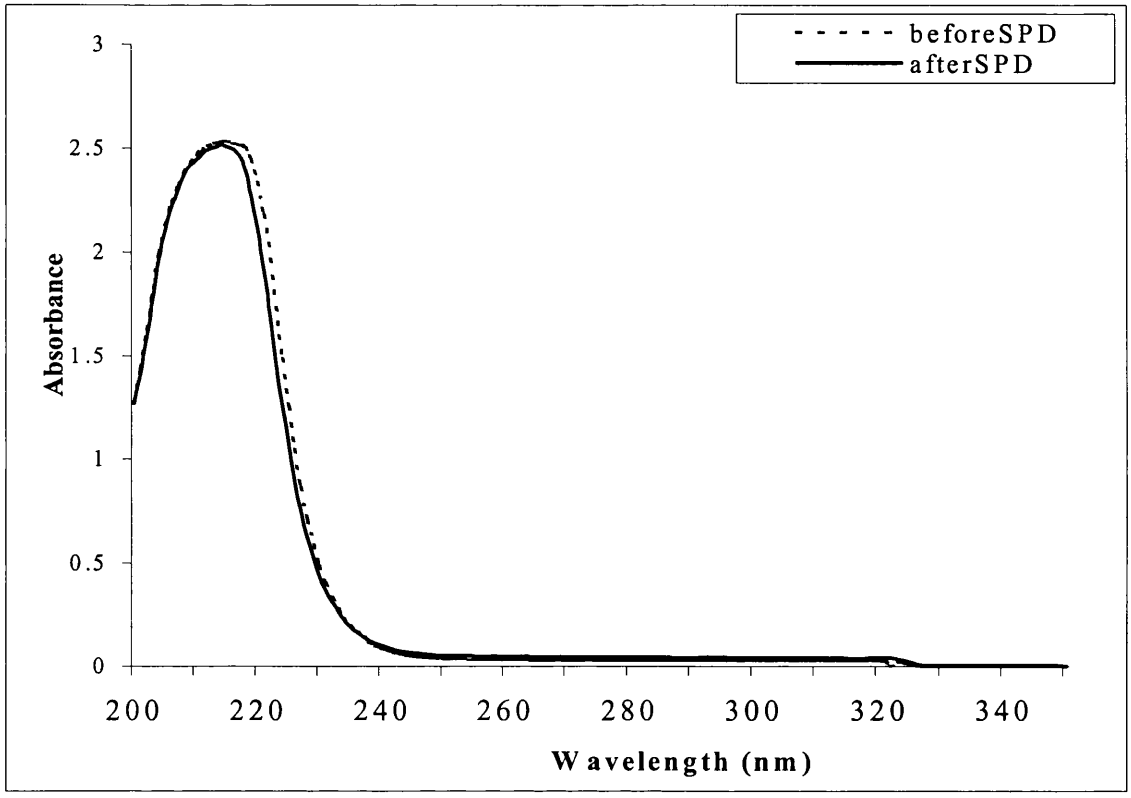

Figure 4.10 Ultra violet absorption spectra of liposomes made of DSPC and equimolar CHOL before and after spray-drying (SPD) and rehydration

4.3.5 Effect of the lipid composition on the stability of spray-dried and rehydrated liposomes: retention of the encapsulated compound

This part of the study was aiming at understanding the parameters which affect the physical stability of liposomes during the spray-drying process, and at achieving a dry product with good properties so that it could be delivered by inhalation or by reconstitution in water before administration. A significant aspect of the physical stability of liposomes is the improvement in the retention of an entrapped low molecular weight water soluble compound by adding carbohydrates as thermoprotectants.

The most convenient method to assess the stability of the liposomes is to encapsulate a small molecular weight water soluble compound and to assess their size distribution and leakage upon spray-drying and rehydration.

Figure 4.11 shows the retention values for the encapsulated glutathione before (dotted bars) and after (grey bars) spray-drying in the presence of the phosphate buffer saline salts (PBS pH=7.4) and rehydration as function of the lipid composition. Generally speaking, drying liposomes preparations from an aqueous medium in the presence of salts results in large vesicular systems after rehydration. This is in agreement with the findings of Goldbach and coworkers (Goldbach *et al.*, 1993a and 1993b).

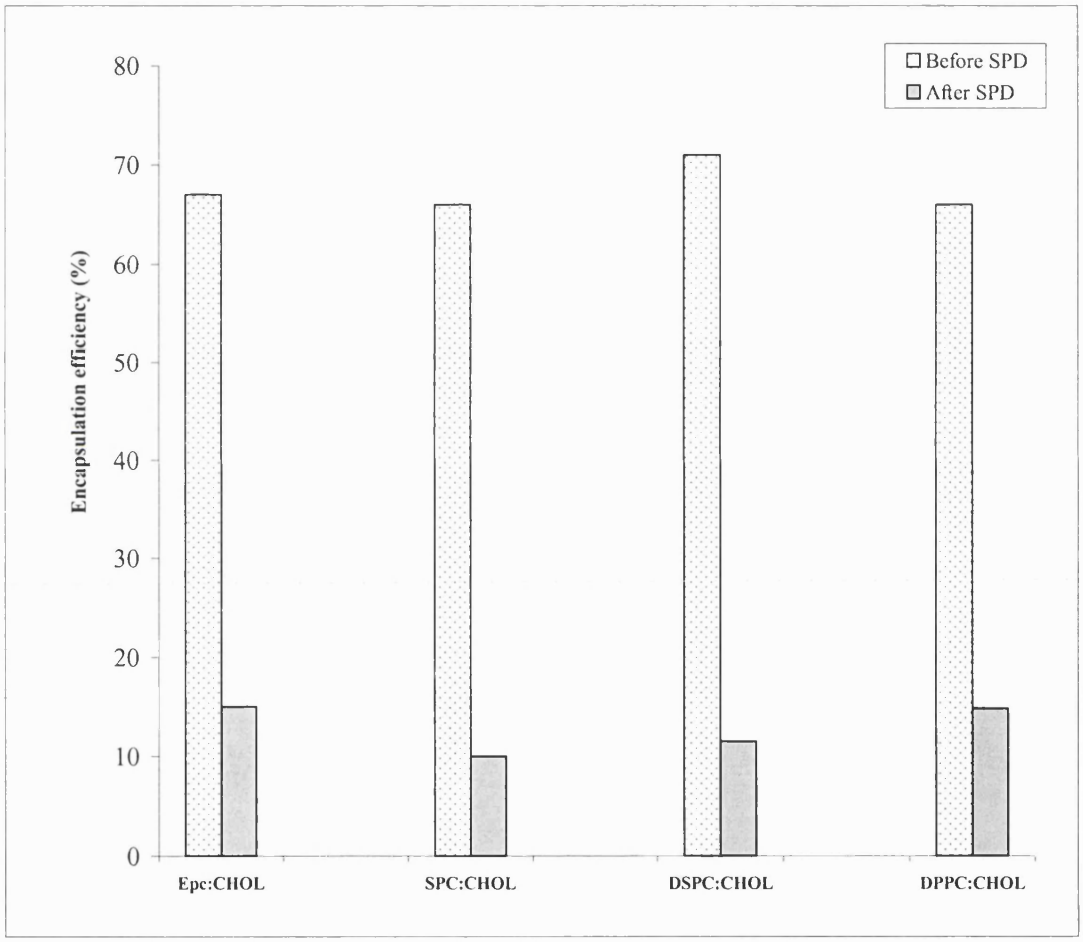

Figure 4.11 Retention of glutathione after spray-drying (SPD) and rehydration in the presence of PBS.

The encapsulated glutathione leaked out of the vesicles independantly of the lipid composition. The retention was around 10-20% of the originally encapsulated glutathione. Parameters other than the bilayer lipid composition could play a role in maintaining the integrity of the dried liposomes. Since all the samples were spray-

dried at an inlet temperature of 110°C, all the samples could be similarly damaged above their Tc.

As a first step, the effect of the bilayer composition on the retention upon the hydration of riboflavin and glutathione was investigated.

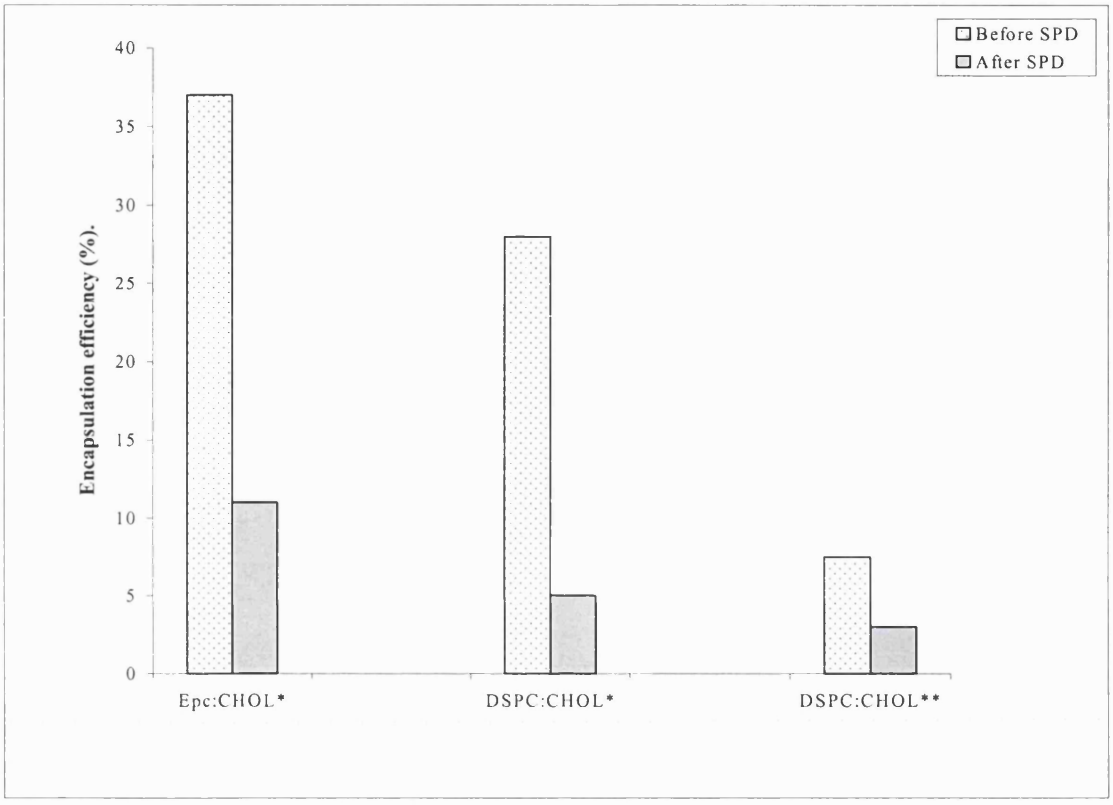

Figure 4.12 Retention of riboflavin before and after spray-drying (SPD) and rehydration in the presence of lactose (* equimolar ; coentrapment of lactose)**

It was not possible to check the effect of the inlet temperature at which the spray-drying was processed because it was difficult to obtain any powder using a lower inlet temperature.

The reconstitution of the vesicles during rehydration constitutes a decisive step during which the leakage phenomena occurs. Glutathione is a water soluble compound that does not interact strongly with the bilayers. The removal of water and the subsequent rehydration affect the liposome integrity to a certain extent. Indeed, the bilayer permeability to encapsulated solutes depends on the degree of disorder in the bilayer

structure and its phase behaviour (Quinn, 1989). Disaccharides were largely reported to protect against damage which occurs during the drying and rehydration cycle (Crowe *et al*, 1987). Lipids in the gel state form vesicles that are less permeable to entrapped compounds than those in the liquid crystalline state. However, varying the lipid bilayer composition from EPC to SPC, DPPC and DSPC in the presence of equimolar cholesterol did not improve the retention of glutathione. Figure 4.12shows the retention of riboflavin of the described liposome preparation upon spray-drying in the presence of lactose and rehydration. The spray-dried powder did not show any shrinkage or collapse during the time of study and it could be easily reconstituted. These data show that despite the presence of carbohydrate, the spray-drying of liposomes causes leakage of the water soluble entrapped riboflavin. Moreover, the influence of the bilayer composition (EPC or DSPC) on the retention was not significant. The difference between the retentions observed after spray-drying and rehydration (around 33%) suggests that the bilayer structure and its integrity is affected during this cycle. The co-entrapment of an excess of lactose led to a poor encapsulation efficiency but to almost 50 % of retention. Quantitatively, there was no overall advantage in using such an approach. The final amount of drug retained was very low.

All the liposome preparations contained equimolar cholesterol as the incorporation of cholesterol into liposomes is known to stabilise phospholipid bilayers and to decrease their permeability to entrapped solutes (Raffy and Teissie, 1999). The leakage was higher when the liposomes were spray-dried in the presence of the phosphate buffer saline salts. Lactose provided a better protection than salts as it was expected. A possible explanation of this leakage is that under the effect of the heat generated by the drying temperature (inlet T= 110°C), the lipid bilayers undergo a phase transition

to the liquid crystalline state. This transition fluidised the membrane which then became very permeable to the entrapped solute. It is indeed well established that gel to liquid crystalline phase transitions of the lipid bilayers result in an increased permeability of the liposomes membrane to the entrapped solutes upon drying and rehydration (Crowe *et al*, 1994).

4.3.6 Effect of the nature and amount of the disaccharide used on the stability of spray-dried liposomes

The amount of carbohydrate used (mass disacharide/mass lipid) was also investigated in an attempt to quantify its impact and the liposome size reduction was checked as means of improvement of the retention upon rehydration.

The nature of the carbohydrate used was reported (in the freeze-drying case) to have an effect on the quality of protection (Tanaka *et al,* 1992, Crowe *et al*, 1987). Here we investigated the effect on three commonly used carbohydrates (trehalose, sucrose and lactose).

It has been reported that when drying, both the amount and the nature of the saccharide used for liposome stabilisation are important factors influencing the retention of the entrapped solutes (Crowe and Crowe; 1988b, Crowe *et al.*, 1988 and Crowe and Crowe, 1993). These studies concerned the investigation of the percentage of solute retention by liposomes as well as their fusion in the presence of various additives. It was reported that the disaccharides trehalose, sucrose, and maltose were in general superior to other sugars in terms of improvement of the retention of the entrapped material.

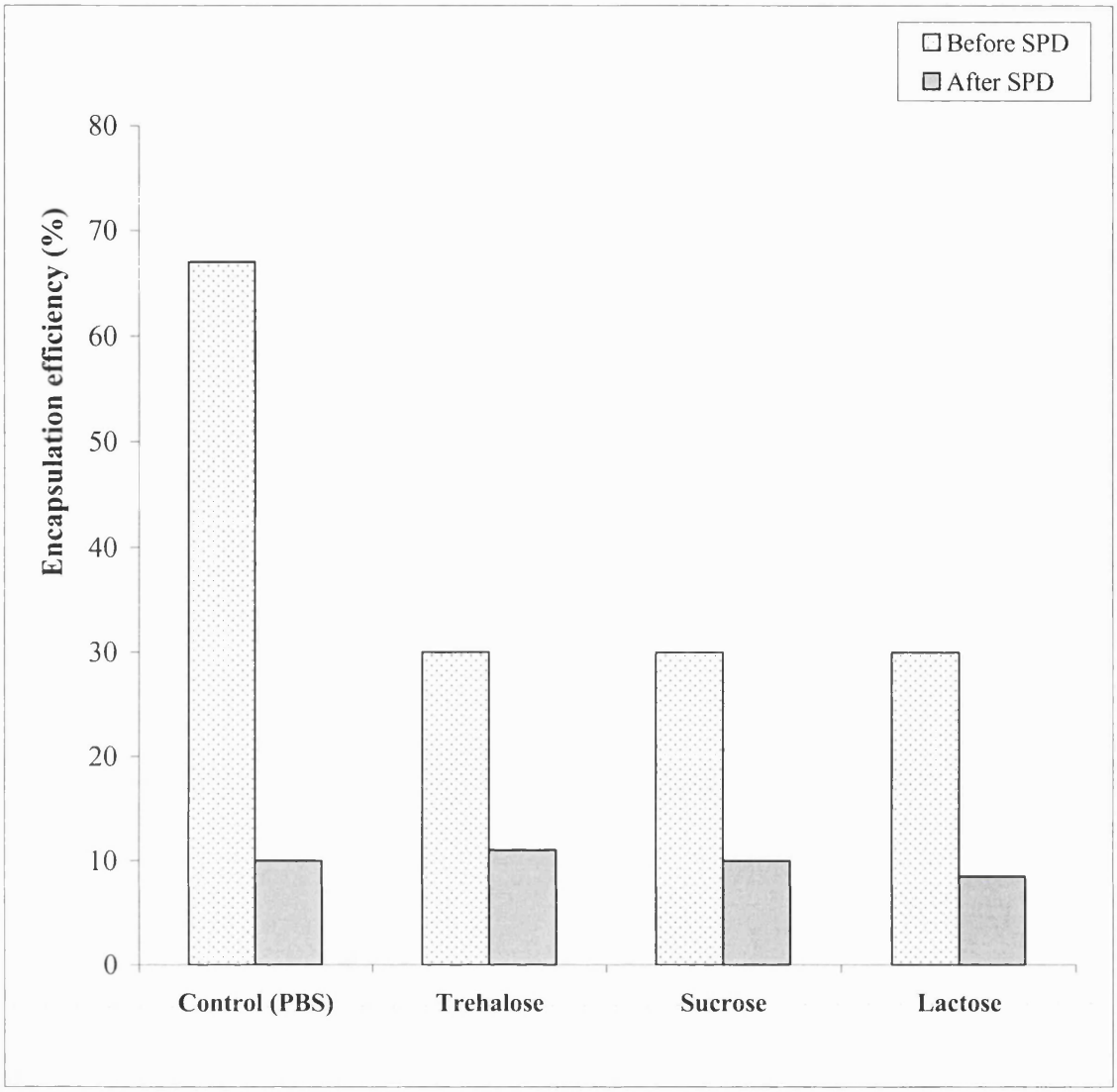

Fig 4.13 Effect of the nature of carbohydrates on the stability of spray-dried (SPD) liposome (PC and equimolar CHOL) in terms of glutathione retention.

Tanaka (Tanaka *et al*, 1992) reported similar finding when trehalose, sucrose and maltose were added during the freeze-drying of sonicated small unilamellar vesicles (mean diameter around 30 nm). Trehalose, sucrose and maltose showed the same protecting effect on these small unilamellar liposomes in terms of retention. Monosaccharides such as glucose, manose and galactose were less effective in improving the retention of the encapsulated solute.

In this part of the study, trehalose, sucrose and lactose were added to the MLV liposomes entrapping glutathione. These preparations were then subjected to spray-

drying . An amount of the obtained dry product was then rehydrated and the amount of glutathione retained by the liposomes assessed after separation from the free drug by ultracentrifugation.

As shown in Fig 4.13, no significant difference was observed among the three dissacharides used. Trehalose, sucrose and lactose were of an equal efficiency in terms of improvement of the retention of the entrapped glutathione (retention around 30 %). The spray-drying process differs from the freeze-drying one as it bears two consequent phenomena: the atomisation of the liposomes suspension into small droplets followed by the removal of water from the droplets to produce particles. In freeze-drying, a crystallisation occurs on freezing which is followed by a drying step. The whole procedure takes a longer period of time and is by far different from the spray-drying one. The fixation of the liposome structure in the matrix of the additive when spray-drying occurs in a fraction of a second. Morever, important considerations of the properties of the vesicles before spray-drying have to be taken into account. The first one is the vesicle size. It may be possible that the drying of the atomised droplets imposes a mechanical shrinking force due to the rapid removal of water which is responsible for the destruction of the arranged structure of the vesicle even before rehydration. An attempt to confirm this hypothesis was carried out by spray-drying liposomal suspensions containing a higher amount of sucrose. The resulting increased viscosity of the suspension could allow the production of dense particles in which the liposomes were conferred a better protection.

In another aspect of this study the liposome suspension was spray-dried in a set of experiments where an increasing ratio of sucrose to lipid were used. The liposome suspension was not extruded prior to spray-drying and exhibited an average diameter of around 1 μm.

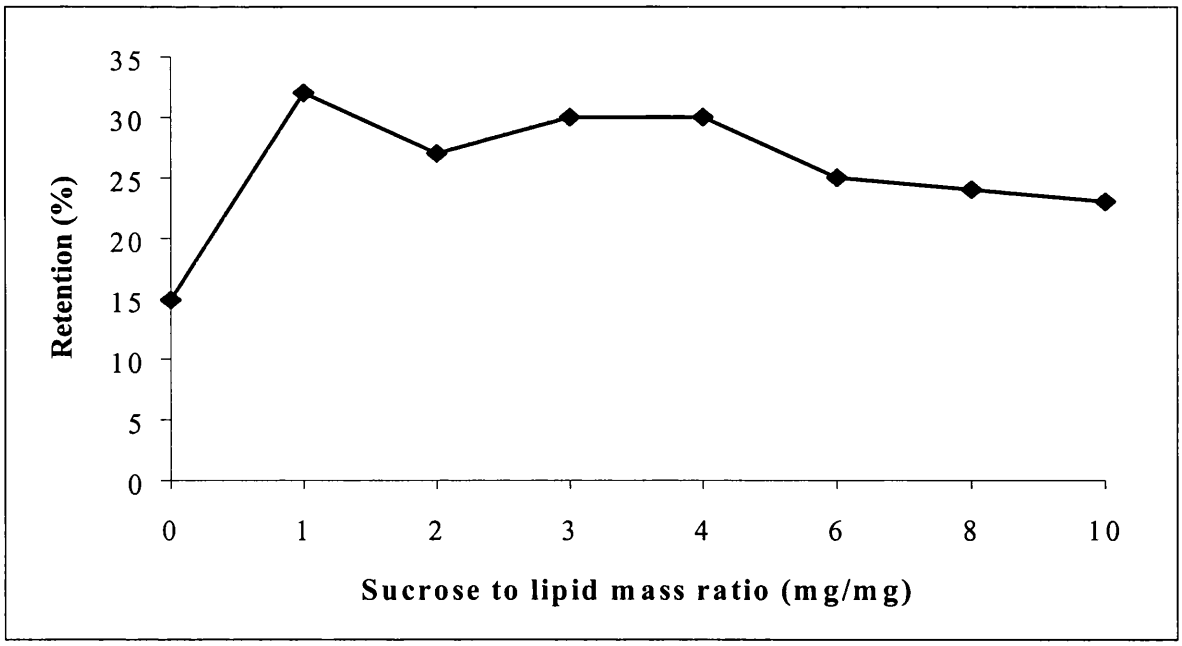

Figure 4.14 Effect of sucrose to lipid mass ratio on the retention of glutathione upon spray-drying and rehydration (unextruded PC and equimolar CHOL liposomes)

Table 4.3 Effect of sucrose to lipid mass ratio on the percent retention of glutathione by liposomes and on vesicle size after spray-drying and rehydration.

Sucrose to lipid mass ratio (g/g)	% Retention	Initial size (nm) (±SD)	Final size (nm) (±SD)
1	32	522(23)	371(43)
2	27	439(52)	505(42)
3	30	810(34)	843(30)
4	30	702(6)	675(35)
6	25	1039(208)	1091(105)
8	24	1194(304)	624(36)
10	23	832(44)	637(43)

As shown in Figure 4.14, increasing the amount of sucrose did not lead to any improvement in terms of retention of the encapsulated glutathione.

Using one gram of sucrose per gram of lipid was as efficient as using ten fold more sucrose (table 4.2) to the same amount of lipid. An average of 30 % of the originally entrapped glutathione was retained.

A possible explanation of this phenomenon is that the vesicle structure is not maintained during the spray-drying and rehydration cycle and that the amount of thermoprotectant is not a determinant in maintaining the integrity of the liposomes.

By contrast, in the freeze-drying situation, studies focusing on the thermal behavior of carbohydrates in the frozen state showed that the thermal and physical properties depend on the concentration of the carbohydrate solution. (Roos and Karel, 1991; Sahagian and Goff, 1994) .

Two thermal phenomena take place when a frozen solution of sucrose is heated. The first one is a glass transition of the sucrose water-system. A second one occurs at a slightly higher temperature and is attributed to the melting of ice crystals. In this temperature region, a collapse of the freeze-dried cake can happen in the early drying stage (Shalaev and Franks, 1996; Mackenzie, 1975). Freeze-drying at a temperature lower than this glass transition temperature hence resulted in the preservation of the porous cake structure.

In the reported freeze-drying studies concerning liposomes/sucrose mixtures, the temperature has to be maintained quite low (- 40°C) and so below the melting or collapse temperature of the frozen-concentrated sucrose (-34°C). The glass will not soften into a viscous liquid, with adverse consequences on the liposomal product.

Generaly, these conditions are considered to be appropriate for the preservation of the physical integrity of the freeze-dried product.

For the liposomes processed by spray-drying, the thermal behavior of an atomised droplet travelling from the drying chamber to the cyclone is different from a frozen-

concentrated system subjected to a long drying stage. It is highly probable, that at some stage, between the atomisation zone and the separation zone (cyclone) the liposome/sugar mixtures undergo a thermic event that is not favorable for the preservation of the liposomal structure and integrity.

4.3.7 Effect of vesicle size reduction on the retention of the encapsulated compound

In earlier studies on spray-drying liposomes (Hauser and Strauss, 1987; Goldbach *et al*, 1993a and 1993b), the liposomes were processed before spray-drying for size reduction. Hauser (Hauser and Strauss, 1987) reported that sonicated liposomes (SUVs arround 70 nm in diameter) were spray-dried in the presence of 0.1 M sucrose. A reconstitution of the dry product in water led to liposomes which conserved their characteristics in terms of size and retention of the encapsulated material.

The size of liposomes was also reported as an important parameter influencing their stability upon freeze-drying in the presence of cryoprotectants and reconstitution in water.

In order to check the effect of the liposomal size on the stability upon spray-drying and reconstitution, the preparations were processed for size reduction, by extrusion, before spray-drying in the presence of sucrose.

Table 4.4 shows the retention values of the encapsulated glutathione after spray-drying and rehydration of liposomes of identical lipid compositions, but of different average sizes. The smaller vesicles retained more marker than the liposomes with an average vesicles size of around 1μm.

Table 4.4 Effect of the liposomal size reduction on the retention of encapsulated compounds.

Lipid composition	Encapsulated compound	Sucrose/lipid mass ratio	% Retention	Initial size (nm)	Final size (nm)
PC:CHOL (1:1 molar ratio)	**riboflavin**	1	17.6	1362	1967
		1	37.2	184	232
		4	36.5	206	196
DPPC:DPPG: CHOL (1:0.2:0.8 molar ratio)	**riboflavin**	1	25	132	204
		4	38.6	178	158
PC:CHOL (1:1 molar ratio)	**glutathione**	1	32	522	371
		1	72.5	142.5	177

The damage caused to the liposomes by drying and rehydration is more dependent on the size of the vesicles than on any other parameter.

Reducing the liposome (PC:CHOL 1:1 molar) size from about 1 μm down to 184 nm resulted in the improvement (by a factor of 2) of the retention of the originally entrapped riboflavin.

Varying the lipid bilayer composition of the vesicles exhibiting an average diameter of around 130 nm in size from PC and CHOL (1:1 molar ratio) to DPPC, DPPG and CHOL (1:0.2:0.8 molar ratio) did not lead to any improvement in the retention of riboflavin. This may be due to the fact that riboflavin does not show any form of interaction with the bilayers. Indeed, the addition of DPPG could improve the retention of compounds thanks to electrostatic interactions between the lipid (DPPG) and compounds that exhibit a positive net charge. When the size of equimolar PC:CHOL liposomes encapsulating glutathione was reduced to around 140 nm, the retention was of 72% of the originallyentrapped glutathione compared to only 32 % for 0.5 μm PC:CHOL liposome spray-dried in the same conditions.

No significant improvement in the retention of riboflavin in PC and equimolar CHOL liposomes was noticed when the ratio of sucrose to lipid was increased from 1g/g to 4g/g.

These findings are in agreement with early reports (Tanaka *et al*, 1992; Crowe and Crowe, 1988b) on freeze-drying. Indeed, Van Winden and Crommelin (Van Winden and Crommelin, 1999) reported that CF retention after freeze-drying and rehydration was higher for the 0.1μm (100nm) vesicles than for liposomes with an average size of 0.2 μm (200nm). This conclusion was valid for a range of lipid compositions. In some experiments, the 200 nm vesicles showed a retention value of CF as low as 15%.

It appears from both these reports and our study that a minimum size of 150 nm is needed in order to obtain a substantial retention of encapsulated water-soluble compounds.

A size of a 100 nm of the liposomes could be considered as optimal for a satisfactory retention as well as an optimal stability in general terms.

It is highly difficult to prepare vesicles of a size of around 100 nm that passively encapsulate a water-soluble compound. Indeed, usual approaches use freeze-thawing as well as freeze-drying and rehydration as a loading step followed by a homogenisation using high pressure homogenizers, such as the microfluidizer. These methods are cumbersome, require many steps, not always reproducible and need high lipid to drug ratios. Furthermore, the encapsulation efficiencies are quite poor. Stabilising such liposomes seems to be pointless because of the many steps involved and of the poor quantities of drug involved. The whole problem of the stabilisation of liposomes resides in the reliability of a novel method to prepare vesicles able to encapsulate an optimum amount of compound and exhibiting an average size of 100 nm. Is it possible to develop a method requiring as few steps as possible but

generating preparations that can be preserved in a dry state and that can exhibit the necessary properties in terms of drug content and size distribution once rehydrated ? Attempts to answer such questions are reported in Chapter 6.

4.3.8 Thermal analysis of spray-dried liposomes with carbohydrates mixtures using Modulated Differential Scanning Calorimetry (MDSC)

As we already discussed, drying is a technique used to improve the stability in systems that are labile when dispersed in an aqueous phase. Liposomes and proteins are dried in the presence of carbohydrate for protection against the dehydration stress and it results in the formation of an amorphous matrix around the liposome or the proteins. There are increasing numbers of investigations concerning the stability of such dried systems especially for protein drugs (Franks and Van Den Berg, 1992; Skrabanja *et al,* 1994). The glass transition temperature (Tg) of the matrix is considered to be a critical parameter regarding the long term stability. A slow chemical and physical degradation is observed at a temperature below Tg. With a temperature above Tg this degradation is accelerated.

The glass transition temperature Tg can be observed as a change in the heat capacity with Differential Scanning Calorimetry (DSC) (Chowdhry and Cole, 1989). But in stability studies other thermal events occur in the same temperature range making the Tg detection difficult.

Modulated DSC (MDSC) has been developped in which a heat capacity corresponding to the heat flow can be distinguished from the total heat flow, making the Tg identification and determination easier (Reading *et al,* 1993 and 1994). With conventional DSC the sample is subjected to a constant heating rate. The heat flow responsible for increasing the sample temperature consists of two components, the first

one is function of the heat capacity of the sample, the second one depends on thermally or kinetically driven processes taking place in the sample. The first component in relation to the heat capacity of the sample is proportional to the heating rate. This particular difference is exploited in MDSC.

In the MDSC technique a small sinusoidal temperature modulation (of a period p and a temperature amplitude T_a) is imposed on the constant heating rate (q). Consequently, the heating rate of the sample is modulated. Within a narrow temperature range the difference in heat flow will only depend on the heat capacity of the sample. A change in heat capacity during the scan will be reflected as a change in the amplitude of the modulated heat flow, making all thermal events visible in the average heat flow.

For extruded and non extruded glutathione liposomes spray-dried in the presence of sucrose, the Tg values obtained from these heat flows records, for a first and a second cooling heating cycles, are presented in Table 4.5 and Figures 4.15.

Table 4.5 The Tg values (cooling and heating) of spray-dried liposomal powders (PC and equimolar CHOL, extruded or unextruded, spray-dried in presence of sucrose)

Sample	Number of cycle	Tg cooling (°C)	Tg heating (°C)
Extruded PC:CHOL (1:1 molar ratio)	First	No transition below 20°C	21.43
	Second	13.78	16.20
Extruded PC:CHOL (1:1 molar ratio)	First	4.31	4.04
	Second	46.24	49.31
Non-extruded PC:CHOL (1:1 molar ratio)	First	10.12	9.91
	Second	5.44	6.56
Non-extruded PC:CHOL (1:1 molar ratio)	First	No transition below 20°C	24.72
	Second	- 8.26	- 9.09

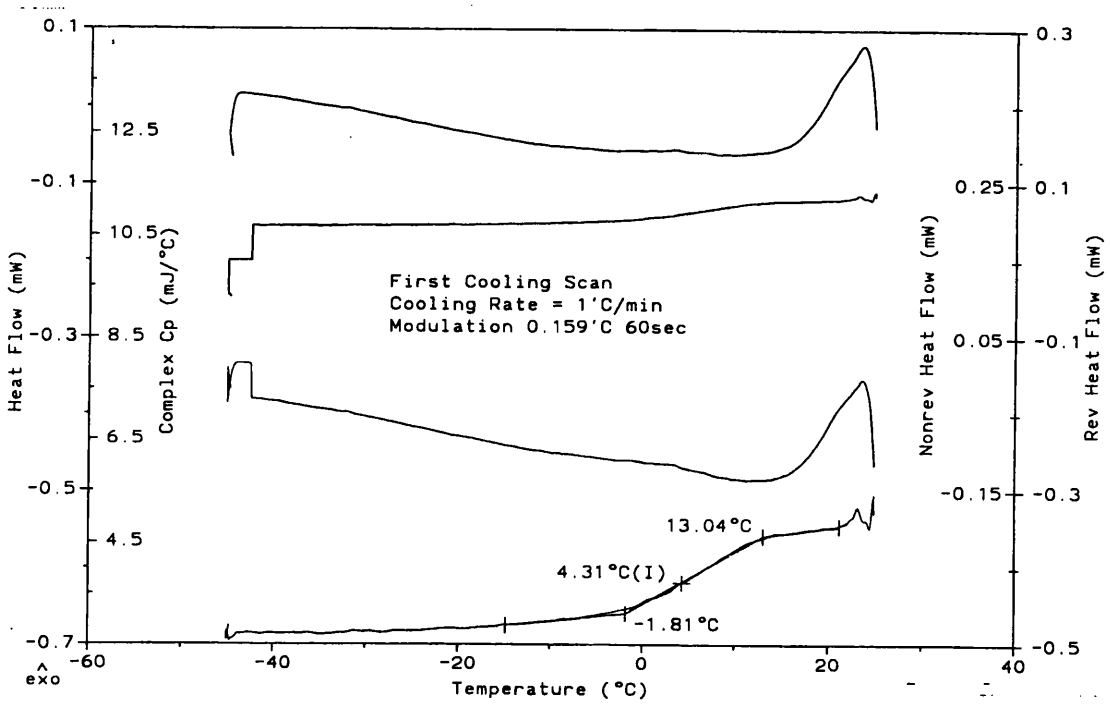

Figure 4.15.1 MDSC profile (first cooling scan) of spray-dried extruded liposomes (PC and equimolar CHOL at sucrose to lipid mass ratio of 3).

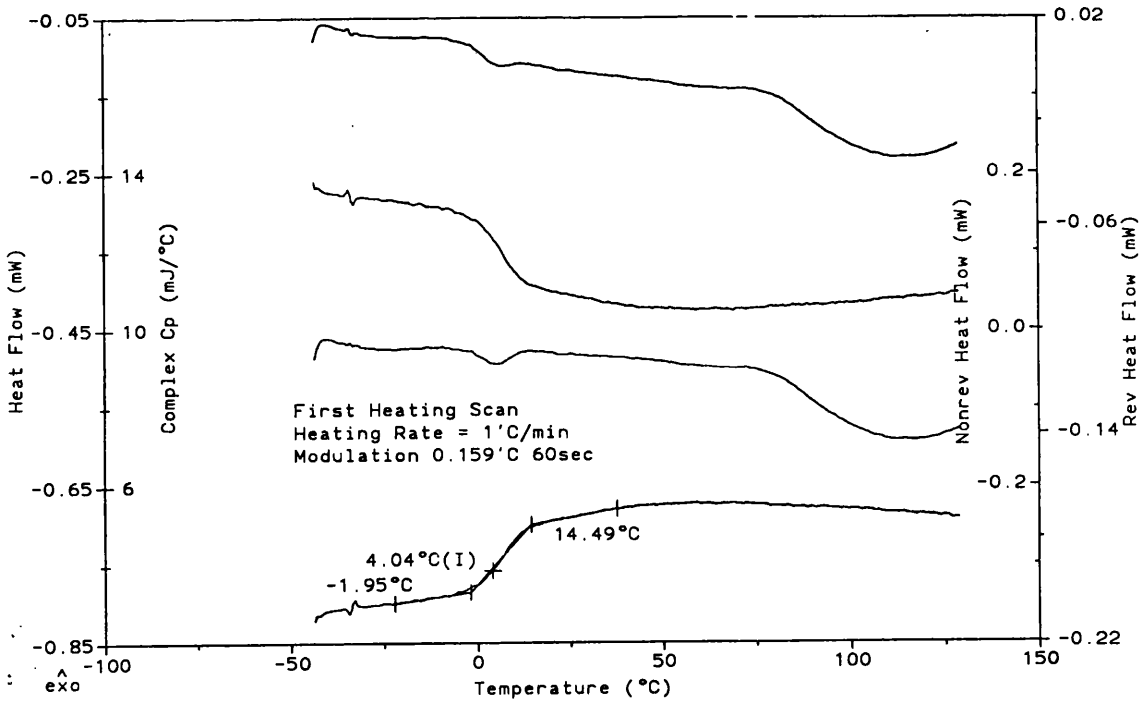

Figure 4.15.2 MDSC profile (first heating scan) of spray-dried extruded liposomes (PC and equimolar CHOL at sucrose to lipid mass ratio of 3)

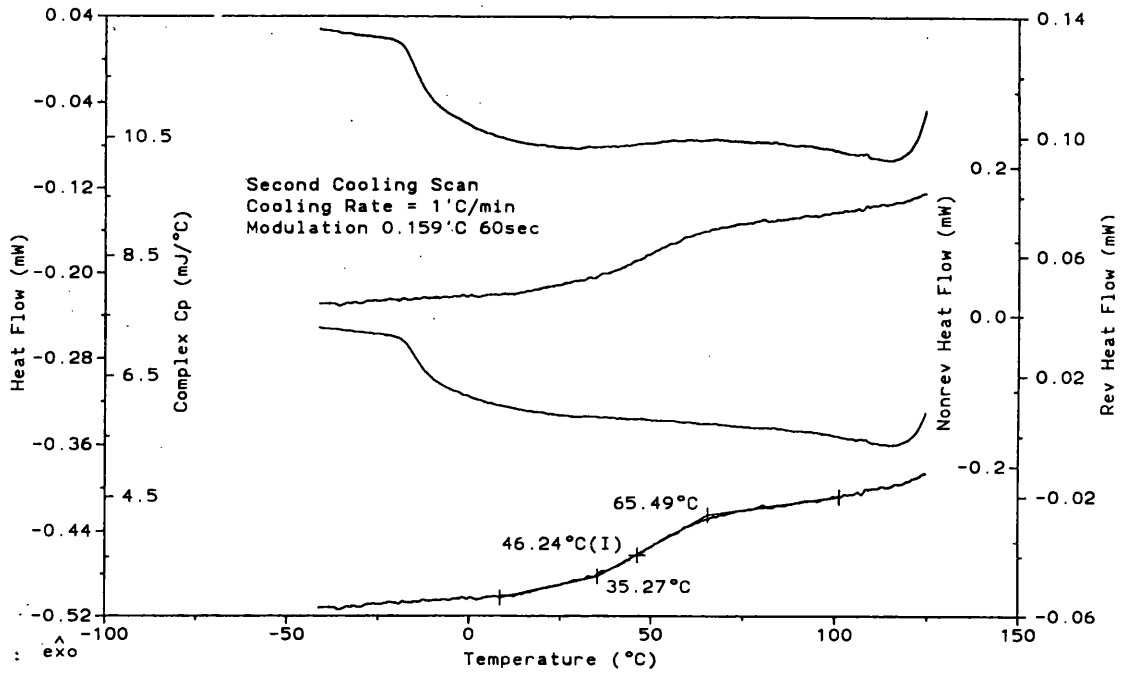

Figure 4.15.3 MDSC profiles (second cooling scan) of spray-dried extruded Liposomes (PC and equimolar CHOL at sucrose to lipid mass ratio of 3)

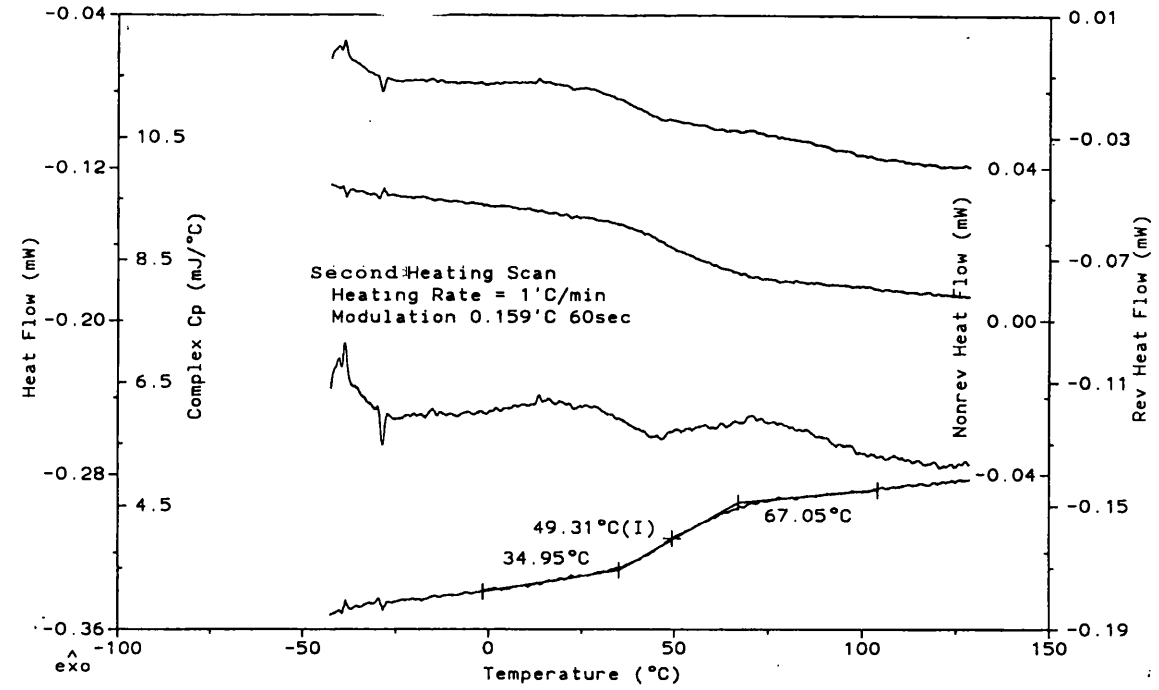

Figure 4.15.4 MDSC profiles (second heating scan) of spray-dried extruded Liposomes (PC and equimolar CHOL at sucrose to lipid mass ratio of 3)

Apparently, the Tg in the total heat flow is a reflection of an endothermal relaxation process which is associated with the glass transition, but it is not correlated with a change in the heat capacity of the sample.

These data reveal that in spite of the carefuly designed sample handling protocol, a major source of variability could be originated by the sampling procedure, the variability of the residual water content in the different vials and between different samples and/or the heterogeneity of the spray-dried powders which occurred upon storage .

Changes in the water content after closing the pans was typically around of 4 to 5% of the sample weight.

These scans were poorly reproducible. They may be artefacts or indicate metastability of the spray-dried powders. It is common that samples are annealed above their Tg to allow the determination of Tg.

Furthermore in the experiment described above, cholesterol containing liposomes had been used and no bilayer transition melting peak was exhibited. It was reported that the presence of cholesterol abolishes the bilayer Tc (Mc Mullen and Mac Elhaney, 1997) and therefore no clear melting behavior could be determined in these samples Cholesterol could possibly act as a plastisizer in the bilayer membrane leading to reversible but non reproducible deformation and transitions in the bilayer membrane.

4.4 Conclusions

In this part of the study, we showed that spray-drying can be considered as an alternative method of drying liposomes in the presence of other additives such as sugars.

Regardless of the possible use of the dry product for inhalation purposes, spray-drying improves the mechanical characteristics of the liposome/sugar mixture, easing its handling. It was reported in a study (Chawla *et al*, 1994) involving planned experiments that the design of the pneumatic nozzle of the spray-dryer was of great importance as it bears the greatest effect on the characteristics of the obtained product. Still, the effect of atomisation was unknown and a set of experiments were conducted in order to check wether it was damaging to the liposomes.

The 0.7 mm pneumatic nozzle of the spray-dryer generates droplets exhibiting a relatively large size (10 – 50 μm) which could explain the results obtained when sizing the liposomes before and after atomization. The overall unchanged size distribution suggests that the thermic phenomena taking place are crucial. Minimising the damage occuring during drying as such requires the consideration of other parameters such as the presence or absence of the thermoprotectant as well as the liposomal characteristics prior to spray-drying.

As shown by the determination of the lipid content, the recovery of the lipid was good. This suggests that there is no seggregation between the various dried compounds and that the powders were homogenous. The dry product obtained was in the form of spherical particles as shown by scanning electron microscopy. The use of lipids exhibiting a relatively high temperature of phase transition can be beneficial as it can confer to the particles a more rigid structure. The DSPC based liposomes products exhibited less aggregated particles than those based on PC.

The hygroscopy of the added thermoprotectant or bulking agent may also have played an important role since it is one of the factors determining the amount of residual moisture. Sugar-based liposomal dry products will exhibit different mechanical

properties from other non-hygroscopic materials used as carriers for spray-drying liposomes.

The mean diameter of the dry product as determined by the Mastersizer was around 5 μm suggesting that an important fraction of the particles, if inhaled, would probably reach the lower airways. At this stage of the study, no definitive conclusion can be drawn about the inhalability of the powders produced. Further investigations involving the measurement of the residual moisture and requiring specific storage conditions should be performed. Here, the sugars were used as thermoprotectant because the dry product was intended for rehydration in water. There is no doubt that the use of other bulking agents will be more beneficial than sugars if the intended use is not resuspension in water. The application for inhalation will require the use of polymers and other materials conferring specific properties to both the mixtures (liposomes/bulking agent) and the liposomes.

Vesicle size distribution determined before and after spray-drying and resuspension in water shows that the unextruded liposomes (MLVs) undergo aggregation/fusion phenomena during which some of the liposome content is lost. Since the liposomes are suspended in water, a liposome extrusion was carried out, bearing in mind the effect of the liposome size on the in-vivo behaviour of injected liposomes. The extruded liposomes contain less encapsulated material and they tend to conserve their mean diameter upon spray-drying and rehydration.

The spray-drying rehydration cycle of liposomes did not induce any oxidation damage by means of a formation of conjugated diene. No second peak appears around the 233 nm wavelength. This suggests that the liposomes were not oxidised before and after use. Normal care, like sonication in ice and under nitrogen, should be taken to avoid lipid oxidation during the handling and the preparation of the liposomes.

The retention of the encapsulated compounds (riboflavin and glutathione) was determined after spray-drying and rehydration of the liposomes.

Generally, drying large liposomes generated aggregated large vesicles from which an important amount of the encapsulated compound leaked out. An improvement in terms of retention could be observed when the liposomes were spray-dried in the presence of sugars such as trehalose, sucrose and lactose rather than in the presence of salts (as PBS pH = 7.4). When the liposomes are subjected to resuspension in water, the carbohydrates play a crucialrole in improving the retention.

The removal of water and the subsequent rehydration affect the liposome integrity. The bilayer permeability to (water soluble) encapsulated compounds depends on the disorder to which the bilayer structure is subjected. Varying the lipid composition entering in the liposome preparation can lead to optimum bilayer structures allowing a better stability. Our study shows that despite the presence of equimolar cholesterol, varying the lipid composition (using lipids exhibiting high Tc) did not lead to the improvement in the retention of glutathione. Despite the presence of lactose, drying and subsequent rehydration made the liposomes (independently of their composition) loose their integrity, generating some leakage of the encapsulated riboflavin or glutathione. The use of salts in the form of PBS (pH = 7.4) as a bulking agent generated more leakage of glutathione than when lactose was used as a thermoprotectant. The drying temperature (inlet temperature) was set at 110°C. The rapid removal of the water from the atomised droplets during spray-drying enables the dry product to stay relatively cool. Normally the dry product never reaches 110°C.

But any possible increase in the temperature of the product affects the lipid bilayer structures as it is forced to undergo a phase transition to the crystalline state. Upon rehydration, this generates a fluidising effect making the membrane more permeable

to the encapsulated solutes. This fluidisation of the membrane also has a dramatic effect even if it is very short in time and whether it occurs before and after rehydration. If the lipid bilayer undergoes this phase transition just before drying, the organisation of the bilayer molecules is brought to a state in which bilayer permeability is increased.

It has been frequently reported in the lit erature that some saccharides are superior to others in terms of improvement of the retention of the entrapped compounds (Crowe *et al*, 1987; Tanaka *et al*, 1992). These studies were about freeze-drying liposomes. Our study drew a comparison between trehalose, sucrose and lactose in their improving effect of the retention of glutathione. No significant difference was observed between the effects of the three disaccharides. All of them maintained the retention of glutathione to the same level. These results could originate from the differences between the drying methods used in our study and the reported work using the freeze-drying technique.

The freeze-drying method involves three steps:

- freezing to a set temperature,
- primary drying at the same set temperature,
- secondary drying at a higher temperature.

All these steps are very sensitive to the composition and the dilution of the formulation subjected to freeze-drying. The variability in the drying conditions from one study to another could explain why some sugars were reported to be better cryoprotectant than others. The number of parameters involved (concentration, cooling rate, lower cooling point, primary and secondary drying time) may also be responsible for the lack of agreement between the various studies. In spray-drying, the fate of liposomes in the matrix of the additive is decided in a fraction of a second. The

drying of the atomised liposome suspension droplets imposes a very rapid removal of the water responsible for the destabilisation of the vesicle structure. The nature of the phenomena involved is probably not sensitive to the nature of the added carbohydrate. Spray-drying liposomal suspensions wtih an increasing amount of sugar does not lead to the improvement of the retention of glutathione. The amount of the thermoprotectant used is not determining in the maintenance of the integrity of the liposomes. The increased viscosity of the suspension resulting in the production of denser particles does not lead to a better protection of the liposomes. Other parameters should be investigated.

The damage caused to the liposomes by the drying/rehydration cycle was dependent on the size of the vesicles. We succeeded in reducing the size to nothing smaller than around 200 nm because of the amount of lipid involved in our experiment and to the use of equimolar cholesterol. This was enough to see the effect of the size reduction on the retention of the marker during spray-drying and rehydration. It was reported (Van Winden and Crommelin, 1997 and 1999) that there was a substantially higher retention of CF in the 0.1 μm vesicles than in the 0.2 μm vesicles. The size reduction is always correlated with a loss of encapsulated material due to the nature of the homogenization technnique used and it therefore leads to stabilized liposomes encapsulating low amount of drugs.

An approach to designing a method of liposome preparation in which the final dry product can be rehydrated to form liposomes exhibiting an adequate size distribution and good entrapment values will be of a great interest. Indeed, the drying and rehydration cycle will induce the loading of the drug in liposomes which will be kept reasonably small. A study was directed towards the development of such a method (see Chapter 6).

In order to evaluate the long-term stability of the dry liposomal product, an MDSC study was conducted to determine the glass transition temperature (Tg) of the matrix. The scans performed were not reproducible. This poor reproducibility could indicate a metastability in the spray-dried powders. Even if the samples were kept at 4°C it is possible that they were annealed above their Tg. Furthermore, the presence of equimolar cholesterol supressed the bilayer transition melting peak. It is unknown whether these two thermic events could interfere and be responsible for the lack of reproducibility.

Chapter five

Stability of spray-dried liposomes after incubation with lung surfactant, Triton X-100, and water

5.1 Introduction

In the literature, liposomal delivery to the lung has been investigated by two general approaches, namely the systemic delivery (Hunt *et al*, 1979, Zachman and Tsao, 1980 Abra *et al*, 1984) and the more direct route of intratracheal or aerosol delivery (Juliano and Mc Cullough, 1980; Oyarzun *et al*, 1980; Wyden *et al,* 1988).

Successful delivery by aerosolisation is dependent on the sustained release of the drug and its availability in the lung after the liposomes have been diluted in the lung fluids. An in vivo determination of the therapeutic efficacy provides an indication of the drug availability and, therefore, its release.

However, it is easier to study drug release from liposome in vitro. Inhaled liposomes will be in contact with the bronchoalveolar fluid. This fluid mainly contains lipids and the specific and very hydrophobic surfactant proteins SP-B and SP-C (Curstedt *et al*, 1990).

The stability of the liposomes is compromised by their interaction with amphiphilic molecules, particularly when the vesicles are delivered into physiological fluids. It is therefore important to consider the way in which the liposomal content is released in the presence of foreign molecules that may interact and destabilise the phospholipid bilayer.

A variety of surfactants effectively perturb the bilayer architecture (Helenius and Simons, 1975 , Lichtenberg *et al*, 1983). Triton X-100 which is of a synthetic origin interacts with the bilayers (Goni *et al*, 1986) and is extensively used to release the content of the vesicles for entrapment determinations.

The lung surfactants act by decreasing the interface surface tension between alveoli and air. In this study, the leakage (release) characteristics of spray-dried liposomes

encapsulating the water soluble marker riboflavin incubated with lung surfactant and triton X-100 were considered.

5.2 Materials and methods

Liposomes of varying lipid compositions and containing riboflavin were prepared and extruded when necessary as described in Materials and Methods. The preparations were spray-dried in the presence of sucrose as already described.

A dialysis technique was used as described in 2.3.20 and as follows under 5.3.1.

5.3 Results and discussion

5.3.1 Methodological aspects

The procedure adopted to assess the effects of surfactants on the release of liposomal contents consisted in treating the liposomal sucrose powders with one amount of detergent to achieve a ratio of 2:1 (w/w) of lipids to surfactants. The mixtures were placed in a dialysis bag and then immediately submerged in 25ml of phosphate buffer saline (PBS pH= 7.4). No time was allowed for equilibration even though it has been reported that these systems undergo a complete equilibrium after a period of time for incubation (Lichtenberg *et al*, 1979; Alonso *et al*, 1987).

However after such equilibrium time, detergent-induced release of liposomal contents would be masked by the concomitant spontaneous diffusion of solutes outside the vesicles. Also, in the real inhalation conditions, the amount of water present corresponds to the amount of water present in the inhaled air, at 37°C, saturated with humidity. The methodology used to test these powders creates an exagerated

condition of rehydration with the presence of an excessive amount of water. Furthermore, water molecules will cross the dialysis bag diluting the surfactants (lung surfactants and triton) and at the same time suspending the liposomes in an aqueous system.

In reality, inhaled liposomes will rather be in a viscous medium composed of lung fluids. Aliquots were taken at time intervals to assess the amount of riboflavin released from the dialysis bag.

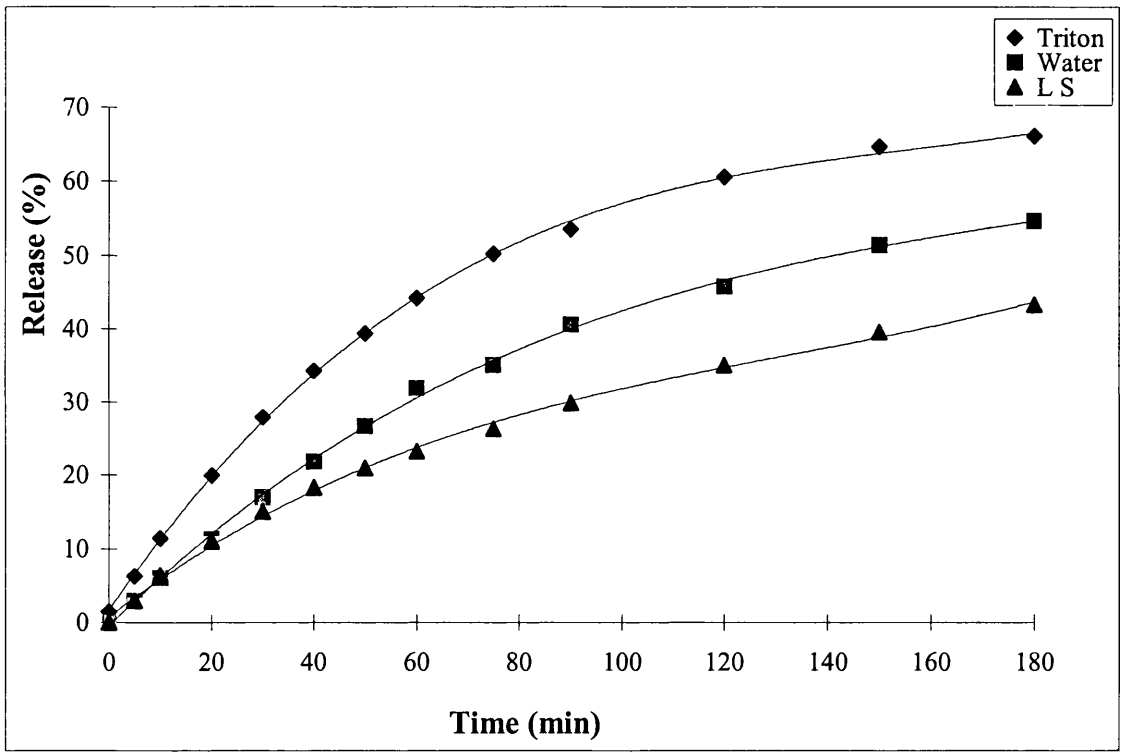

Fig 5.1 Riboflavin release from unextruded PC and equimolar CHOL spray-dried liposomes incubated at 25°C.

According to Fig.5.1, the riboflavin release from unextruded spray-dried PC and CHOL (equimolar) liposomes exhibit a similar profile for triton, water and lung surfactant. In these conditions it is possible that a rehydration step of the powder occurs prior to the interaction of the surfactant molecules with the bilayer membrane.

The liposomal destabilisation occuring in the presence of surfactant can mainly be attributed to either the removal of the phospholipids from the bilayers or to the formation of a loop within the outer monolayer leaflet of the lipid membrane (Jayusira *et al*, 1990). The defects created by the surfactant depend on the chemical structure of this disrupting molecule. Certain molecules will create deeper and larger defects than others (Nagawa and Regen, 1992). In the case of the removal of phospholipid molecules from the bilayer, formation of micelles between the surfactant and the phospholipids is expected to occur.

Concerning the release of riboflavin, the above results show that triton X-100 induces the highest permeability in the membrane. This is in agreement with the reported use of triton to release the content of vesicles. As a general observation, the release of small molecular markers from liposomes such as riboflavin is sometimes spontaneous. A leakage of small molecular weight molecules naturally occurs during storage without the help of any solubilising or destabilising agent In these experiments, we cannot differentiate between spontaneous leakage and leakage due to bilayer solubilisation inducing riboflavin release. Furthermore, the rehydration step constitutes an additional factor generating leakage of the encapsulated riboflavin. When the incubation was carried out at 25°C, 40 % of the liposomal content leaked out in 50, 90 and 180 min after incubation with triton, water and lung surfactant respectively. Triton was the most disruptive agent followed by the situation of simple rehydration with water. The lung surfactant exhibited the least disruptive activity. The conditions of the experiment did not allow the observation of the consequences of the rehydration step separately from those generated by all the other phenomena. An important part of the leakage could occur during the liposomes reconstitution (vesicle formation). Furthermore, the dialysis bag rendered the leakage extended in time. It is

likely that the most important part of the leakage occured instantly and is less time dependant than it appears.

5.3.2 Effect of the temperature of incubation

By increasing the temperature of incubation from 25°C to 37°C (Fig 5.2), the difference between the release profile generated by incubation with Triton, water and L.S. were not significant.

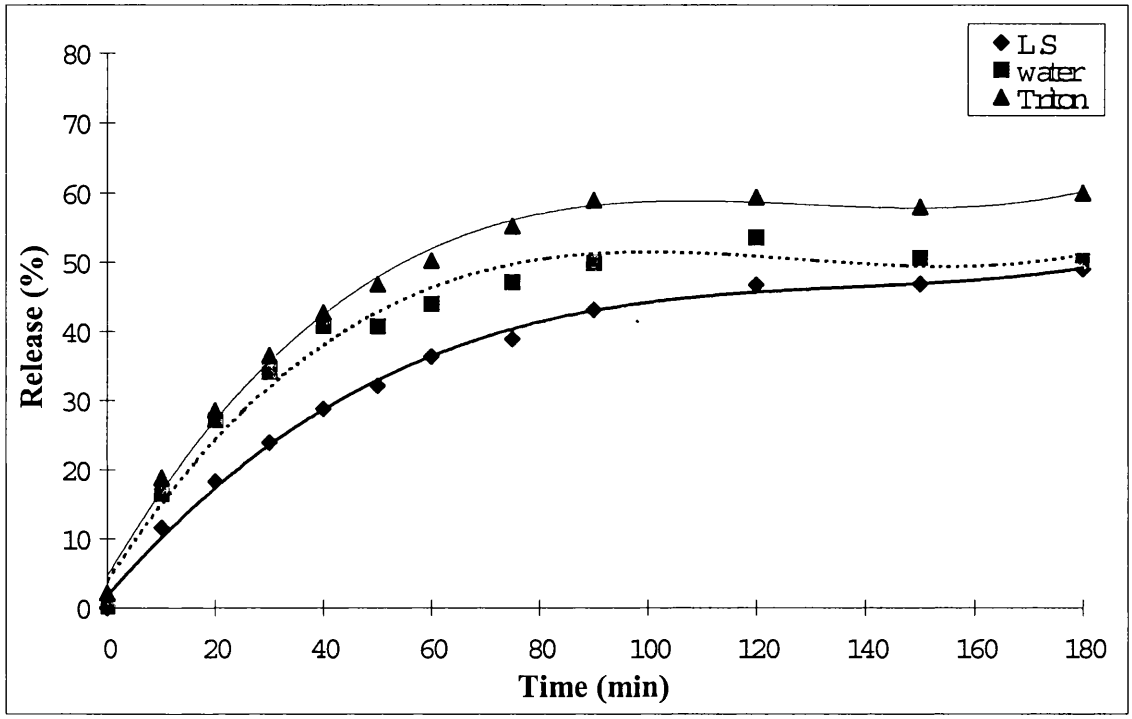

Fig 5.2 Riboflavin release from spray-dried PC and equimolar CHOL unextruded liposomes incubated at 37°C

The time required for the release of a relatively simillar amount of riboflavin varied slightly for lung surfactant water and triton. This situation brings up the consideration of another parameter which is the fluidity of the liposomal membrane. At a high temperature, the fluidity of the membrane of PC and CHOL (equimolar) liposomes is expected to be higher. The rigidity of the membrane seems to be more important in regulating the leakage of the small molecular weight marker riboflavin. At a high temperature of incubation the liposomal membrane exhibited less stability. The

leakage was less dependent on the nature of the destabilizing agent (triton, L.S. and water)

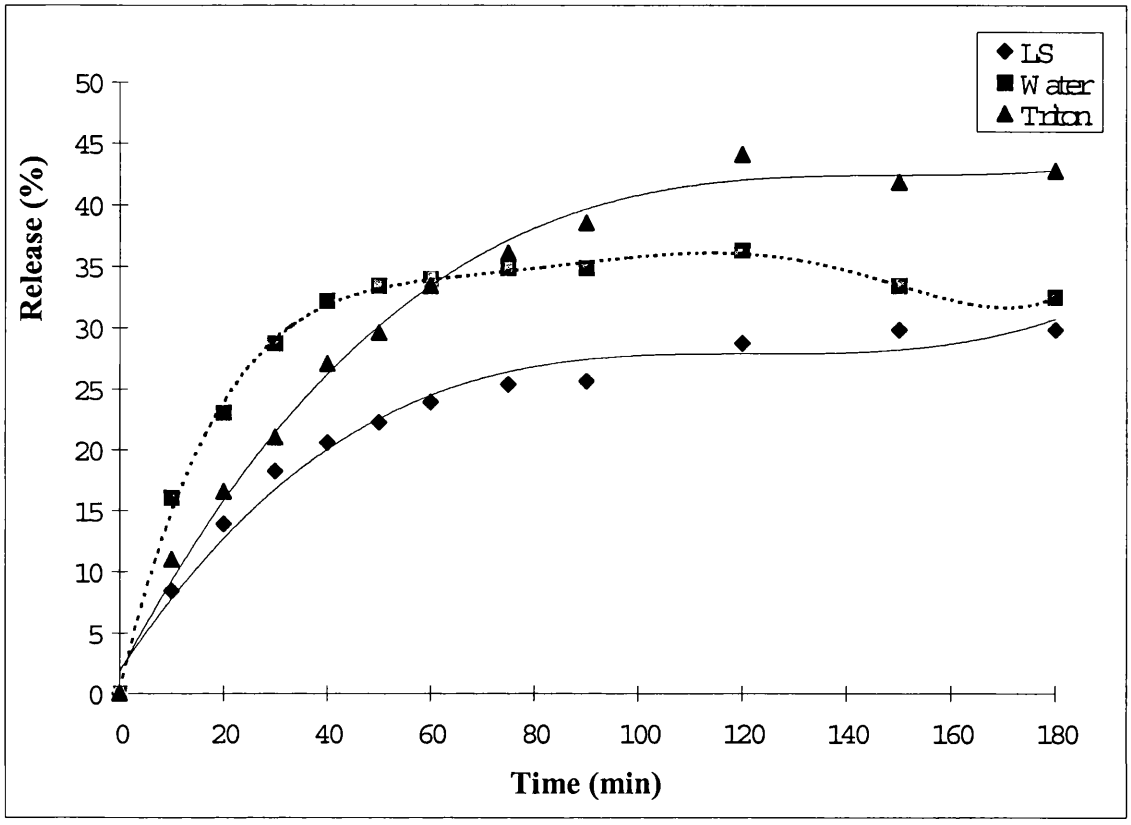

Fig 5.3 Riboflavin release from DSPC and equimolar CHOL spray-dried liposomes incubated at 25°C

The fluidity of the liposomal membrane is regulated by the phase transition temperature (Tc) of the main lipid constituting the bilayer. The phase transition (Tc) of DSPC is around 55°C. DSPC-based liposomal membrane exhibited less fluidity and apparently more stability when in contact with these type of destabilising molecules. At 25°C (Fig 5.3), the rehydrated DSPC-based liposomes are probably not fully reconstituted allowing the riboflavin to be released from the possibly fragmented-liposomes much more easily than when the liposomes are properly and fully reconstituted.

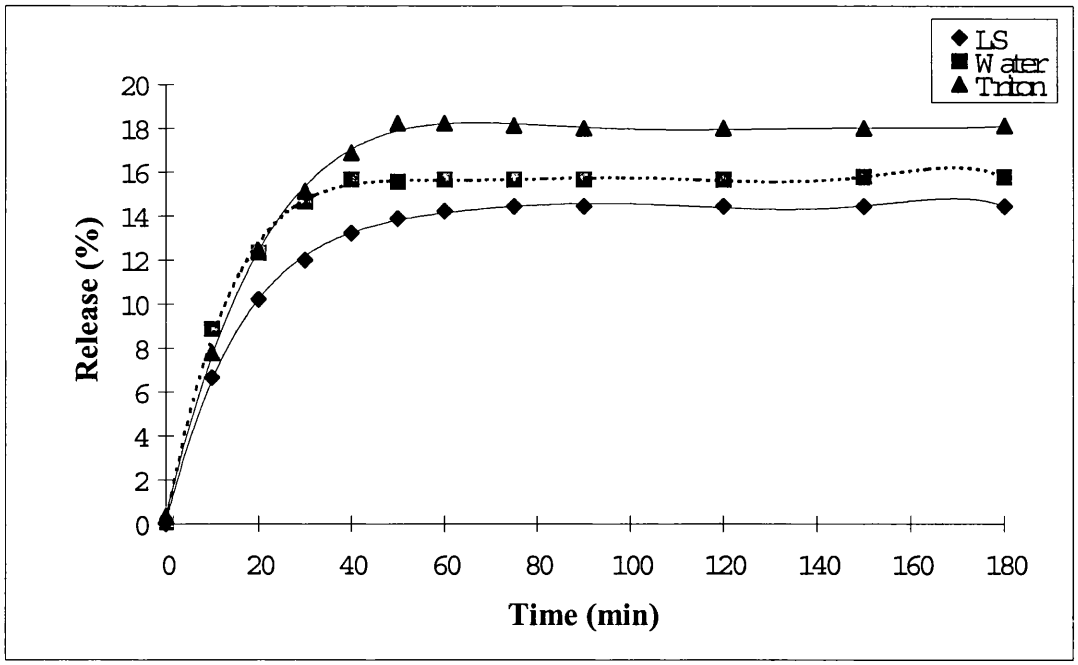

Fig 5.4 Riboflavin release from DSPC and equimolar CHOL spray-dried liposomes incubated at 37°C

Indeed, by increasing the temperature of incubation to 37°C (Fig 5.4), the DSPC and CHOL (equimolar) liposomes released only around 15% of the originally encapsulated riboflavin after 3h of incubation with lung surfactant.

5.3.3 Effect of size reduction by extrusion

Generally, liposomes extrusion generates homogeneous vesicles with decreased lamellarity. This is often considered as one of the factors contributing to the improvement of liposomes circulation half-life after intravenous injection. To the contrary, smaller liposomes are less stable in presence of blood plasma components. The lipid composition is a much more determining factor for stability. In this study, the extruded liposomes incubated with L.S. or Triton at 25°C showed a release profile where the lung surfactant and water exhibited quite similar values and where triton X-100 generated a faster release (Fig 5.5)

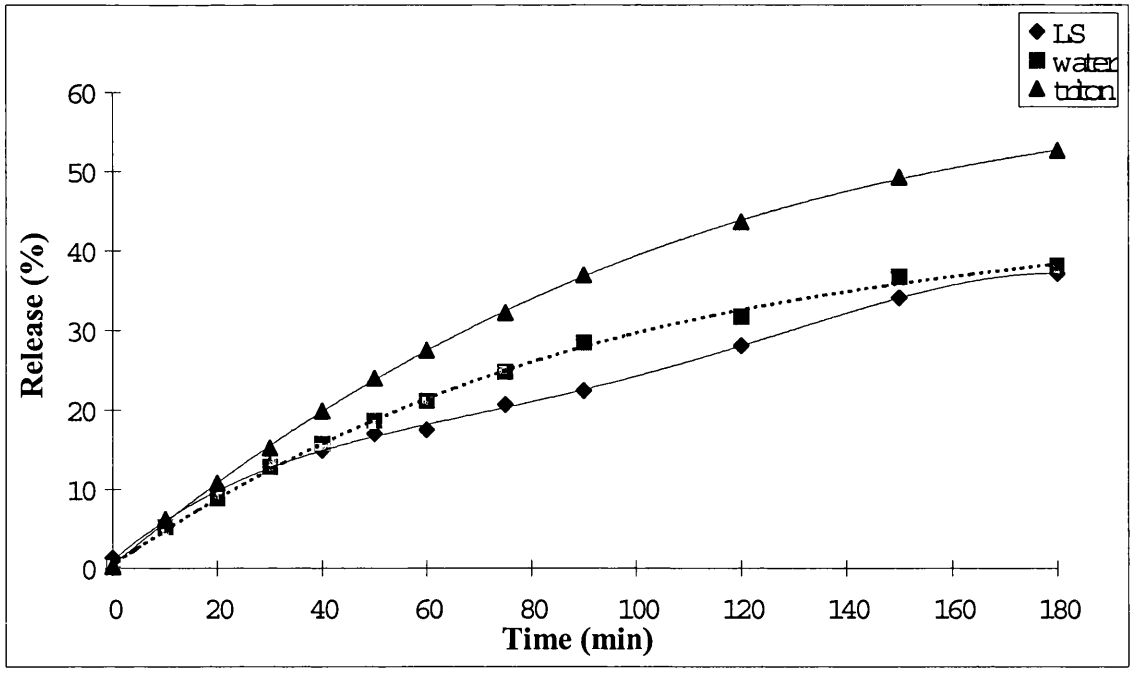

Fig 5.5 Riboflavin release from PC and equimolar CHOL extruded liposomes incubated at 25C

Triton X-100 exhibited a higher destabilising action because of the uni- or ol igolamellarity of the extruded vesicles.

On the other hand the lung surfactant showed a slight difference in the release profile in the case of extruded and unextruded liposomes at 25°C. The mechanism of action of triton is more disruptive than the lung surfactant. It may even be that lung surfactant promotes the reconstitution of vesicles rather than disrupt them. By increasing the temperature of incubation to 37°C (Fig 5.6), the release of riboflavin was more pronounced for the first 60 min. The general trend was maintained, the release profile showing that triton X-100 is still the most disruptive in all the cases.

Figures 5.7 and 5.8 show the release profile of extruded or unextruded liposomes incubated with lung surfactant at 25°C and 37°C.

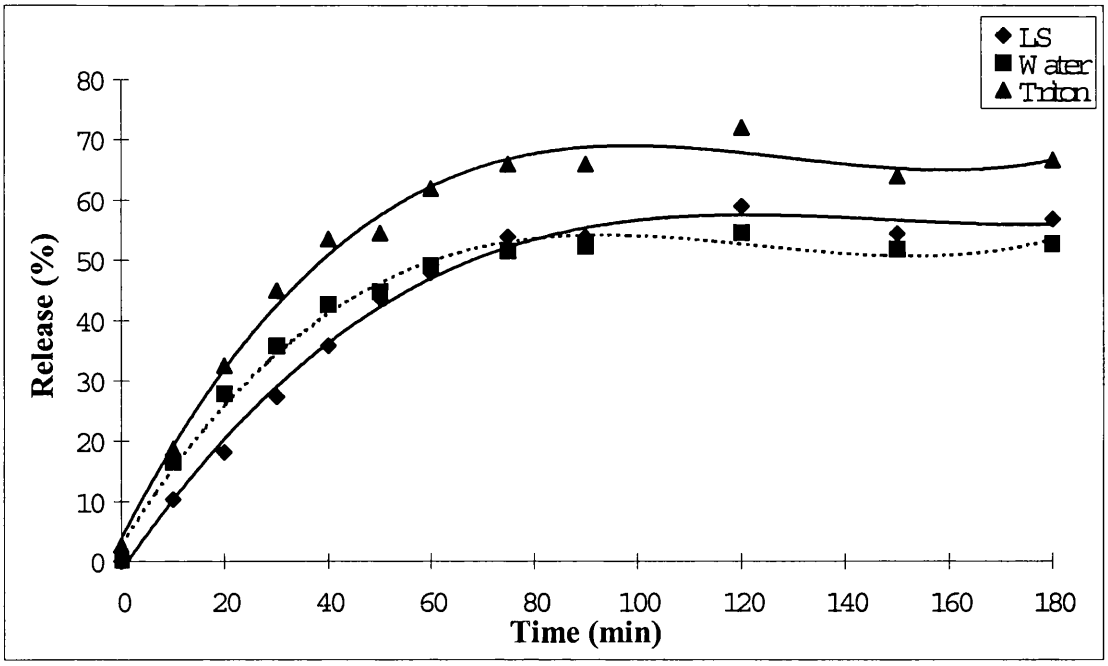

Fig 5.6 Riboflavin release from extruded spray-dried PC and equimolar CHOL liposomes incubated at 37°C

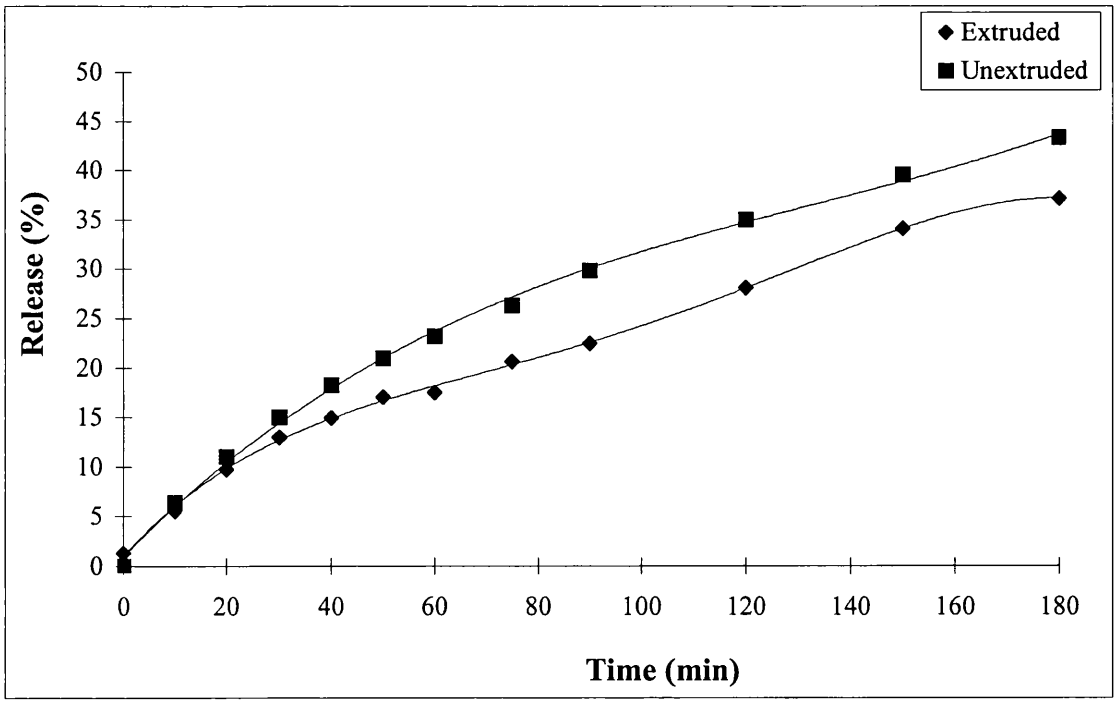

Fig 5.7 Riboflavin release from spray-dried PC and equimolar CHOL extruded and unextruded liposomes incubated with lung surfactant at 25°C

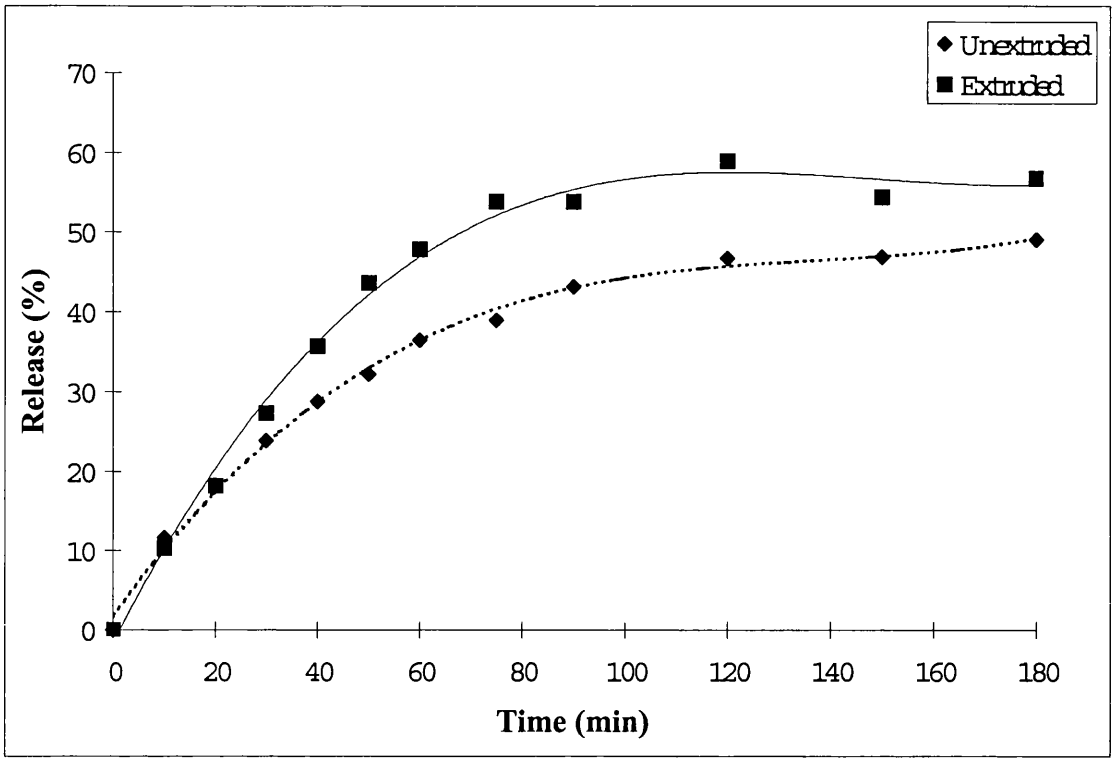

Fig 5.8 Riboflavin release from spray-dried PC and equimolar CHOL liposomes incubated with lung surfactant at 37°C

These results suggest that both the fluidity of the bilayer membrane and the lamellarity are to be taken in consideration as factors affecting the stability of liposomes. At a relatively low temperature (25°C) the effect of the size on the stability is predominant. At a relatively high temperature (37°C), the effect of the membrane stability, overrides the size effect. The multilamellar liposomes become more stable than the unilamellar ones (PC and equimolar CHOL liposomes) incubated with lung surfactant.

5.3.4 Effect of the nature of the destabilising surfactant

In another aspect of this study, we formulated liposomes to contain the lipid dipalmitoyl phosphatidylcholine (DPPC). The lung surfactant used, Survanta® , is a modified bovine lung extract containing mostly phospholipids and modified by the addition of dipalmitoyl-phosphatidylcholine, palmitic acid and tripalmitin. In this

regard, liposomes containing DPPC will possibly present a kind of neutrality towards the lung surfactant used for incubation. Indeed, it has been reported (Ikegami *et al,* 1985) that liposomes, in suspension, constituted of dipalmitoyl phosphatidylcholine associate with natural surfactants such as lung surfactant.

For this purpose, we used two different types of liposomes: DPPC, CHOL and DPPG at a molar ratio of 1:0.8:0.2 respectively, and DSPC with equimolar CHOL aiming at evaluating the effect of the liposome component (DPPC) on the L.S. used for incubation

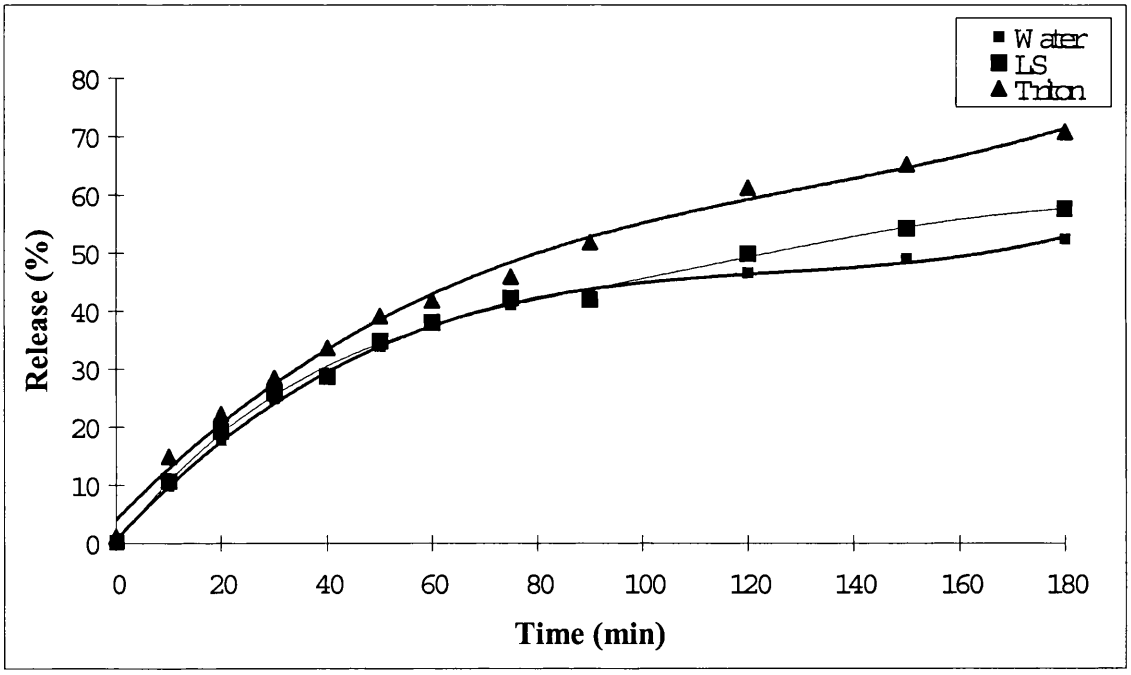

Fig 5.9 Riboflavin release from DPPC:DPPG:CHOL (1:0.8:0.2 molar ratios) liposomes incubated at 25°C.

Figure 5.9 shows the release profile of riboflavin from the DPPC, CHOL and DPPG (1:0.8:0.2 molar) based liposomes incubated at 25°C. No major difference was observed when incubation was carried out in the presence of lung surfactant or simply when water was used to rehydrate the sample. Triton gave slightly higher release rates.

Almost half (50%) of the encapsulated riboflavin was released after 3 h of incubation. This phenomena could be explained by the fact that the DPPC constituent of the liposomes could adopt a kind of neutrality towards the lung surfactant molecules allowing a situation of reconstitution comparable to the one where only water is added.

When the temperature of incubation was increased from 25°C to 37°C , an increase in the amount of riboflavin released was observed for the DPPC based liposomes (Fig. 5.10 and 5.11). The release profile for the simple rehydration (water) was still superposed on the release profile of riboflavin from the same liposomes when incubated with the lung surfactant. The neutrality of the L.S. towards the DPPC based liposomes did not lead to any significant improvement in the release of riboflavin.

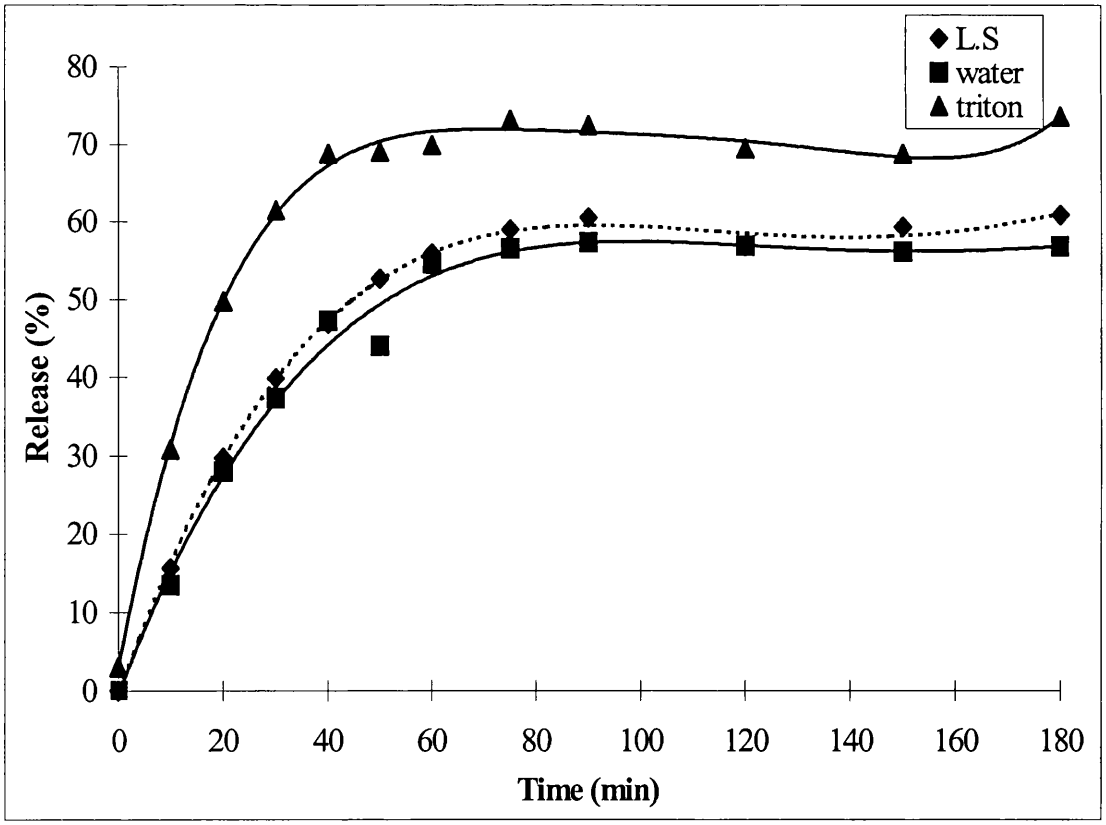

Fig 5.10 Riboflavin release from DPPC:DPPG:CHOL (1:0.8:0.2 molar ratios) spray-dried liposomes incubated at 37°C.

By contrast, the DSPC and equimolar CHOL liposomes (Fig.5.11 and Fig 5.12) were more stable than the DPPC, DPPG and CHOL (1:0.8:0.2) and the PC and equimolar CHOL ones

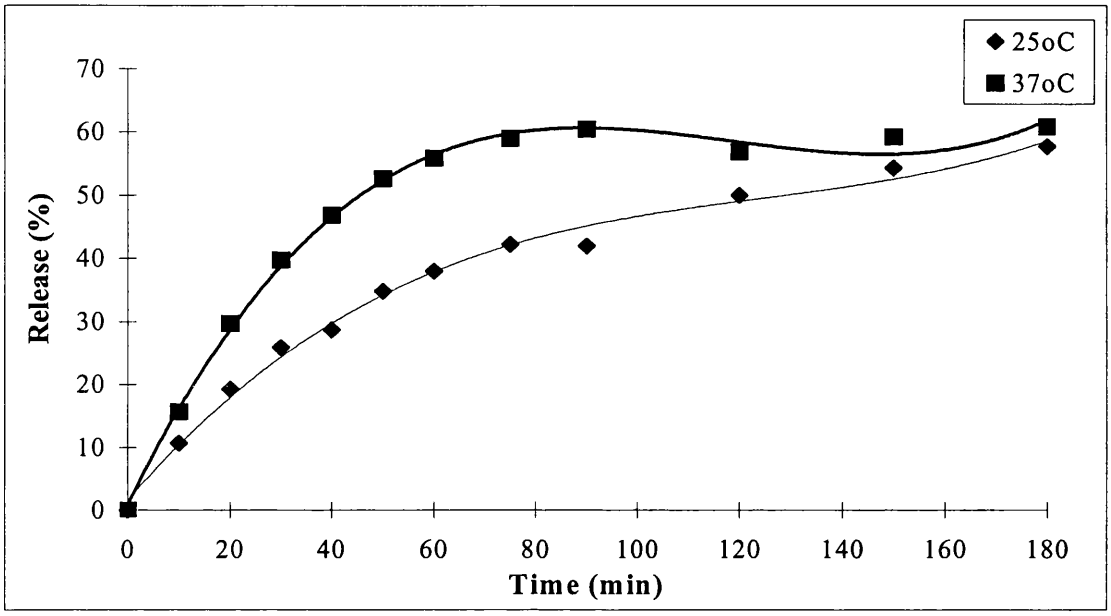

Fig 5.11 Effect of the temperature of incubation on the release of riboflavin from DPPC:DPPG:CHOL (1:0.8:0.2 molar ratios) spray-dried liposomes incubated with lung surfactant at 25 and 37°C.

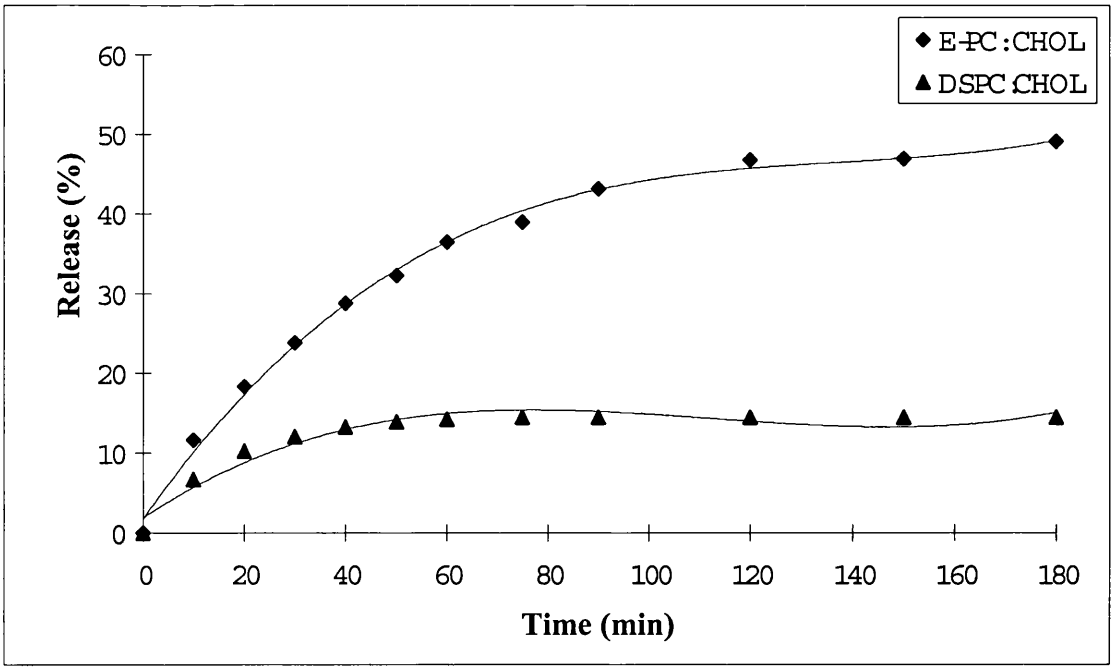

Fig 5.12 Effect of the lipid composition of liposome on the release of riboflavin after incubation with lung surfactant at 37°C.

A possible explanation of this finding is the difference in the temperatures of phase transition of DPPC, DSPC and PC based bilayers. The corresponding phase transition temperatures are 40°C and 55°C and -10°C for each of the lipids respectively. The lipids entering in the composition of the bilayer membrane confers its fluidity The latter influences the leakage of the encapsulated compounds directly. It is possible that the effect of the temperature of phase transition Tc is more important than the suggested compatibility of the destabilising molecule with the membrane lipids. Using lipids exhibiting the highest temperature of phase transition in the liposomal preparation can lead to a very significant improvement in the stability of such systems upon spray-drying and incubation with destabilising surfactant molecules such as those present in the L.S. survanta® . The mechanism by which the dried lipid molecules reorganise to form the bilayer structures could explain the increased leakage. Furthermore, the non-conservation of the integrity of the liposomal structure could explain the difference observed between DPPC and DSPC. This effect could as well be independant of the nature of the molecule used for incubation.

Another consideration of importance is that the natural surfactant used in these experiments, survanta®, contains the highly hydrophobic surfactant proteins (SPA and SPB) which could have a high affinity for anionic lipids such as the DPPG. Indeed, it was reported that these proteins bind rapidly to liposomes and mediate their aggregation (Meyboom *et al*, 1999). Possibly, these proteins interact with the DPPG molecules present in the bilayer membrane. They could create a loop or a defect leading to the riboflavin release. Alternatively, they could associate with the DPPG to form micellar phases also responsible of the bilayer destabilisation. The association of the natural L.S. with DPPC-based liposomes could not generate optimal release

profils of riboflavin. Such association will be progressive and leave enough time for the vesicles to release their content.

5.4 Some comments concerning the methodological aspects

The liposomal powders were treated with an equal volume of L.S. deposited in a dialysis bag that was then submerged in a volume of phosphate buffered-saline (PBS pH= 7.4).

The diffusion of the salts into the dialysis bag induces the dilution of the liposome-lung surfactant mixtures. Furthermore the dialysis bag constitutes a barrier to the instant diffusion of the released riboflavin masking the differences between triton, water and L.S. Some authors (Linchtenberg *et al*, 1979; Alonso *et al*, 1987) reported that liposomes in suspension required several hours to reach a complete equilibrium when treated with surfactant solutions. It is important to note that this dealt with liposomes in suspension. In the study reported by Ruiz (Ruiz *et al*, 1988), an equilibrium time of 30 min was allowed when liposomal suspensions were incubated with different surfactant solutions. This made the comparison possible between different liposomes/surfactants systems for the release of carboxyfluorescein.

Our study focuses on the incubation of a dry system with solutions of surfactant. There was no prior rehydration and resuspension in an aqueous media. In the procedure used there was an overlaying of two phenomena: rehydration leading to the reconstitution of the dried vesicles with release of the encapsulated riboflavin and the release of riboflavin at some stage due to the presence of the used surfactant.

Since these two phenomena occured at the same time it was difficult to assess which of the two was predominant.

With regards to the release kinetics, the dialysis bag could act as a delaying barrier. This could be misleading since it would suggest that there is a controled release of riboflavin rather than an almost instant and catastrophic leakage during the laps of time when rehydration and reconstitution occured.

All these considerations have to be taken into account for the interpretation of the real phenomenon taking place during stability testing using surfactant molecules and water.

5.5 Conclusions

In spite of the numerous investigations concerned with surfactant-lipid bilayer interactions, the precise mechanism by which a surfactant disrupts a lamellar phase remains poorly understood (Ruiz *et al*, 1988; Helenius *et al*, 1975).

An approach that has been used to monitor surfactant-lipid membrane interactions involves the measurement of the release of vesicle entrapped fluorescent markers such as 5 (6) carboxyfluorescein (CF) (Weinstein *et al*, 1984).

This method provided an insight into the disruptive activity of a surfactant molecule but it did not clarify issues concerning the nature of the disruption mechanism. It is possible that the release of the encapsulated fluorescent marker occurred by a catastrophic rupture event where the vesicles rapidly release their entire content. Alternatively the release can occur in the form of a leakage where the marker is gradually released from the vesicles rather than in a catastrophic process. The packing and the structure of the lipid membrane can significantly affect the pathway of release.

Our study concerns liposomal powders that were assessed for the release of their

riboflavin content from a dry state. The procedure used consists of allowing a volume of surfactant solution to be in contact with a dry liposomal powder deposited in a dialysis bag. Two phenomena take place simultaneously: rehydration leading to the reconstitution of the dried vesicles and interaction of the surfactant with the bilayer membrane leading to the release of the encapsulated riboflavin. Furthermore, the dialysis bag can act as a reservoir for the leaking riboflavin inducing a regulation effect.

The procedure used did not allow the distinction between the liposomal powder rehydration step and the proper interaction of the lung surfactant constituents with either the dry product or the reconstituted liposomes in suspension.

Keeping in mind all the limitations imposed by the adopted experimental procedure some conclusions could be made concerning the interaction of the used surfactant with the dry liposomal powders.

Our results suggest that unextruded spray-dried liposomes exhibit a similar release profile after incubation with water and lung L.S. The highest amount of riboflavin released was observed when triton X-100 was incubated with liposomal powders. Single chained surfactant molecules are generally used to disrupt biological membranes in order to extract proteins. It is believed that their disrupting ability derives from a mismatch between their intrinsic geometry and that of the lipid forming the lamellar structures. Most of the single chained surfactants are conical in shape and self-assembled into spherical micelles (Jayasuriya *et al*, 1990). In the presence of the lipid bilayer these surfactants are incorporated within it and provoke a lamellar to micellar phase transition and the release of the vesicle content.

Naturally occurring leakage of the small molecular weight markers encapsulated within liposomes can be an additional factor to be taken in consideration.

We used DSPC and PC based-vesicles with equimolar cholesterol in order to evaluate the effect of the membrane fluidity on the stability. A PC-based liposomal membrane is more fluid at a high temperature (T>Tc). By increasing the temperature of incubation to 37°C we increased at the same time the membrane fluidity and consequently its permeability to encapsulated solutes. We think that the increased permeability in PC and CHOL liposomes is more related to the increase of the temperature than to the presence of the surfactant molecules. On the other hand we could observe a more adequate reconstitution of the DSPC and CHOL-based liposomes at 37°C leading to higher stability in terms of riboflavin release. Indeed, the Tc of DSPC is equal to 55°C. The incubation being performed at 37°C was advantageous for a better reconstitution of the dried liposomes. The high temperature of incubation was then favorable for the retention of markers within the liposomes prepared out of lipids exhibiting high Tcs.

On the one hand extruded liposomes exhibited a faster release of riboflavin when incubated with triton X-100. On the other hand, the L.S. and water exhibited a relatively similar and lower release of riboflavin. The triton X-100 showed a more disruptive activity than the natural based lung surfactant. The L.S. promotes the conditions for rehydration and reconstitution of the vesicles better than the conditions for disruption of the bilayer membrane and subsequent leakage of the encapsulated compound.

At a low temperature (room temperature), the disrupting activity of the tested surfactants was found to decrease with the increased lamellarity of the vesicles. At a relatively high temperature (37°C) small vesicles prepared using high phase transition lipids seemed to be more stable. By increasing the temperature of incubation we influenced the fluidity of the membrane directly.

Both the fluidity of the membrane and the size of the liposomes affect their stability. Liposomes were formulated to contain dipalmitoyl phosphatidylcholine. DPPC is used as an additive to bovine lung extract to form the lung surfactant survanta® used for lung surfactant replacement therapy. The L.S. exhibited less disruptive property of the DPPC-based liposome bilayer membrane. L.S. and water generated the same release profile when incubated with liposomes allowing optimal rehydration and reconstitution. In contrast the DSPC-based liposomes released different amounts of riboflavin in presence of water and L.S.

The use of DPPC was not advantageous in terms of improving the marker retention or delaying the release. This is due to the presence of other components in the lung surfactant used such as surfactant proteins that may be more active than the main constituant DPPC. The concept of neutrality was hard to establish because of the complexity of the L.S. composition and of the mechanism of action of its constituent ,. Furthermore, any association of the L.S. with the membrane is progressive and will constitute an opportunity for leakage.

For a possible application in inhalation, other additives should be used. These could be polymers exhibiting a barrier to water by forming particles upon drying and by reconstituting the vesicles within their matrix upon inhalation. High phase transition lipids such as DSPC should be used with equimolar cholesterol for the suitability of its high phase transition. Their multilamellar structures will allow an optimal reconstitution within the lung fluids.

Chapter six

A novel method for high yield entrapment of solutes into small liposomes.

6.1 Introduction

A variety of liposome preparation techniques on a laboratory scale have been described in chapter 1 and elsewhere (Crommelin and Schreier, 1994; Barenholz and Crommelin, 1994). As we know, if liposomes are to be used in clinical practice, they must be tailored to exhibit specific properties such as bilayer fluidity, vesicle size, charge and hydrophilicity of the external surface.

Consequently, the fate of the encapsulated drug after parenteral administration is determined by the behaviour of the vesicles in the body, by the release kinetics of the drug from the carrier and by the in-vivo behaviour of the drug itself.

The circulation time of liposomes in blood after intravenous injection controls the fate of the encapsulated material. The sustained release of the drug in tissues with an increased blood vessel permeability is correlated to the circulation time of liposomes.

The rate and extent of liposome uptake by the cells of the mononuclear phagocyte system (MPS) and, therefore the circulation time, depend on the size of the liposomes. Bilayer rigidity, hydrophilicity and the charge of the liposome surface also play important roles in the improvement of the circulation time.

Large vesicles are rapidly eliminated from the blood stream and small liposomes circulate much longer. Thus, when targeting to the macrophage is desired, the use of large liposomes may be advantageous.

Several steps exist in the process of liposome preparation. The minimum number of steps involved are the hydration of the lipids resulting in vesicle formation, the drug encapsulation and the reduction of the liposomal size.

High shear homogenization techniques such as microfluidization applied on a large scale are used for sizing liposomes, whether empty or loaded. In Chapter 3, we

reported the use of the microfluidizer to size preformed DRV liposomes encapsulating the marker ^{14}C sucrose. Homogenization constitutes an additional step during liposome preparation. On the one hand this resulted in liposomes of around 100 nm in size retaining about 25% of the originaly entrapped sucrose, on the other hand the problems of stability in suspension of these liposomes remained unresolved.

A further step in our work consisted in an attempt to identify parameters influencing the stability (in terms of size of liposomes and retention of the marker) after spray-drying of the preformed liposomes and their rehydration. It appeared that the initial size of liposomes was the most decisive factor in improving the retention of the entrapped solutes. Size reduction of liposomes became itself a prerequisite for their stability during the drying-rehydration cycle. As the drug leakage was dependent on the starting size of liposomes, we thought of an approach where a substantial amount of drug could be loaded into a relatively small sized liposome upon drying and rehydration. The freeze-drying of a liposome dispersion from an aqueous solution without adding sugar or other additive for protection resulted in the formation of large vesicles after rehydration, independently of the vesicle size prior to drying. The process consists in a freezing step followed by water sublimation on vaccum application. This phenomenon of size enlargement upon water removal and rehydration was exploited to produce liposomes with a high-yield drug entrapment known as the DRV method (Kirby and Gregoriadis, 1984). The volumes used for the rehydration of lyophilised material were very low (ten times smaller than the original solution volume). Optimum encapsulation efficiencies by the DRV method were achieved when the lipids were fully hydrated as small unilamellar vesicles (SUV) prior to the freeze-drying step.

The relative contribution of the three distinct processes of freezing, dehydration and rehydration to the overall event of vesicle fusion is not known. The juxtaposition of vesicles and solute molecules prior to rehydration is an important factor for optimum entrapment values.

In this novel method, SUV liposomes are freeze-dried or spray-dried in the presence of the solute destined for encapsulation together with appropriate amounts of sucrose as an agent to control the fusion/aggregation process allowing the formation of relatively small drug loaded liposomes. The product obtained (prior to rehydration) can be stored in the dry state avoiding problems of hydrolysis, size growth and leakage of the encapsulated drug. When needed, the cake or the powder can be reconstituted by rehydration in minutes before being administered to the patient.

A comparison of the results obtained with this novel method with the results obtained whith the extrusion technique was carried out.

6.2 Materials and methods

Described in Chapter 2. For materials see 2.1.1, 2.1.4, 2.1.5, 2.1.6, 2.1.7, 2.1.8, 2.1.9, 2.2.3, 2.2.4, 2.2.6, 2.2.7, 2.2.8, For methods see 2.3.1, 2.3.3.1, 2.3.3.2, 2.3.10, 2.311, 2.3.12, 2.3.13, 2.3.19.

6.2.1 The novel method

Liposomes were prepared by the dehydration-rehydration method with the following modifications:

- the generated SUV suspension (see methods) was transferred into a vial in which the correct amount of drug in solution was added together with an amount of sucrose to achieve the desired mass ratio of sucrose to lipid.

-the desired volume, in some cases, was then adjusted to obtain the correct molarity of sucrose and the preparation was frozen and then freeze-dried for a sufficient period of time (generally 24-48 hours).

6.3 Results and discussion

6.3.1 Radioiodination of the epidermal growth factor (EGF)

The separation of ^{125}I labelled EGF from free ^{125}I was achieved using Sephadex G-50 gel. As shown in Figure 6.1, the first peak represents the labelled EGF followed by a second peak representing the free ^{125}I. The labelled EGF fractions were stored in the deep freezer. Aliquots of ^{125}I-labelled EGF representing enough counts were added to the cold EGF for labelling.

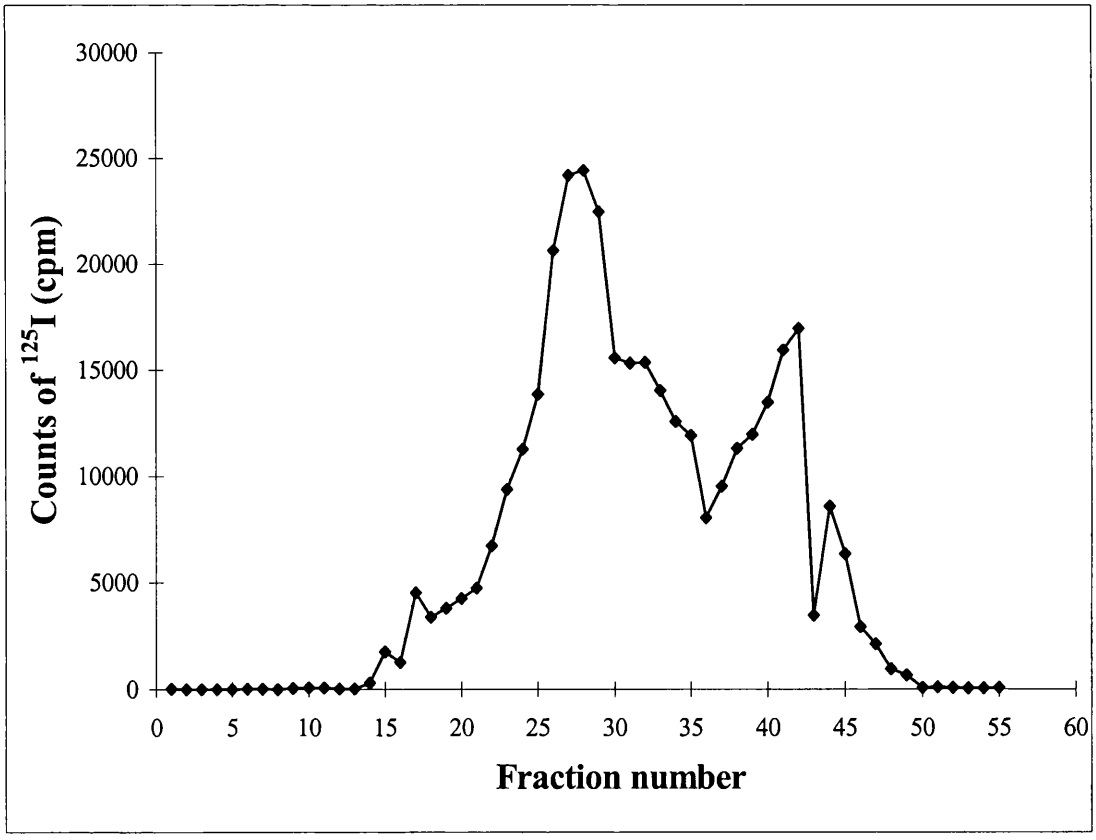

Fig 6.1 Gel filtration on Sephadex G-50 of EGF after radioiodination with ^{125}I.

6.3.2 Effect of the presence of sucrose on the entrapment efficiency and on the liposome size

As discussed, the freeze-drying of a liposome dispersion in the absence of cryoprotectant (saccharides) leads to the formation of large vesicular structures upon rehydration (Casals *et al*, 1996; Zingel *et al*, 1996). In early studies, dehydration/rehydration vesicles (DRV s) were preformed without using sugars as stabilisers, the procedure being based on the induction of fusion of preformed small unilamellar vesicles upon controlled rehydration. This generally leads to the entrapment of high amounts of the drug, but formed vesicles exhibit large sizes (up to 5μm). A subsequent sizing of the preparation was then necessary to reduce the vesicles to the desired size allowing its administration by the intravenous route (Gregoriadis *et al*, 1990). This implied an additional step and, consequently, more restrictions for the fulfillment of the aseptic conditions. Furthermore, the final preparation exhibited a relatively low encapsulation value because of the leakage occuring during this additional step of size reduction. Besides, these final liposomal suspensions are subject to instability and damage during storage. Fusion and aggregation, lipid oxidation and drug leakage are some of the problems that could be encountered during storage of the microfluidized preparations. Freeze-drying of such preparations was promoted as an interesting option.

A classical approach to improve the stabilization of liposomes consisted in freeze-drying small (150 nm) preformed vesicles encapsulating the drug, in the presence of carbohydrates or of other compounds (Crowe *et al*,1987; Van Winden and Crommelin, 1999). A simil ar approach was attempted in Chapter 4 where a water soluble compound was encapsulated in liposomes. The preparation was spray-dried in the presence of sugars and the properties of the resulting liposomes were monitored

(size and % entrapment) after rehydration. It appeared that the size was the most decisive parameter in improving the retention of the aqueous marker.

As shown in Figure 6.2, the use of sucrose in the drying steps could control the fusion when preparing liposomes using the novel method. This also lead to a substantial encapsulation of the compound used.

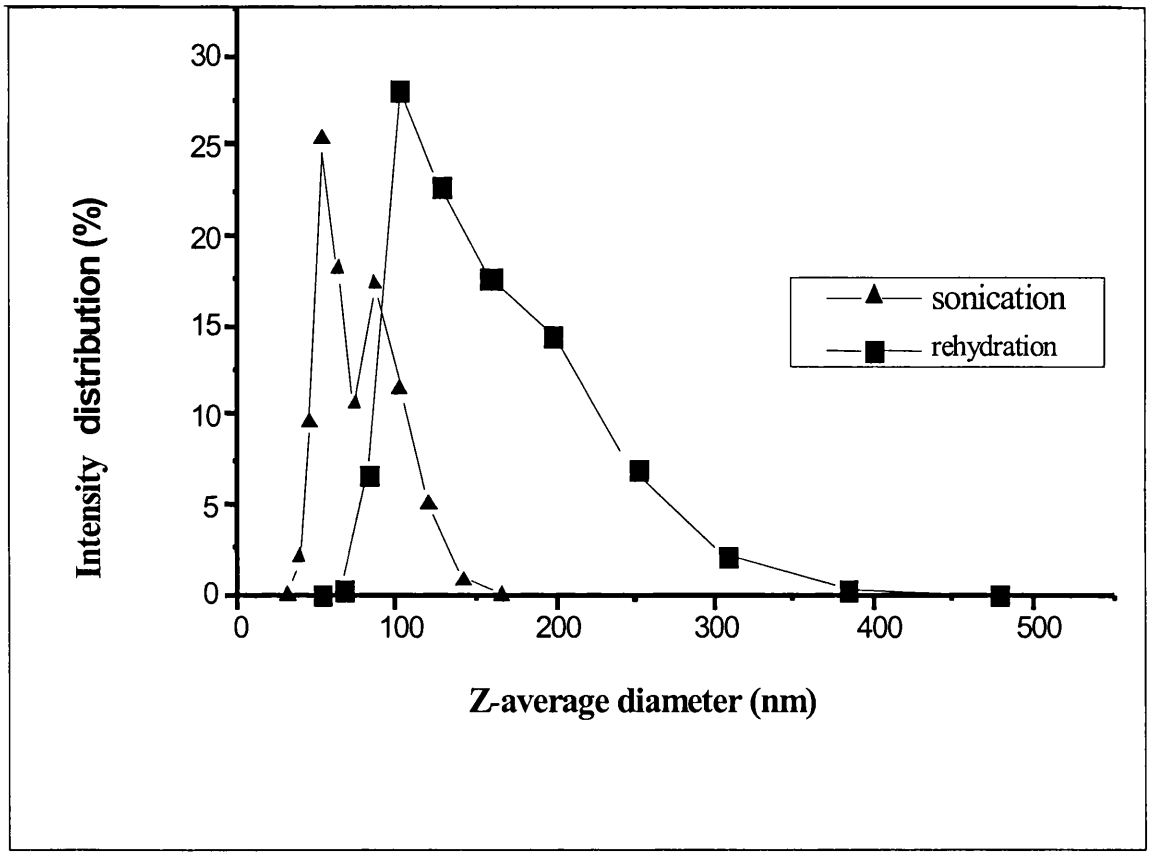

Fig 6.2 Size evolution of DPPC and equimolar CHOL liposomes from sonication to freeze-drying in the presence of 35.7 mM sucrose and rehydration.

The sucrose added to the SUV preparation together with the compound to be encapsulated permitted a limited degree of fusion after the controlled rehydration step was performed. This allowed the encapsulation of the compound into the vesicles exhibiting small Z-average diameters and not requiring any further sizing.

The formation of ice crystals during freezing of the liposomes dispersion may damage the liposomes in terms of size conservation (Ausborn *et al*, 1994) as it may rupture the

bilayers (McDonald *et al*, 1994). A high concentration of the remaining unfrozen solute can be responsible for a similar damage. The reported amount of water that remained uncrystallised in a frozen liposome dispersion is between 0.1 and 0.35 g water/g phospholipid (Bronshteyn and Stepenkus, 1993; Crowe *et al*, 1990). The addition of sucrose allowed the formation of an amorphous glass upon freezing which could minimize this damage. Space was created between the vesicles, which prevented the fusion. On this basis, we notice that the presence of sufficient amounts of sucrose during drying permits a substantial liposome stabilization that leads, upon reconstitution with water, to an adequate size and to substantial entrapment values.

The percentage of entrapment of various compounds and the corresponding Z-average diameters of the liposomes prepared in the presence or absence of sucrose are presented in Figures 6.3 – 6.9 and Table 6.1.

It appears that at a moderate degree of sucrose stabilization, the reconstitution of liposomes with a minimum amount of water allowed a certain extent of fusion leading to a good percentage entrapment.As shown in Fig 6.3 this procedure permitted the encapsulation of a substantial amount of FITC-albumin in the presence of sucrose at different molarities.The use of sucrose resulted in a drastic reduction of the liposome size (from 5μm in the absence of sucrose down to 287 nm) and the same percentage of encapsulation was achieved independently of the presence or absence of sucrose (87% and 84% respectively). Carboxyfluorescein could also be successfully encapsulated (Fig. 6.4). Both the final size and the entrapment values remained unaffected when a solution of sucrose at different molarities was used.

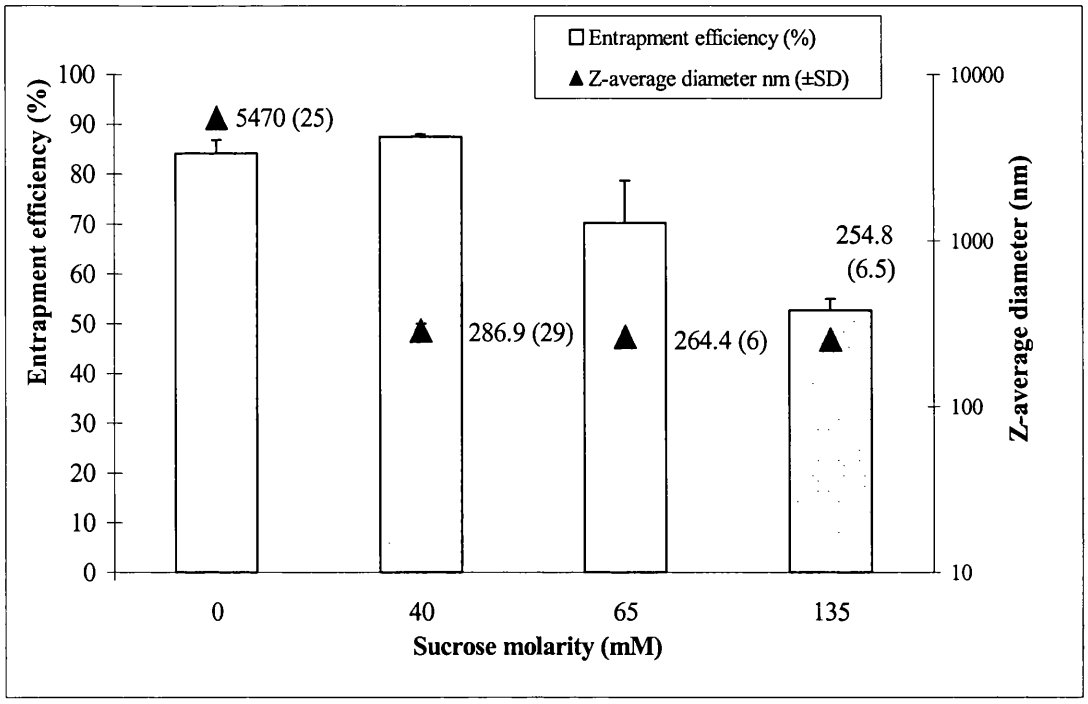

Fig. 6.3 Entrapment efficiency and Z-average diameters of liposomes (25 mg PC and equimolar CHOL) entrapping FITC-albumin (1 mg used) prepared using the novel method in the presence of various sucrose molarities.

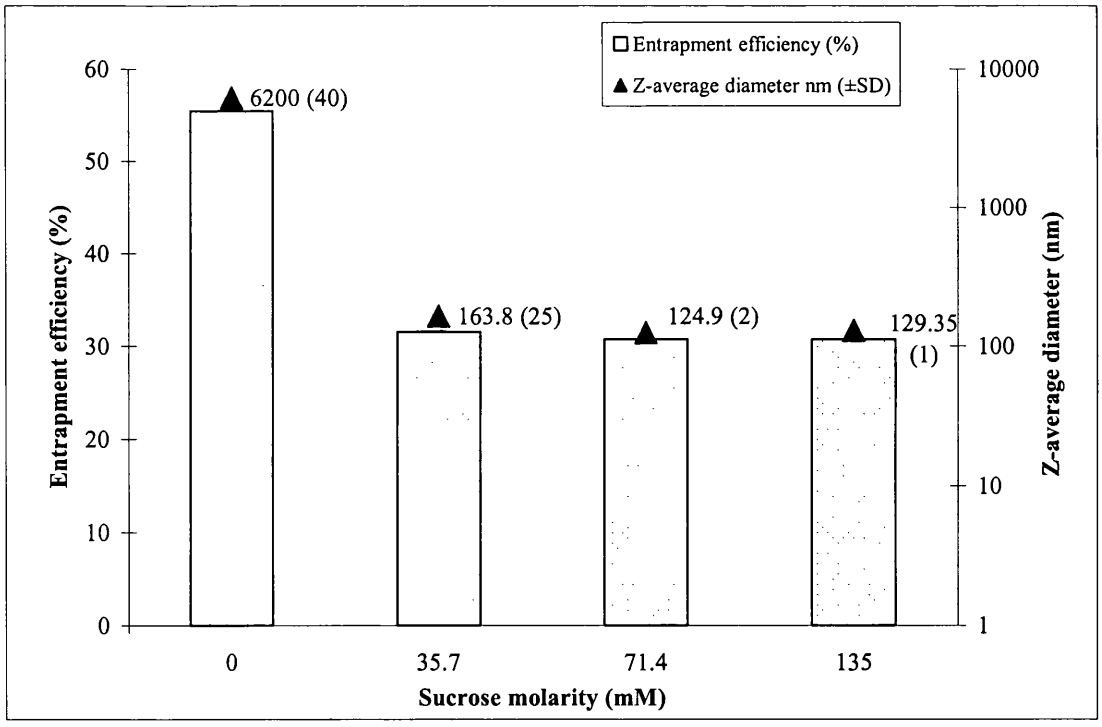

Fig. 6.4 Entrapment efficiency and Z-average diameters of liposomes (25 mg PC and equimolar CHOL) entrapping carboxyfluorescein (1mg used) prepared using the novel method in the presence of various sucrose molarities.

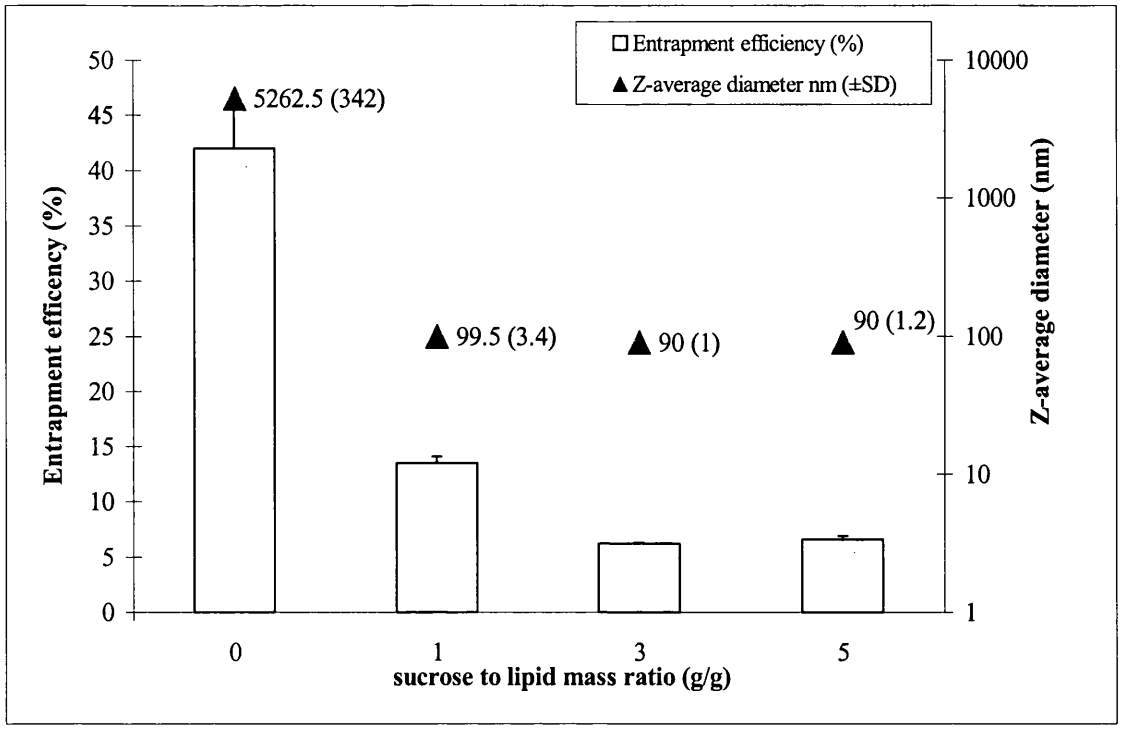

Fig.6.5 Entrapment efficiency and Z-average diameters of liposomes (25 mg PC and equimolar CHOL) entrapping ^{14}C-penicillin (5 mg used) prepared using the novel method in the presence of various mass ratios of sucrose to lipid at a final sucrose concentration of 68.7 mM.

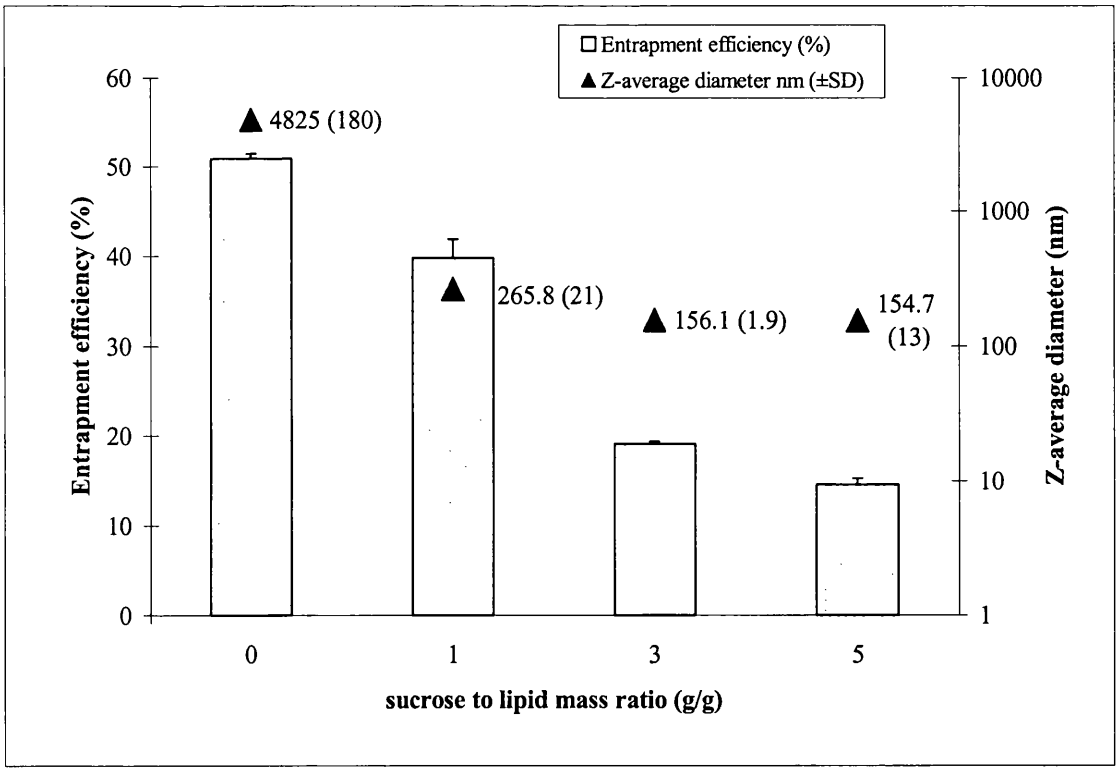

Fig.6.6 Entrapment efficiency and Z-average diameters of liposomes (25mg DSPC and equimolar CHOL) entrapping ^{14}C-penicillin (5 mg used) prepared using the novel method in the presence of various mass ratios of sucrose to lipid at a final sucrose concentration of 71.1 mM.

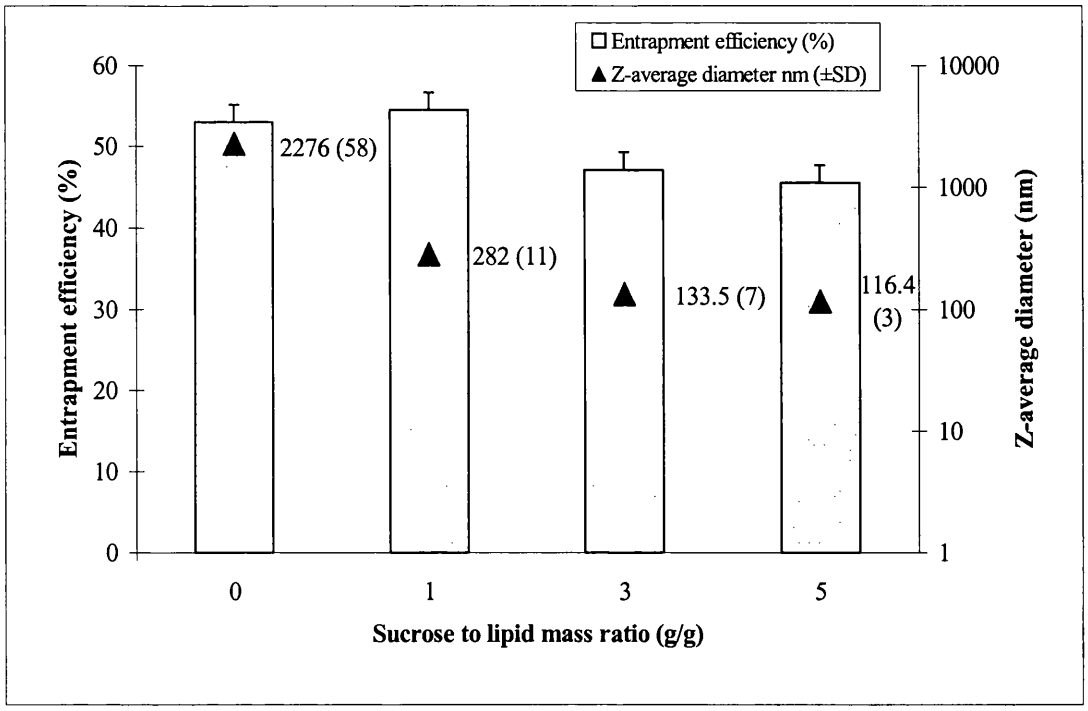

Fig 6.7 Entrapment efficiency and Z-average diameters of liposomes (25 mg PC and equimolar CHOL) entrapping doxorubicin (1 mg used) prepared using the novel method in the presence of various mass ratios of sucrose to lipid at a final sucrose concentration of 74mM

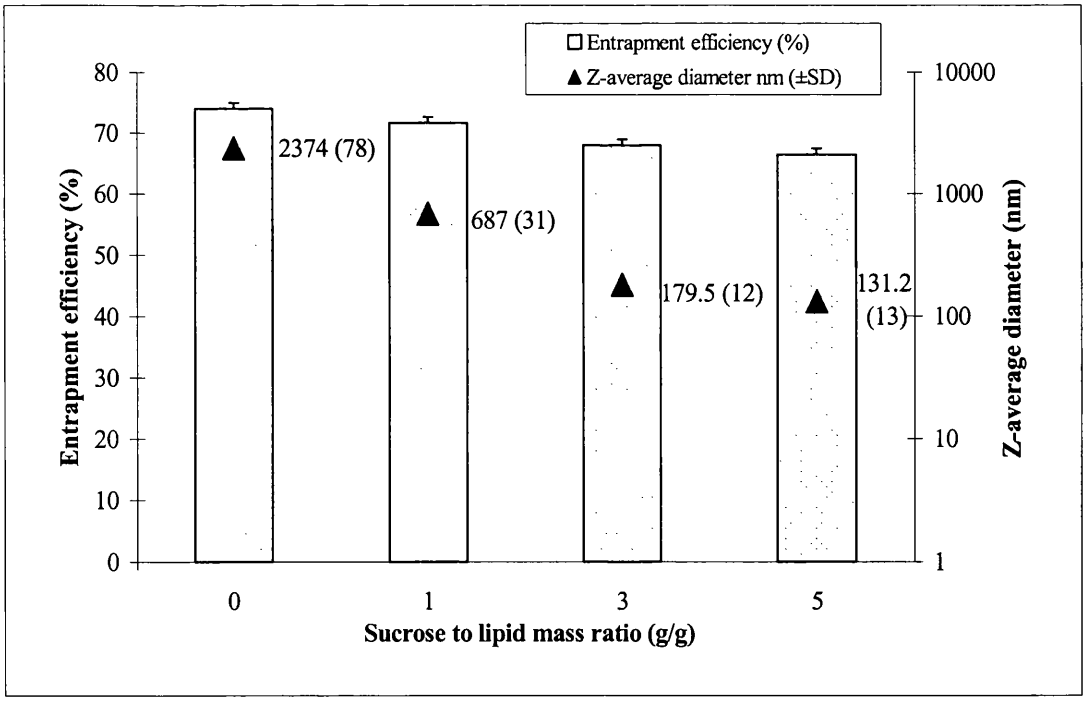

Fig 6.8 Entrapment efficiency and Z-average diameters of liposomes (25 mg DSPC and equimolar CHOL) entrapping doxorubicin (1 mg used) prepared using the novel method in the presence of various mass ratios of sucrose to lipid at a final sucrose concentration of 74mM

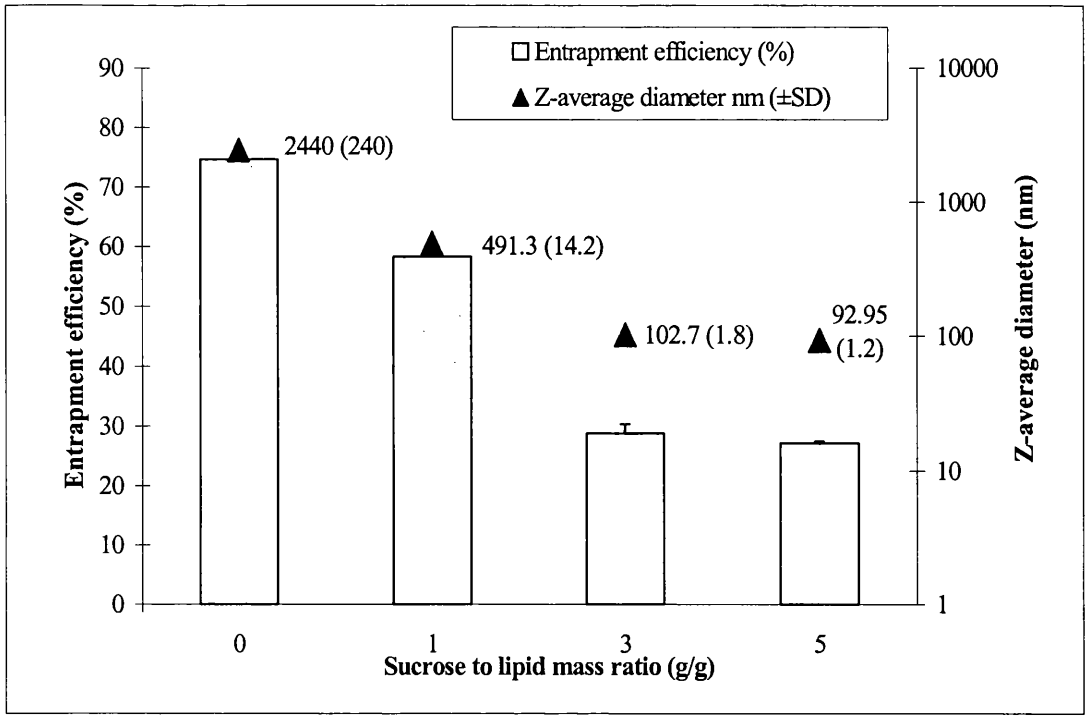

Fig.6.9 Entrapment efficiency and Z-average diameters of liposomes (25 mg PC and equimolar CHOL) entrapping daunorubicin (1 mg used) prepared using the novel method in the presence of various mass ratios of sucrose to lipid at a final sucrose concentration of 51.7mM.

Table 6.1 Entrapment efficiency and size of liposomes encapsulating EGF (150μg used) prepared by the novel method in presence of various sucrose molarities at a 3:1 of sucrose to lipid mass ratio.

Lipid composition	Sucrose molarity	Entrapment efficiency (%) (±SD)	Size (nm) (±SD)
PC:CHOL[(a)]	none	22.3 (2.1)	1276.7 (100)
	35.7	33.5 (7)	144.8 (32)
	126	29.6 (1)	146.4 (1.3)
PC:CHOL[(b)]	64.4	42 (1.5)	167.4 (9)
DPPC:CHOL[(b)]	None	31 (0.9)	2495 (329)
	35.7	25.5 (0.2)	127.6 (3)

a, 16 μmoles of PC and equimolar cholesterol

b, 64 μmoles of PC or DPPC and equimolar cholesterol

DSPC and equimolar cholesterol liposomes encapsulated higher amount of ^{14}C-penicillin than PC based ones. The Z-average diameters ranged between 90 to 265 nm (Figs 6.5 and 6.6). Doxorubicin and daunorubicin exhibited substantial entrapment values (Figs 6.7 - 6.9) correlated with Z-average diameters as low as 100 nm when the mass ratio of sucrose to lipid was around 5. Finally, similar conclusions could be drawn (Table 6.1) for the encapsulation of EGF into small liposomes, where entrapment values of EGF were similar to those obtained when the unmodified DRV procedure was used. The presence of sucrose generated vesicles exhibiting a Z-average diameter of around 120-160 nm independently of sucrose molarity.

6.3.3 Comparison of characteristics of liposomes generated by the novel method and DRV extrusion

Figures 6.10 and 6.11.show that a drastic reduction in the size of liposome is obtained when the liposomal preparation are extruded unwashed (in presence of unentrapped solute) through polycarbonate filters. The liposome size is reduced from a submicron size range down to around 300 nm when passed through the 0.4 μm pore size filter. Subsequent extrusion through the 0.2 μm and 0.1 μm pore size polycarbonate filter resulted in further size reduction. As a general rule, the PC and CHOL (equimolar) based liposomes extruded through the 0.2μm and 0.1 μm pore size membranes lead to final preparations exhibiting Z-average diameters slightly larger than the pore diameter of the membranes. A possible explanation for this, is the presence of equimolar cholesterol which inserts between the phospholipid molecules, imposing a defined vesicle curvature and maximum rigidity. However, reduction of the liposome size was correlated (Fig 6.10 and 6.11) with a loss of the entrapped drug. Figure

6.10.shows that this leakage (due to size reduction) is less pronounced for an encapsulated large molecule such as the protein BSA.

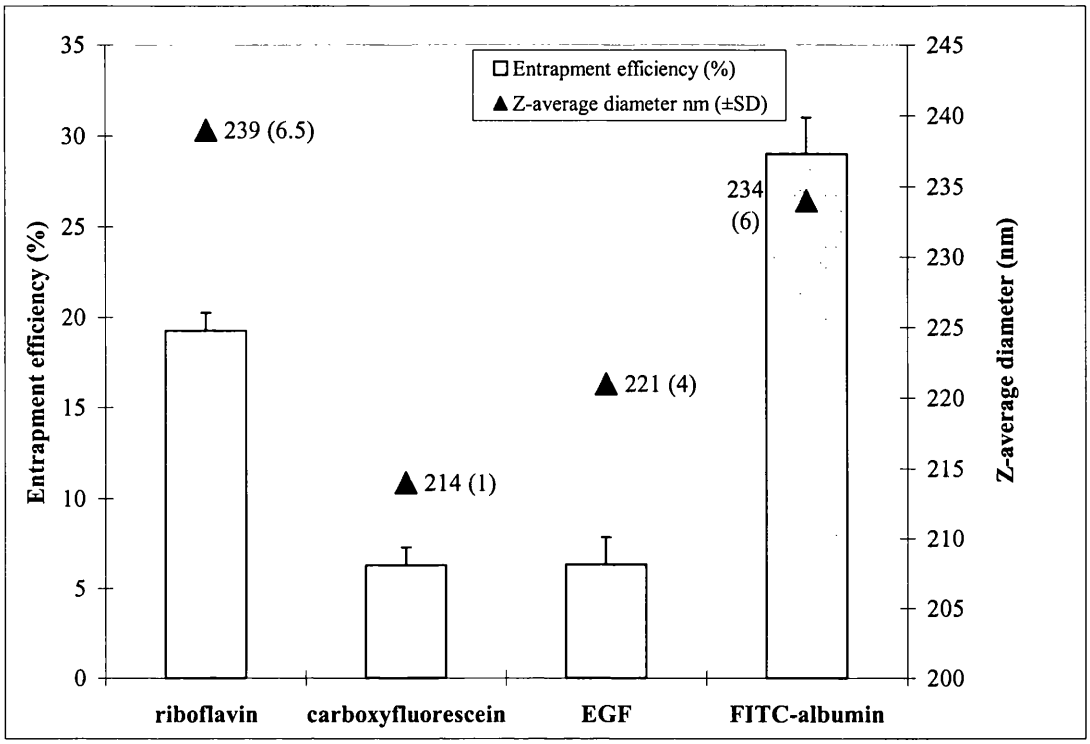

Fig 6.10 Entrapment efficiency and Z-average diameters of extruded (200nm final pore size membrane) DRV liposomes (PC and equimolar CHOL) entrapping various compounds.

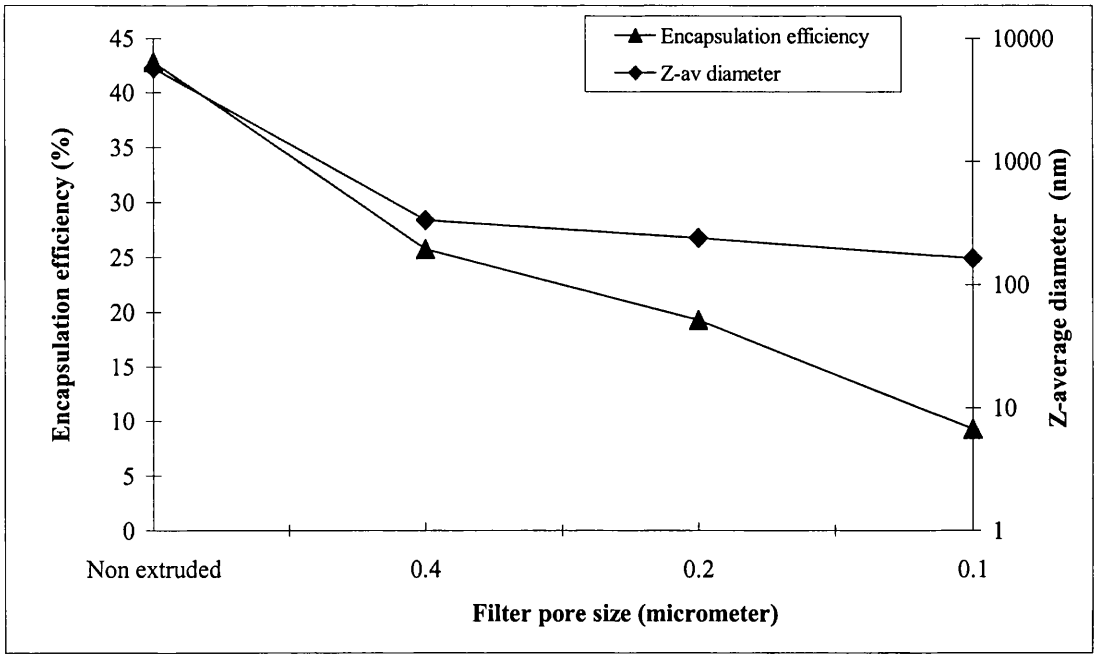

Fig 6.11 The effect of extrusion of 25 mg PC and equimolar CHOL liposomes on size reduction and on the entrapment efficiency of riboflavin (1 mg used) (100nm final pore size membrane)

Even though the large molecular weight molecules were reported to be less likely to escape through bilayers membranes, liposomes encapsulating FITC-albumin could have their size reduced down to 230nm with a loss of 50% of the amount originally entrapped.

On the other hand, small molecular weight compounds such as carboxyfluorescein and riboflavin, could be encapsulated using the unmodified DRV up to approximately 55 and 43% of the originally added amount. After processing the liposomes through the 100 nm pore size filter, these percentages of entrapment were found to be 6% and 9%. Only 25% of the originally entrapped riboflavin was retained within the vesicles. This finding is in agreement with the results in Chapter 3 related to the microfluidization of ^{14}C sucrose-containing PC and CHOL (equimolar) liposomes. A minimum of 27% of the originally entrapped ^{14}C sucrose is retained within the liposomes after microfluidization in the stated conditions. Those liposomes exhibited a Z-average diameter of around 120 nm.

The leakage of the entrapped material is related to the nature of the process. A very high pressure is used to push the vesicles through the pores of the polycarbonate membranes. Certainly, the most external bilayer forming the liposomes will be dismantled in favour of other possibly newly formed vesicles. The extrusion of preformed DRV liposomes generated small vesicles but leakage of the encapsulated drug occurs during this procedure. Moreover this constitutes an additional step in liposome formulation. Using sucrose allows a virtual one-step method where the rehydrated product exhibits higher entrapment values than the ones obtained at the final stage of the size reduction procedure. The Z-average diameter exhibited by the rehydrated liposomes will be in the same range of those obtained after extrusion of the preformed DRV.

6.3.4 Size distribution of liposomes prepared by the novel method.

Performing the dehydration-rehydration procedure (dehydration being accomplished by freeze-drying or by spray-drying) in the presence of sucrose generated liposomes encapsulating high amount of the originally added material. These liposomes exhibited relatively small sizes depending mostly on the mass ratio of sucrose to lipid than on the molarity of sucrose. Using sucrose did not only affect the Z-average diameter, it also affected the size distribution (width and polydispersity). Liposomes encapsulating FITC:albumin exhibited a narrower size distribution when the molarity of sucrose used to freeze-dry was increased from 40mM to 135mM (Fig 6.12)

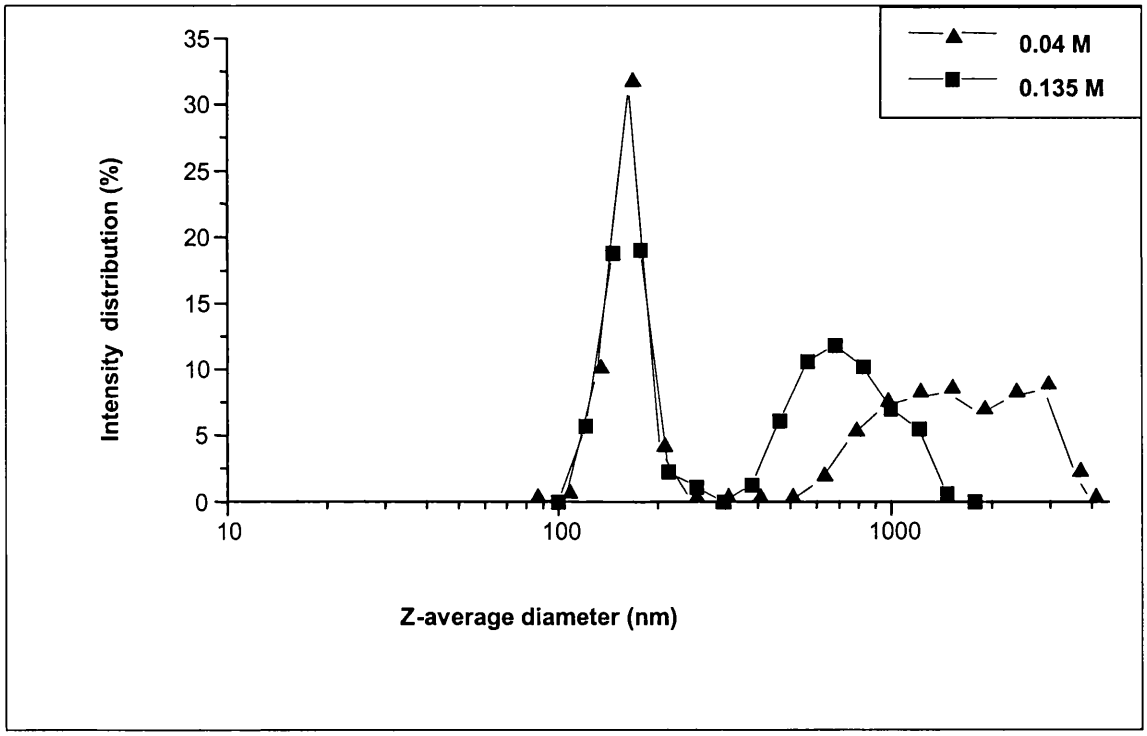

Fig 6.12 Effect of sucrose molarity (mass ratio of sucrose to lipid 3/1) on the vesicle size distribution of 25 mg PC and equimolar CHOL liposomes entrapping FITC-albumin (1mg used) prepared using the novel method.

The first population of the vesicles was situated in the region of 100-200 nm for both molarities. However, in terms of size distribution, the liposomes prepared in the presence of 135 mM of sucrose exhibited a second population of larger vesicles.

Liposomes prepared in the presence of a 40 mM solution of sucrose exhibited a similar second vesicles population of even larger size. The adsorbant property of the albumin may play an important role in the generation of a second vesicle population situated in the region of relatively larger vesicle diameters. For instance Fig 6.13 suggests that the albumin adsorbed on the liposome surface can link the SUV's to one another facilitating their fusion and aggregation and thus the generation of a population exhibiting larger diameters.

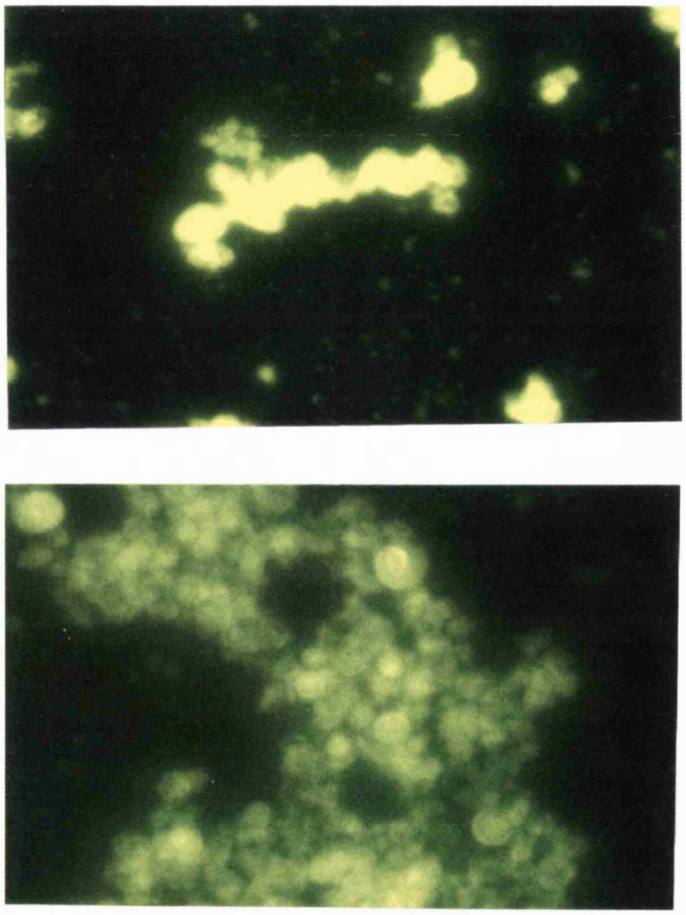

Fig 6.13 Photographs of fluorescent microscopy showing the aggregation of PC and equimolar CHOL liposomes encapsulating FITC-albumin prepared by the DRV method (5 μm).

The same lipid composition (equimolar PC and CHOL) was used to prepare liposomes encapsulating EGF. The freeze-drying step was carried out in the presence of 35.7 mM or 126 mM solution of sucrose. When the rehydration was performed (Fig 6.14) the liposomes prepared in the presence of the higher molarity (126 mM sucrose) exhibited slightly smaller Z-average diameters and a narrower size distribution. The mass ratio of EGF to lipid (150μg/18 mg) was almost 3 fold lower than the ratio of albumin to lipid (1mg/36 mg). At this EGF to lipid ratio, we could not observe any aggregated vesicles.

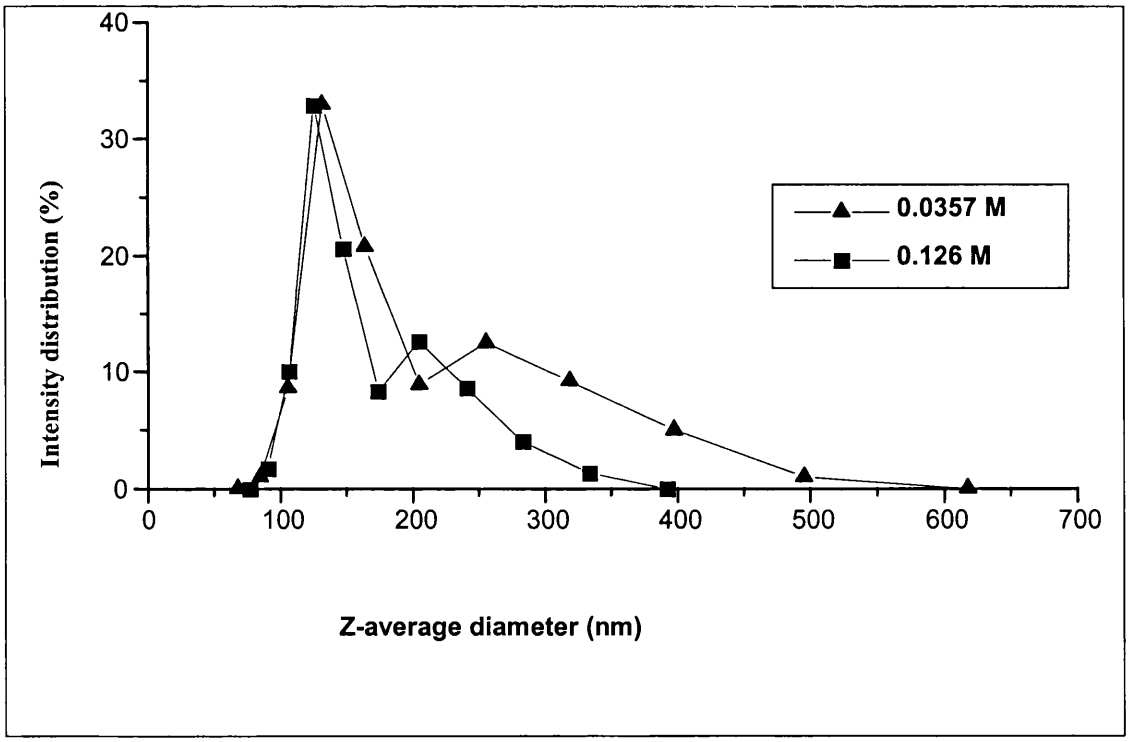

Fig 6.14 Effect of the sucrose molarity (sucrose to lipid mass ratio of 3 to 1) on the vesicle size distribution of PC and equimolar CHOL liposomes entrapping EGF (150 μg used) prepared using the novel method.

The extruded liposomes exhibited a narrower size distribution. The nature of the phenomena involved in the forcing of the vesicles through pores of an equal diameter could easily explain these results.

It was reported that the microfluidization (Sorgi and Huang, 1996; Vemuri *et al,* 1990) as well as the extrusion (Schneider *et al*, 1995) techniques generally lead to a monodisperse vesicle distribution. The vesicles obtained using the present novel method exhibit a small Z-average diameter but in some cases a broad vesicle size distribution. Indeed, in the particular case of the small molecular weight marker carboxyfluorescein, liposomes prepared in the presence of a 135 mM solution of sucrose exhibited a size distribution profile almost identical to the one obtained with liposomes prepared by the extrusion of preformed classical DRV s (Fig 6.15).

The cumulative percentage distribution of vesicles prepared in the presence of 135mM sucrose overlays the cumulative percentage distribution of vesicles extruded through the 100 nm pore size filter (Fig 6.15) For this particular compound (CF), the homogeneity of the vesicles obtained upon rehydration is better than those obtained for other compounds (albumin and EGF). It is comparable to that obtained when preformed vesicles are extruded.

Changing the lipid compositions used for the liposome preparation in the presence of 35.7mM sucrose from lipids exhibiting a low Tc (PC) to lipids exhibiting a high Tc (DPPC) generated, upon rehydration, vesicles with narrower size distribution (Fig 6.16).

On the one hand, DPPC and CHOL (equimolar) liposomes exhibited a Z-average diameter of 127 nm. On the other, the PC and equimolar CHOL liposomes showed an average diameter of 145 nm with a slightly broader size distribution. In this case the use of high phase transition lipids instead of low phase transition could promote the production of a narrower vesicle size distribution when the liposomes are freeze-dried in the presence of sucrose.

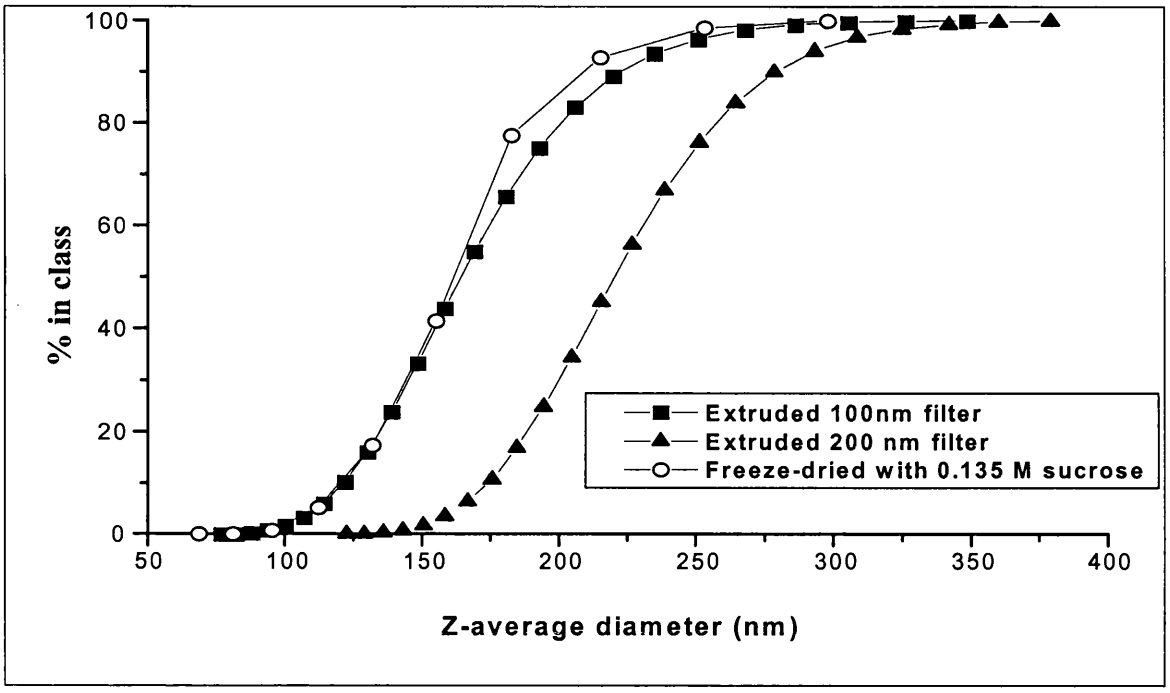

Fig 6.15 Size distribution (cumulative frequency) of 25 mg PC and equimolar CHOL liposomes encapsulating CF (1 mg used) prepared by freeze-drying in presence of sucrose or extruded through polycarbonate membranes (preformed DRVs).

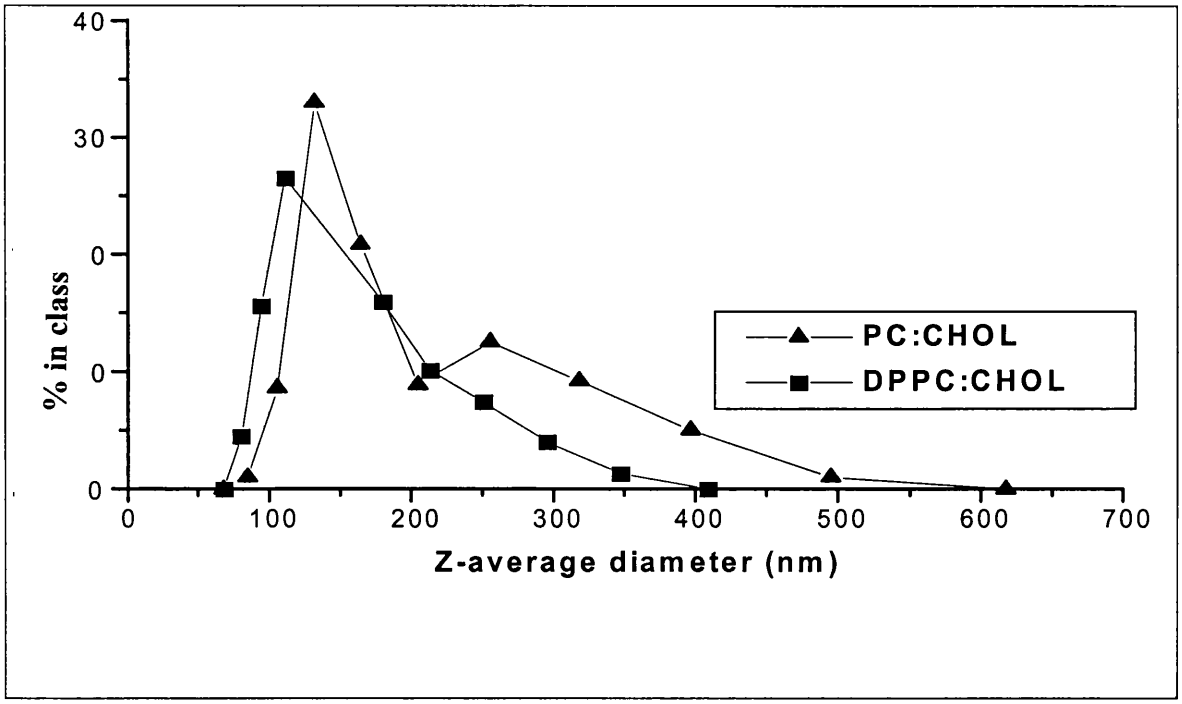

Fig 6.16 Effect of the bilayer composition on the size distribution of liposomes prepared by the novel method in the presence of a 35.7 mM sucrose solution at a mass ratio of sucrose to lipid of 3/1.

6.3.5 Spray-drying as an alternative method for DRV liposomes

The same approach was adopted as with freeze-drying: riboflavin was added to the SUV in suspension together with sucrose. The preparation was then spray-dried at an inlet and outlet temperature of 100°C and 70°C respectively.

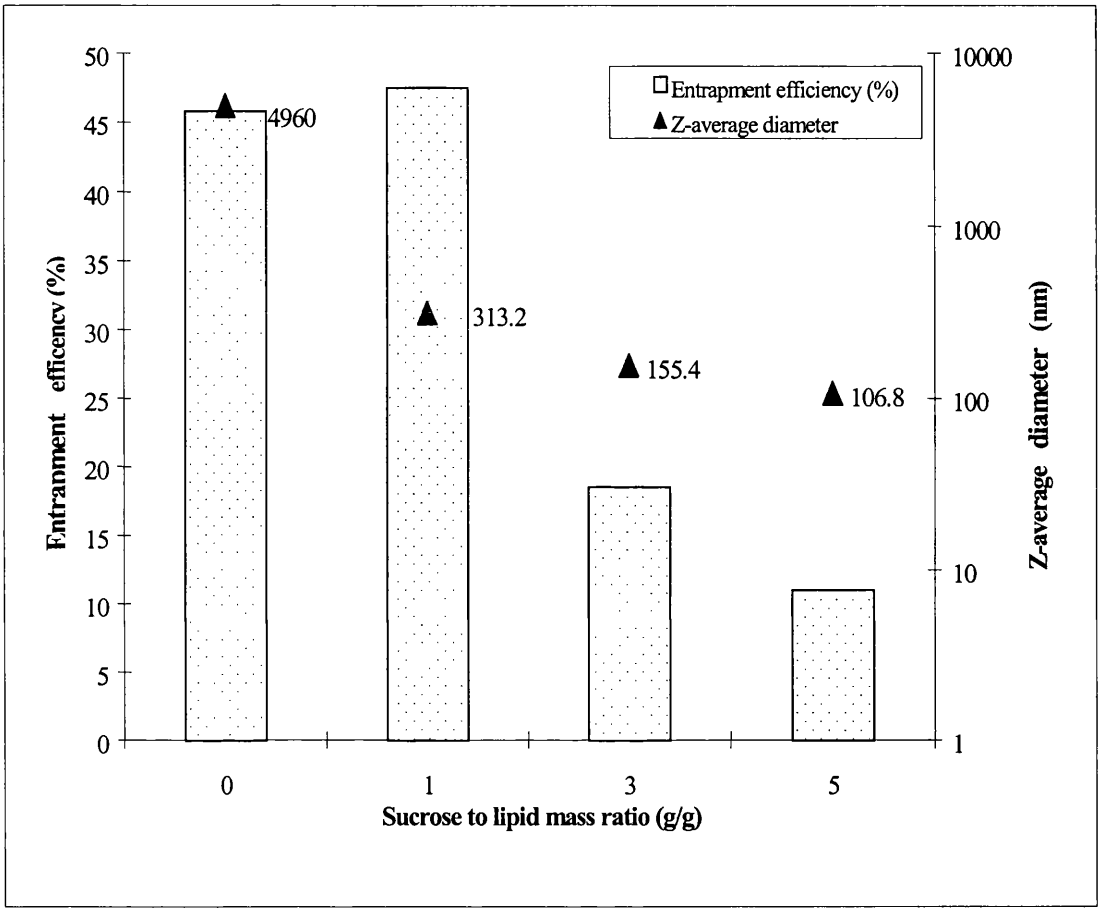

Fig 6.17 Riboflavin entrapment efficiency and Z-average diameters of liposomes (300 mg PC and equimolar CHOL) prepared by spray-drying (and subsequent rehydration) of SUV's in presence of riboflavin (12 mg used) and various sucrose to lipid mass ratios.

An amount of powder was rehydrated to obtain 37 mg of liposomal suspension corresponding to 1 mg of added riboflavin. The relative Z-average diameter and the entrapment efficiency for each sucrose to lipid mass ratio used are shown in Figure 6.17. Upon rehydration, SUV liposomes spray-dried in the absence of sucrose exhibited a large size (5μm). The encapsulation efficiency was around 45% of the originally added riboflavin.

Adding an amount of sugar equal to the lipid amount (ratio of 1) did improve the characteristics of the finally obtained vesicles by decreasing their sizes down to 313 nm without affecting the amount of riboflavin entrapped (47.5%). The use of a higher amount of sucrose lead to further size reduction but to a reduced entrapment efficiency. The sucrose used served to control the fusion of the liposomes during drying and rehydration. It appears that in the unmodified DRV, only part of the fusion phenomenon taking place is responsible for the encapsulation. The spray-drying technique could constitute an interesting approach to producing large batches of liposomal powders. (Kikuchi *et al*, 1992; Redzniak and Perrier, 1996). The use of the spray-drying technique to prepare large batches of preliposome powders has been reported (Kikuchi *et al*, 1991). The approach consists of spray-drying emulsified (in organic solvent) lipids with additives. In some cases the powders obtained are rehydrated with a solution containing the compound to be entrapped. The liposomal suspension obtained is then subjected to high pressure homogenization. Our approach seems to be more advantageous as it is almost a one-step method. It does not involve the use of any organic solvent and does not require any further size reduction through high pressure homogenization either.

6.3.6 Effect of the sucrose molarity on the formulation of penicillin using the novel method

In these experiments, a relatively high amount of ^{14}C-penicillin (5 mg) is added to the SUV's in the presence of a specific mass ratio of sucrose to lipid. The preparations are then diluted in order to obtain the desired molarity.

It appears from Fig 6.18 that dilution prior to freeze-drying leads to the improvement of the encapsulation efficiency. Indeed, the sizes relative to each preparation tend to

be slightly larger. This could be attributed to the fact that the fusion phenomena taking place during drying and rehydration are more pronounced with diluted solutions of sucrose.

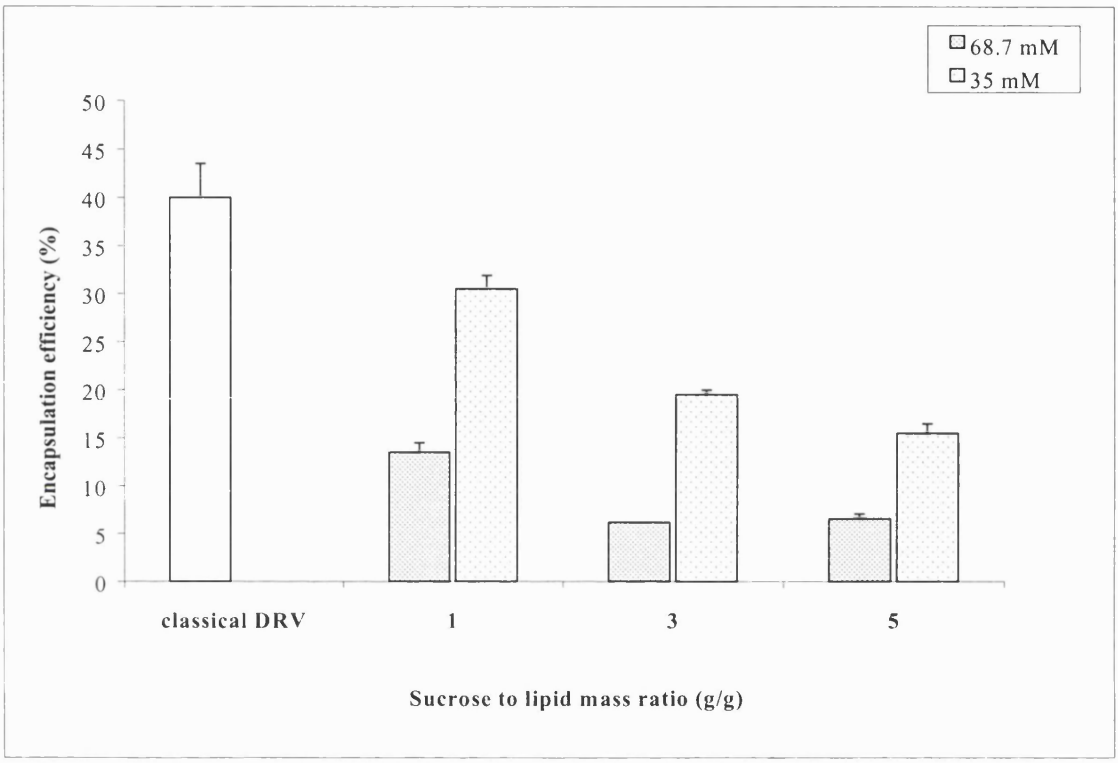

Fig 6.18 Effect of the sucrose molarity on the entrapment of ^{14}C-penicillin into liposomes (PC and equimolar CHOL) prepared by freeze-drying in the presence of various sucrose to lipid mass ratios.

The first preparation (68.7mM sucrose) exhibited a final Z–average diameter between 90 and 100 nm. Almost no fusion occurred since the starting SUV s size was around 60-80 nm. The preparations achieved using the unmodified DRV method exhibited a Z-average diameter around 5µm and an encapsulation efficiency of 40%.

Th optimum achieved for the formulation prepared at high molarity (ratio of 1/1) were of 13% of the encapsulated ^{14}C-penicillin (Z_{-av}=100nm). The encapsulation efficiency improved to become 30% with liposomes exhibiting a Z-average diameter of around 200nm. The dilution to the appropriate sucrose molarity can constitute for some compounds an interesting approach to optimize the outcome of this novel method in terms of loading efficiency and of corresponding liposomal size.

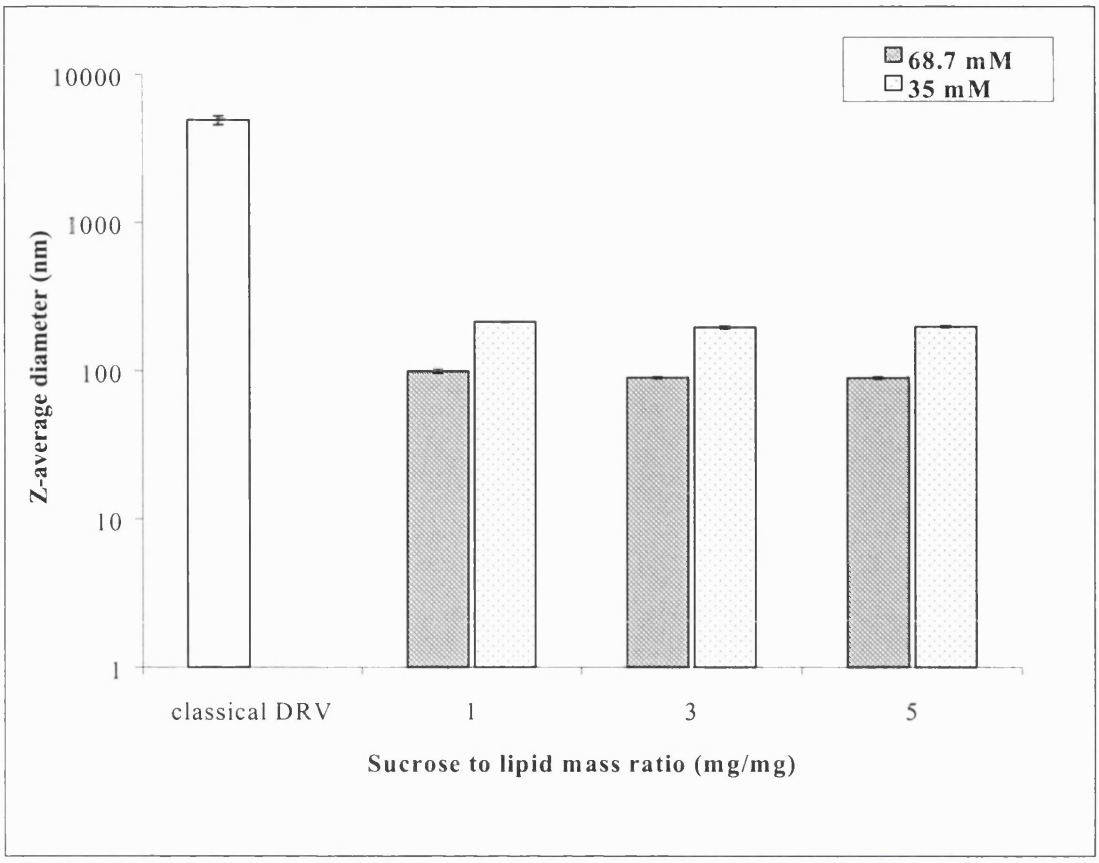

Fig 6.19 Effect of the sucrose molarity on the Z-average diameters of liposomes (25 mg PC and equimolar CHOL) encapsulating ^{14}C-penicillin (5 mg used) prepared by freeze drying in the presence of various sucrose to lipid mass ratios.

6.3.7 Effect of the rehydration temperature

Heating a liposomal suspension generally leads to the fusion of the vesicles and to aggregation as well as to the leakage of encapsulated material. In order to improve the percentage of entrapment of ^{14}C–penicillin, we applied heat to some preparations during the rehydration step. In this procedure the vial containing the preparation was preheated and an amount of water (for rehydration) added. As shown in Figures 6.20 and 6.21 heat application to the rehydrated gel-like preparation led to significant fusion and brought at the same time the diameter of the reconstituted vesicles up to a micrometer range when a ratio of 1g sucrose/g lipid was used. Naturally, the corresponding entrapment were significantly improved (37%).

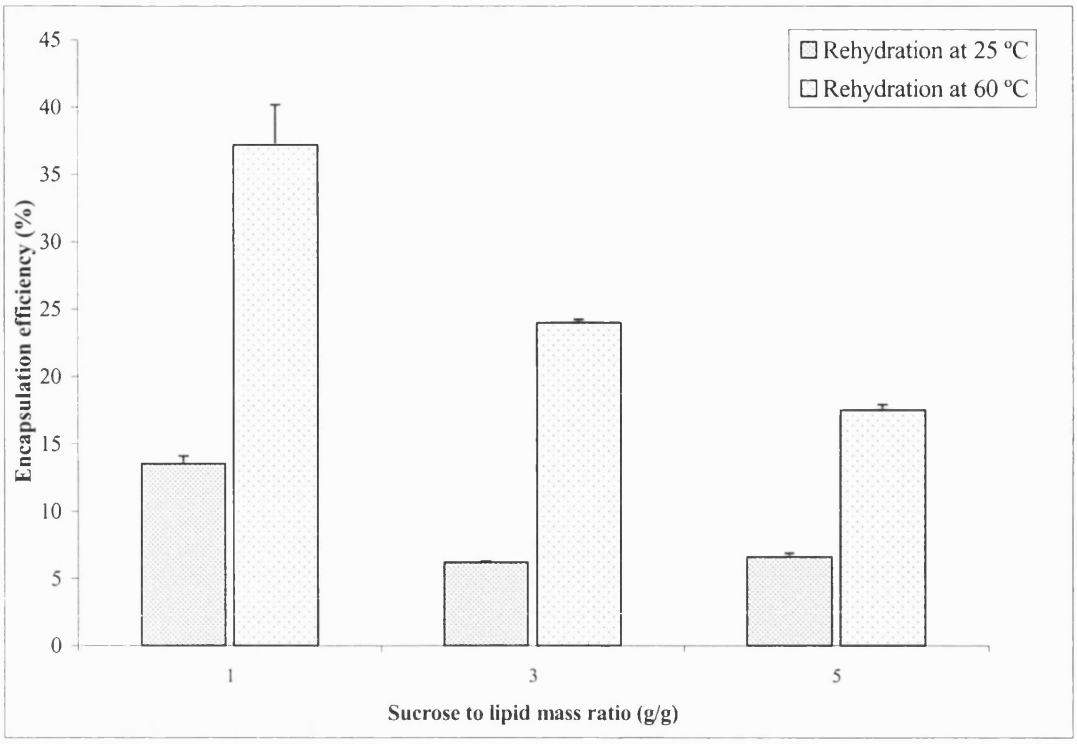

Fig 6.20 Effect of rehydration temperature on the entrapment efficiency of ^{14}C-penicillin (5 mg used) into liposomes (25 mg PC and equimolar CHOL) prepared by freeze-drying in the presence of various sucrose to lipid mass ratios.

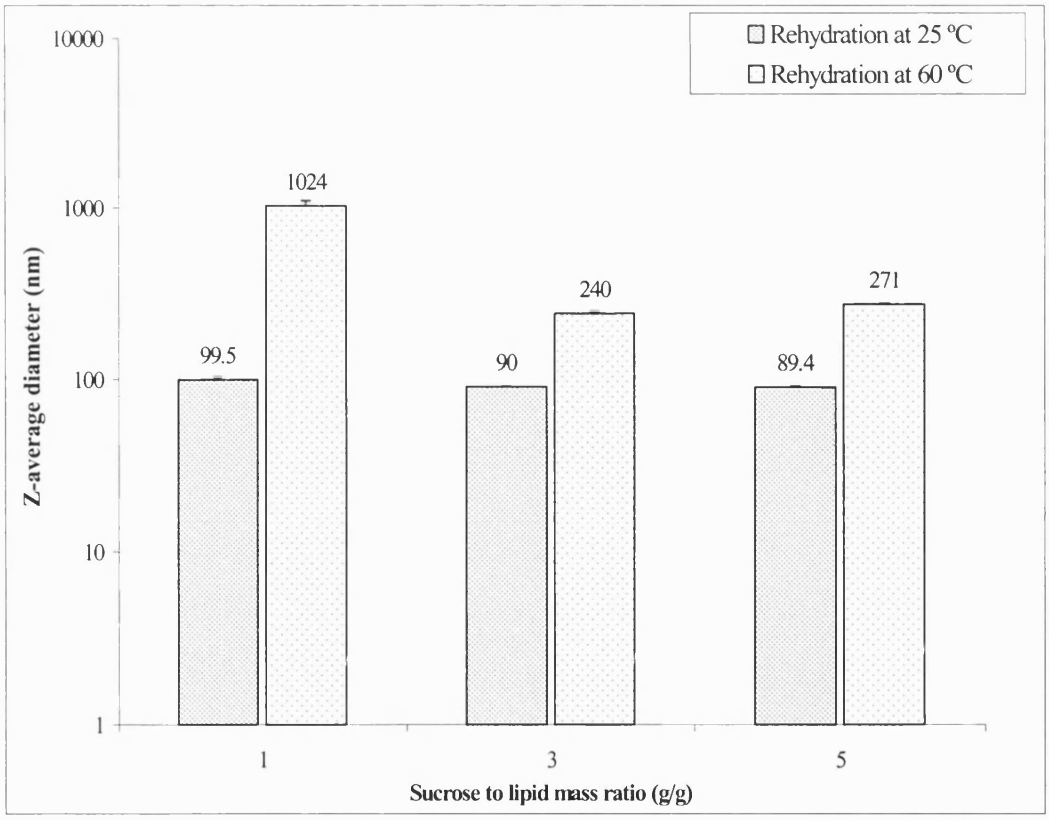

Fig 6.21 Effect of the rehydration temperature on the Z-average diameters of liposomes (25 mg PC and equimolar CHOL) entrapping ^{14}C-penicillin (5 mg used) prepared by freeze-drying in the presence of various sucrose to lipid mass ratios.

At a ratio of 3g sucrose/ g lipid the fusion generated upon heat application was less pronounced leading to a substantial amount of encapsulated ^{14}C-penicillin (24%) and to Z-average diameters of around 240 nm.

This approach does not seem to be beneficial since there is no real control on the extent of fusion. Indeed, a mass ratio of sucrose to lipid of 5 led to even larger Z-average diameters and to relatively lower entrapment values than the ratio 3/1.

When heat application was used as an alternative to improve encapsulation efficiency, the ratio of sucrose to lipid seemed to play a less important role.

On the other hand, the use of a high phase transition lipid (DSPC instead of PC) seemed more appropriate for penicillin formulation. The application of heat for a few minutes was inherent to the necessity of rehydration above the temperature of phase transition (Tc) which was 55° C for DSPC. The use of DSPC (with equimolar cholesterol) and equal to lipid mass of sucrose (mass ratio of sucrose to lipid of 1/1) for ^{14} C-penicillin entrapment, led to vesicles encapsulating up to 40% of the originally added ^{14} C-penicillin and to a Z-average diameter of around 260 nm (Fig 6.7). In similar conditions, the PC based preparation (Fig 6.20 and 6.21) led to vesicles exhibiting Z-average diameter of 1024 nm and to entrapment value around 37%. If this approach has to be considered for the improvement of the encapsulation it is most appropriate to use high phase transition lipids such as DSPC.

6.3.8 Effect of the incorporation of anionic or pegylated lipids into the liposome formulation

6.3.8.1 Effect of the incorporation of the anionic lipid (DMPG)

In these experiments we investigated the effect of the incorporation of the anionic lipid DMPG into the lipid bilayers of liposomes. It is known that the incorporation of charged lipids leads to electrostatic repulsion of bilayers and of vesicles. This generally generates smaller vesicles. The incorporation of DMPG at 0.5 molar ratio into PC and CHOL liposomes (1:0.5 molar) could allow the use of a lower mass ratio of sucrose to lipid. Indeed, at 1g/g of sucrose to lipid the presence of 25% molar (of the total lipid) of DMPG allowed the formation of liposomes with a Z-average of 145 nm encapsulating 30% of the originally added riboflavin (Fig 6.22).

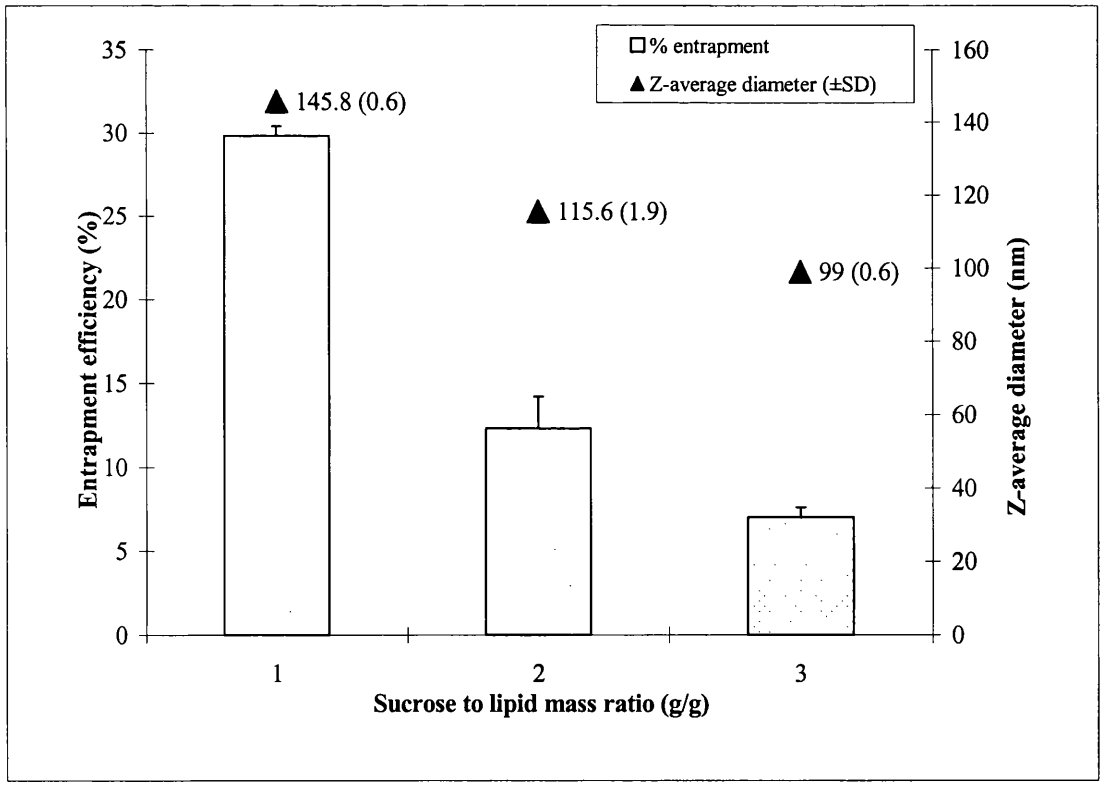

Fig.6.22 Effect of the incorporation of DMPG on the entrapment efficiency and the Z-average diameters of liposomes (PC:CHOL:DMPG 1:0.5:0.5 molar ratios) entrapping riboflavin (2mg riboflavin input to 37 mg of lipids) and prepared in the presence of various sucrose to lipid mass ratios.

Increasing the mass ratio of sucrose /lipid to 2 and then to 3 led to smaller vesicles encapsulating relatively low amounts of the originally added riboflavin (12 and 7 %) for vesicles with a Z–average diameter of around 100 nm. By adding an amount of anionic lipid to the lipid bilayer we reduced the amount of sucrose needed to achieve relatively small vesicles. The electrostatic repulsion allowed a better size control in the presence of a minimum amount of sucrose. Furthermore, in some circumstances, there is a need to use anionic and cationic lipids to help the incorporation of drugs into the inner aqueous phase or to the lipid bilayer surface. Cationic lipids are frequently used to generate plasmid DNA-containing liposomes (Gregoriadis *et al*, 1997). In order to optimize the amount of sucrose needed to prepare liposomes encapsulating doxorubicin, we decided to test the effect of the incorporation of the anionic lipid DMPG. Two experiments were conducted . The first consisted of incubating the anticancer drug doxorubicin with preformed SUV's in the dark at room temperature. The second experiment consisted of preparing liposomes encapsulating doxorubicin using the novel method. Identical SUV's compositions and amount of lipids and drug were used. The separation of the free dug was accomplished by ultracentrifugation as usual. The results in Fig 6.23 show the amount of doxorubicin complexed after incubation with the liposomes as well as the Z-average diameter obtained at different molar percentage of DMPG. On the other hand, when similar amounts of lipids and doxorubicin were used to prepare liposomes by the novel procedure where sucrose was present at a fixed mass ratio of 3 mg per 1 mg of lipids, liposomes exhibited the characteristics shown on Figure 6.24. It appears from Fig 6.23 that substantial amount of doxorubicin complexed to the liposomes which exhibited large sizes. The preparation using the novel method exhibited high percentage entrapment but smaller sizes (Fig 6.24).

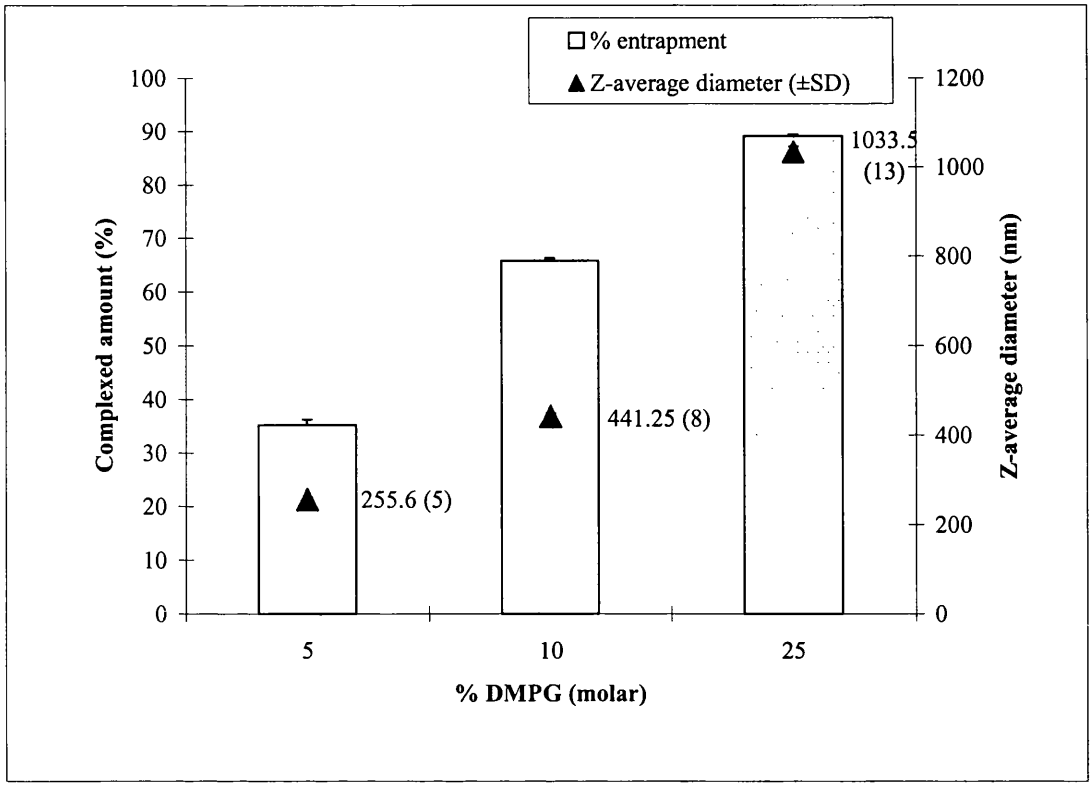

Fig 6.23 Effect of the incorporation of DMPG on doxorubicin-liposomes complex formation (PC:CHOL and DMPG) and on their Z-average diameters after incubation at 20°C for 1 h (1mg of doxorubicin to 18 mg of lipids).

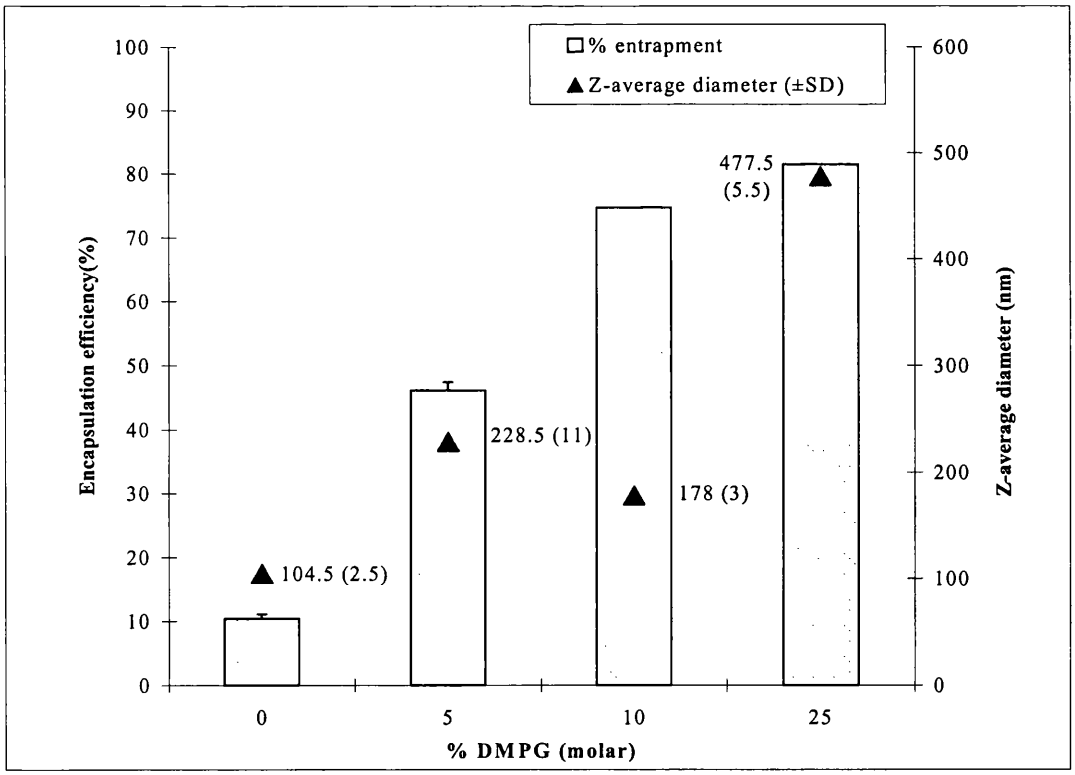

Fig. 6.24 Effect of the incorporation of DMPG on the entrapment efficiency and Z-average diameters of liposomes (PC:CHOL:DMPG) (1mg of doxorubicin input to 18 mg of lipids) prepared by freeze-drying in the presence of 3mg of sucrose per mg of lipid.

An optimum formulation was obtained when 10% molar of DMPG was used. Indeed, 75% of the added doxorubicin was encapsulated within the vesicles prepared in the presence of 3 g of sucrose/g lipid. These liposomes exhibited a small Z-average diameter (180 nm). It appears that not only the variation of the amount of sucrose used is important in performing the novel method but that the lipid charge is also a determinant. We should always take into account the nature of the molecule to be encapsulated as well as its physico-chemical properties and tailor the lipid composition to adapt the requirement of encapsulation using optimum amounts of sucrose.

6.3.8.2 Effect of the incorporation of pegylated lipids

This part of the study was devoted to the examination of the possibility of incorporation of an amount of DSPE-PEG into the lipid bilayer using formulations prepared by the novel method. It has been reported (Senior *et al*, 1991; Torchilin *et al*, 1994, Hosoda *et al*, 1995) that the incorporation of pegylated lipids leads to a reduced clearance of such systems by the RES and thus to an increased blood circulation time. Liposomes were formulated using the novel method and designed to contain an amount of pegylated lipid.

The results (Fig 6.25) indicate that conventional liposomes (equimolar PC and CHOL) exhibited the same encapsulation efficiency of riboflavin as with liposomes containing an amount of DSPE (only) or of PEG conjugated to DSPE (47 to 50% of the originally added riboflavin). The results obtained for the Z-average diameters of these formulations were different.

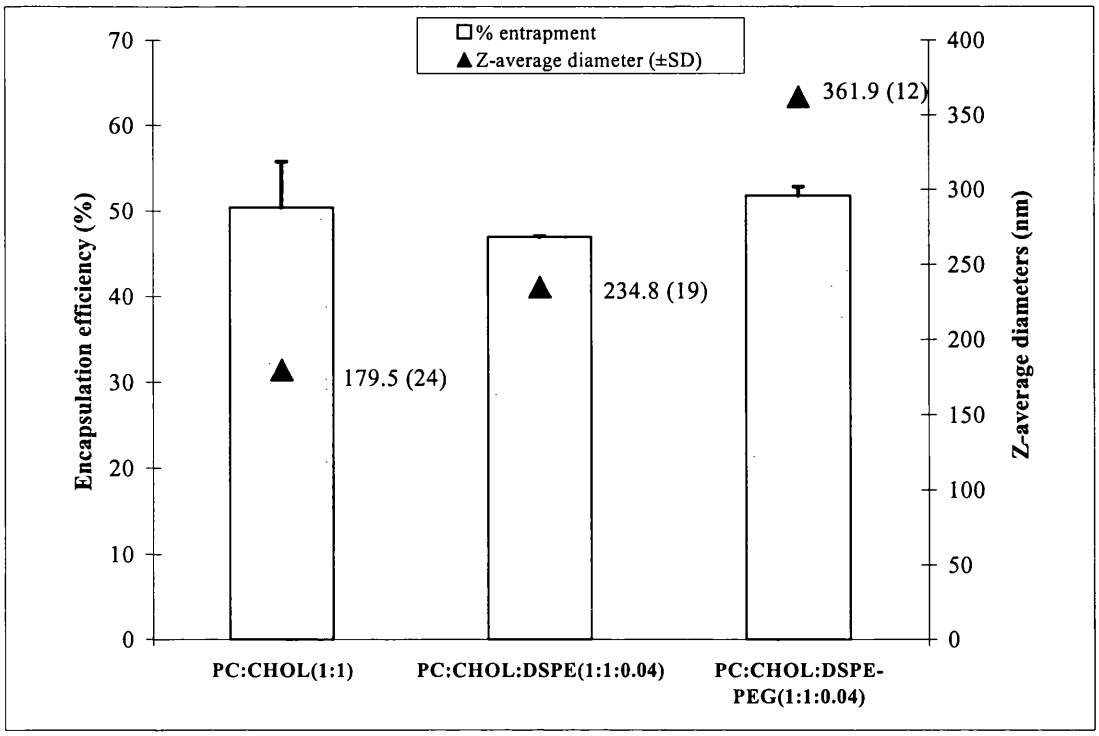

Fig. 6.25 Effect of the incorporation of DSPE or DSPE-PEG (4 % molar) into the bilayers of liposomes (25 mg PC and equimolar CHOL) prepared using the novel method (3mg of sucrose per mg of lipid) on the encapsulation efficiency of riboflavin (1mg used) and their Z-average diameters

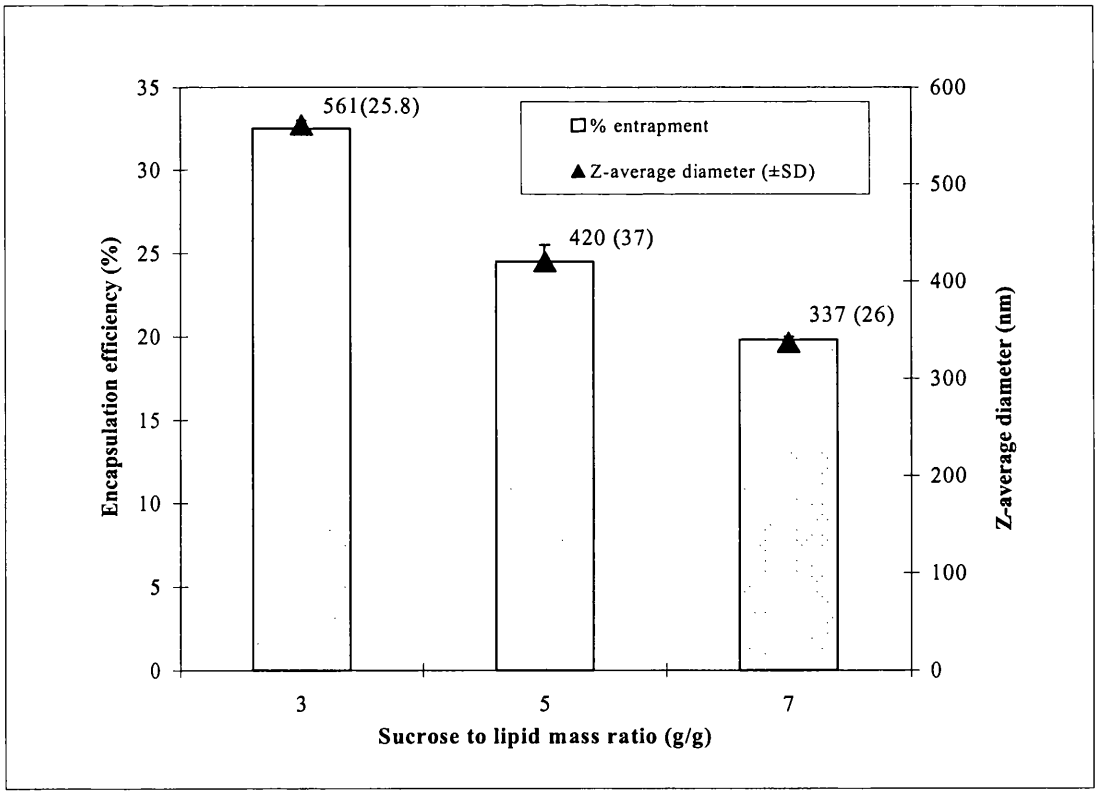

Fig 6.26 Encapsulation efficiency and Z-average diameters of liposomes (25 mg DSPC and CHOL:DSPE-PEG 1:1:0.04 molar ratios) entrapping riboflavin(1 mg used) and prepared (rehydration at 65 °C) in the presence of various sucrose to lipid mass ratios.

The addition of DSPE only to equimolar PC and CHOL liposomes resulted in an increase of the Z-average diameter from 180 nm (PC and equimolar CHOL) to an average of 235 nm (PC:CHOL:DSPE; 1:1:0.04 molar).

The incorporation of DSPE led to more fusogenic liposomes upon freeze-drying in the presence of 3 g of sucrose /g of lipid and rehydration When the DSPE-PEG was incorporated, even more pronounced fusion occurred upon freeze-drying and rehydration leading to larger Z-average diameters. The final Z-average diameter was around 360 nm for the DSPE-PEG formulation (DSPE-PEG at 4 % molar).

In an attempt to improve those results, we formulated similar liposomes using the novel method containing the lipid DSPC which exhibits a high phase transition temperature Tc (55°C). These liposomes were prepared in the presence of increasing mass ratio of sucrose to lipids (3, 5, 7 g of sucrose per g. of lipid used). The formulations were rehydrated at a temperature of 65°C. Increasing the sucrose to lipid mass ratio from 5 to 7 g/g led to decreased encapsulation efficiencies of riboflavin (Fig 6.26). This also lead to a substantial size reduction. At a ratio of 7 mg of sucrose to 1 mg of lipid used, the final Z-average diameter is still substantially high compared to that with equimolar PC or DSPC and CHOL devoid of DSPE-PEG. The PEG moiety conferred some fusogenic properties to these bilayers upon freeze-drying and rehydration. A possible explanation is that the sucrose does not interact with the bilayer membrane containing the DSPE-PEG in a simil ar way to the one involving bilayers made of PC or DSPC and equimolar cholesterol only. Besides, other additives or lipid compositions in conjunction with the sucrose could lead to improved results upon drying and rehydration.

In order to improve these results, the rehydration step for similar liposome preparations (DSPC: CHOL: DSPE-PEG; 1:1:0:4 molar) prepared in the presence of

sucrose (Fig 6.27) was carried out at the lower temperature of 45°C. The presence of DSPE-PEG could enhance the fusion and the rehydration of the liposomes when performed at a lower temperature, thus being beneficial for the overall procedure.

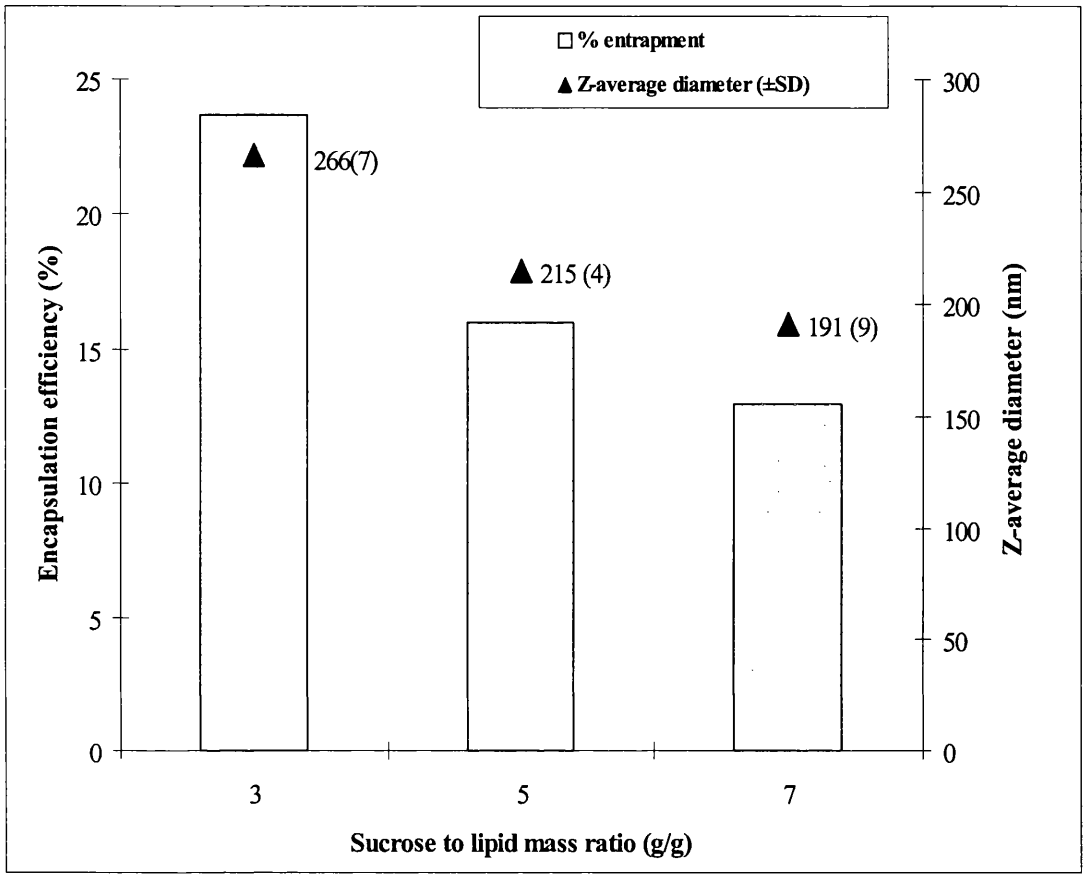

Fig 6.27 Encapsulation efficiency and Z-average diameters of liposomes (25 mg DSPC and CHOL:DSPE-PEG 1:1:0.04 molar ratios) entrapping riboflavin(1mg used) and prepared using the novel method (rehydration at 45°C) in the presence of various sucrose to lipid mass ratios.

Indeed, such an approach was revealed to be beneficial. As shown by the Z-average diameters (Fig 6.27), we noticed less fusion when the rehydration was achieved at 45° C. The formulations prepared at a mass ratio of 3 mg of sucrose for each mg of lipid exhibited a Z-average diameter of around 265 nm with around 24% of the originally added riboflavin entrapped. The same ratio gave a percentage entrapment of 32% and a Z-average diameter of around 560 nm at a rehydration temperature of 65°C. This approach could be interesting in improving the outcome of the novel method when pegylated lipids are used for long circulating liposome formulations.

6.3.9 Effect of the substitution of sucrose by glucose

In this experiment, sucrose was substituted by glucose in order to find out whether another carbohydrate (for instance glucose) could allow the preparation of liposomes exhibiting characteristics similar to those exhibited by identical liposomes prepared in the presence of sucrose.

The liposomes were prepared in the presence of increasing mass ratios of sucrose or glucose to lipid.

It appears from (Fig 6.28 A and B) that the use of sucrose generates liposomes exhibiting smaller Z-average diamcters than those prepared in presence of glucose. Furthermore, the entrapment efficiency (for riboflavin) achieved at the ratio of 1mg of glucose/mg of lipid is lower than the one achieved at the same ratio of sucrose to lipid.

Visual examination of the vials containing the liposome preparation prior to the rehydration step revealed that the cake obtained exhibited a collapsed structure.

For these liposome carbohydrate mixtures, as the temperature was lowered, the system undercooled before freezing had occurred. Solute crystalisation may or may not have happened. Depending on the formulation details, it may also have been incomplete. At a characteristic temperature and composition, freezing stops; at least at a measurable rate. Then the mixture undergoes a glass transition where the frozen concentrated aqueous solution of a very high viscosity transforms into a brittle solid. Operationally, this is characterized by a glass transition temperature Tg'. Tg' is therefore the temperature at which freezing (at a measurable rate) is complete. Knowing the Tg' is paramount when it comes to designing a successful freeze-drying procedure. Table 6.2 shows the values of Tg' of a few excipients commonly used for freeze-drying.

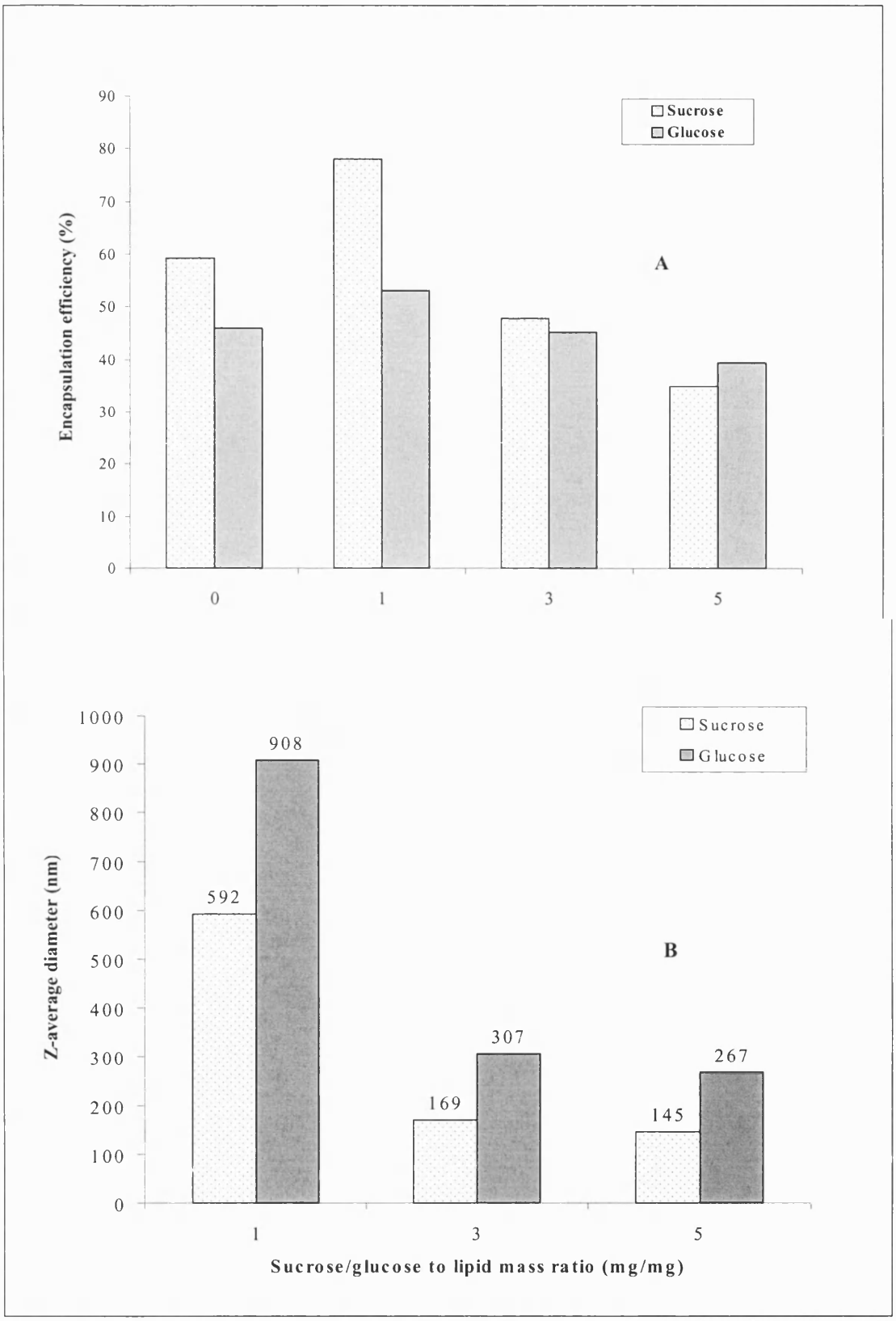

Figure 6.28 Effect of the substitution of sucrose by glucose on the liposome (25 mg PC and equimolar CHOL) characteristics: (A) encapsulation efficiency of riboflavin (1 mg used), (B) Z-average diameters.

Table 6.2: Values of Tg' for various excipients used for freeze-drying

Compounds	Tg' (°C)
Glycerol	-100
Ribose	-47
Xylose	-47
Arabinose	-48
Sorbitol	-43
Mannitol	-40
Fructose	-42
Glucose	-43
Galactose	-42
Sucrose	-32
Maltose H_{20}	-29
Lactose	-28
Trehalose	-30
Raffinose	-26
Dextran	-14

Bringing the temperature of the frozen concentrate below the Tg' (-42°C for glucose not mixed with liposome or any other additive) is a prerequisite for good freeze-drying conditions. At this temperature (Tg'), the solution phase of the frozen mixture still contains a certain amount of water which is unfrozen and which has to be removed by diffusion and desorption during secondary drying. In our experiment, we used a freezing step apart from freeze-drying. The sample was frozen in a freezer at a temperature that never went lower than an average of -40°C. It was then subjected to vacuum without any possible control over the shelf temperature of the vial. The sample dried under constant conditions of pressure (vacuum) and temperature. The temperature of the glucose/liposome mixtures was probably not lowered enough i.e. to the temperature range of Tg'. The solution of the mixture was still containing an amount of water in an unfrozen state. These conditions brought the frozen system containing glucose to collapse during the course of the procedure. This early collapse allowed an undesired fusion generating larger size and no possibility of getting

optimal entrapment efficiency. This was never observed for the system containing sucrose. Some control over the freezing cycle and proper freeze-drying conditions (freezing of the system below Tg', control over the temperature of the freeze-drying chamber) could permit successful use of glucose and other excipients in the freeze-drying procedure involved in the preparation of liposomes.

In the literature, contradictory reports on the efficiency of different excipients used for liposome stabilization are noted. Special care has to be taken when conducting freeze-drying experiments for the evaluation of a carbohydrate efficacy in maintaining the integrity of a liposome structure.

Using the same dilution and the same mass ratio of carbohydrate to identical lipid compositions does not constitute enough conditions for comparisons. Other sources of variability are imposed by the protocols involved, such as the freezing temperature, freezing rate as well as the period of time chosen for the primary and the secondary drying.

6.4 Conclusions

The use of sucrose (amount expressed in mass ratio of sucrose to lipid and in molarity) in the presence of the SUV s and of the compound destined for encapsulation permitted the control over the extent of fusion (as judged by changes in vesicle size) during the rehydration step. This also led the good encapsulation efficiencies of different compounds such as proteins, anti-cancer and antimicrobial drugs and low molecular weight markers. These liposomes exhibited small Z-average diameters upon rehydration.

The controlled rehydration served as a drug loading step in which the sucrose played a major role in maintaining the Z-average diameters of the vesicles relatively low. The results are correlated with the amount of sucrose, which is expressed both in terms of mass ratio and molarity. With this approach, similar entrapment values of compounds to those obtained with the classical unmodified DRV procedure could be achieved. The corresponding small Z-average diameters obtained with the novel method supports the superiority of the method to the unmodified DRV procedure leading to vesicles of micrometer range.

Extruding preformed DRV liposomes generates vesicles exhibiting small Z-average diameters and low entrapment values. The high pressure used to force vesicles through the pores of the membranes resulted in the leakage of the encapsulated compound.

Moreover, extrusion or any other sizing techniques constitute an additional step in liposome formulation. Using sucrose allows a virtual one-step method where the rehydrated liposome exhibit higher entrapment values and similar Z-average diameters to those corresponding to the extruded preparation. The size distribution of liposomes was narrower when the extrusion was employed than when sucrose was used. A possible explanation is the use of high pressure to force the vesicles through pores of equal diameters. An increased molarity of sucrose generates liposomes exhibiting a narrower size distribution, which in some cases was very similar to the size distribution of extruded liposomes. Using lipids exhibiting high phase transition Tc promotes the formation of a narrower vesicle size distribution for liposomes prepared by the novel method.

It was also shown that spray-drying could constitute an alternative drying technique for the preparation of liposomes using the novel method. Indeed, larger amounts of

lipids are used in the presence of adequate amounts of sucrose. Large batches could easily be obtained.

The fusion phenomena that must be occuring during the drying and rehydration cycle are more pronounced for diluted solutions of sucrose. Both the mass ratio of sucrose to lipid and the final molarity of sucrose prior to drying could be adjusted so as to optimize the liposome loading efficiency and the Z-average diameters.

Increasing the temperature (above 20°C) upon rehydration does not seem to be beneficial for the preparation of liposomes employing lipids exhibiting a low temperature of phase transition (Tc). Indeed, high sucrose to lipid mass ratios did not lead to the Z-average diameters usually obtained. Use of high temperature during rehydration is preferred for formulations employing high phase transition lipids, due to their enhanced stability at high temperatures.

The incorporation of anionic lipids (up to 0.25 molar ratio) allowed the use of lower mass ratio of sucrose to lipid. It was possible to achieve liposomes exhibiting a Z-average diameter of around 145nm by using 1mg/mg of sucrose to lipid ratio. The use of the anionic lipids in conjunction with low mass ratio of sucrose to lipid allows the encapsulation as well as complex formation of molecules exhibiting specific properties such as electrostatic interactions

Liposomes prepared by the novel method were also formulated to contain an amount of pegylated lipid (DSPE-PEG). The incorporation of DSPE alone into PC and equimolar CHOL liposomes led to a slight increase in the Z-average diameters upon rehydration. This was even more pronounced when the DSPE-PEG was incorporated. The PEG moiety conjugated to the lipids appeared to confer a fusogenic property to the bilayer membrane upon freeze-drying in the presence of sucrose and rehydration. Rehydration at a lower temperature (45°C instead of 65°C) of DSPC based liposomes

containing DSPE-PEG led to smaller Z-average diameters. The incorporation of the pegylated lipids into the liposome bilayers used to encapsulate riboflavin by the novel method, involved in some experiments the use of high mass ratio of sucrose to lipids as well as high molarities of sucrose. In this particular case, the efficiency of sucrose in maintaining the Z-average diameters around 100-200 nm upon rehydration was lower.

The use of glucose instead of sucrose generated liposomes with slightly larger Z-average diameters. Considerations concerning the stability of the frozen-concentrate (liposome glucose mixtures) has to be taken in account for successful freeze-drying. The estimation of the temperature at which freezing of the mixture stops (Tg'), is a prerequisite for the production of small liposomes entrapping considerable amounts of the added compound. Collapsed or partially collapsed cakes are a source of premature fusion prior to rehydration which is in this case very disadvantageous. This results in the formation of larger vesicles exhibiting lower encapsulation efficiencies. Furthermore, the adequate control of the temperature in the freeze-drying chamber is also important. Conducting proper freezing, primary and secondary drying cycles could be essential in the optimization of the characteristics of the liposomes prepared by the novel method.

The novel method also allowed the production of a dry cake that could be stored until needed, thus solving the problem of stability of liposomes in suspension. More studies are needed to evaluate the long-term stability of the liposomal dry cake.

Chapter Seven

General conclusion and prospects

The present generation of parenterally administered liposomes encapsulate drugs in sufficient amounts to exhibit the correct therapeutic index. The preparation of these formulations must be reproducible and the drug must be chemically stable and totally encapsulated. The vesicle size must be small and to remain so during storage. The first part of our study was an attempt to quantify the effect of the operational parameters during the microfluidization of preformed DRV s leading to smaller sizes and allowing for parenteral administration.

An attempt using mathematical tests was made to predict the outcome of this approach. The aqueous liposomal dispersions exhibited small sizes. Significant aggregation, fusion, leakage of the encapsulated compound and/or chemical degradation of the lipids may occur.

Spray drying was considered as a possible tool to facilitate the production of a dry liposomal product starting from preformed vesicles (eg. DRV s) containing water-soluble compounds. This dry product could be rehydrated or inhaled as such. However, the reconstituted liposomes were leaky and required sizing (before spray drying) to obtain sufficient retention values of the encapsulated compounds upon spray drying and rehydration. Their incubation with lung surfactant led to rapid release of the encapsulated compound except for a specific DRV liposome composition based on DSPC.

The reduction of the size of the liposomes was a prerequisite for both parenteral administration where reduced clearance by the RES is required and also for any attempt to convert the suspension to a dry product which upon resuspension in water exhibits good characteristics in terms of retention of drug and vesicle size.

An attempt was also made to design a novel method that combines both of the following two considerations:

-the production of a dry liposomal formulation constituted of SUV s, the drug and an amount of carbohydrate ideal for storage,

-rehydration of the formulation will constitute the loading step leading to optimal characteristics in terms of entrapment and vesicle size.

7.1 Size reduction using the microfluidization technique of preformed DRV: possibilities and limitations

The statistical method adopted showed that the nature of the phenomena involved during microfluidization overrides the effect of the operational parameters. This is because the air pressure in the intensifier pump determines the energy transmitted to the liposome suspension in order to achieve size reduction. Adequate microfluidization should be carried out at high pressure. A compromise has to be found between the number of cycles employed for microfluidization and the optimum percentage entrapment and size values required for further use. Generally, the first few cycles will substantially reduce the DRV s Z-average diameters. Further processing will lead only to more pronounced leakage of the encapsulated compound. Processing unwashed liposomes was beneficial for the improvement of drug retention and for the production of small sized liposomes.

The mathematical modeling designed for the prediction of the vesicle characteristics can be used to optimize the microfluidization step allowing better control over the final liposomes characteristics. This also could be used as a tool for quality control.

At time intervals, aliquots were taken from a formulation for the estimation of entrapment and sizing . Having set the operational conditions, the values of Z-average diameters and the percentage entrapment could be obtained from a validated

mathematical model. These values were then compared with the real, measured characteristics to evaluate the efficiency of the procedure. A substantial difference between the measured and the predicted data will inform of a problem with either the formulation or the conditions of microfluidization. For instance, clogging in the interaction chamber or a drop in the pressure of the intensifier pump can lead to inefficient microfluidization. Proper action can then be taken.

Microfluidizing preformed DRV s had the limitation of having the final product in suspension containing both the encapsulated and the free drug which has to be removed in most cases.

Because of the dilution involved, processing high amounts of lipid in the presence of high amounts of drug could be an interesting alternative. The increased viscosities (high amount of lipids) will lead to the reduction of the efficacy of the microfluidization. To use such an approach, the highest possible lipid concentration allowing efficient microfluidization should be determined. The new extruder Maximator® (maximum working pressure 140 bars) was designed to operate continuously at high pressure. This involved the use of high lipid concentrations (400 mg/ml). A test carried out on the same equipment in our laboratory involving 400mg of lipid in 30 ml of PBS (liposomes prepared for spray-drying) was shown to be very difficult with, sometimes, loss of some of the material from an evacuation valve designed for security. If the operational pressure goes beyond a set limit of security, this valve opens to by-pass the extrusion device. Processing liposomes based on lipids exhibiting high phase transition temperature was also shown to be very difficult. An additional major limitation when using high pressure homogenization is the production of a final liposomal suspension where the problems of stability still exist (hydrolysis, oxidation, aggregation, fusion, leakage of compound).

7.2 Stabilisation of preformed liposomes

This was attempted by spray drying preformed unwashed liposomes. The atomization did not affect the liposome characteristics as an aqueous suspension. Spray drying involves atomization and water evaporation that take place at the same time. Understanding of these complex phenomena will help the design of a more successful spray-drying process.

The size distribution of the spray-dried product was adequate for use for inhalation. The use of high phase transition lipids conferred better mechanical properties to the powder as examined by SEM. The heat employed did not induce oxidation or any other damage.

The liposome bilayer composition did not influence the retention of the water-soluble markers. It is possible that the damage to the liposome structure occurs during spray drying because of the phenomena involved (eg. shear forces of atomization and subsequent water removal). The removal of water contributes to most of the damage. All the carbohydrates used (trehalose, sucrose, lactose) were equally protective in all the cases, more so than the salts contained in the PBS buffer. It was not possible to employ a lower inlet temperature and in the same time achieving an adequate outlet temperature for an optimal collection of the the hygoscopic mixtures of lipids and sugars used. A higher inlet temperature should be tested. This may induce the formation of specific amorphous particles of carbohydrate conferring better protection to the liposomes. Increasing the amount of the thermoprotectant sucrose did not lead to any improvement in the retention of the water-soluble glutathione.

Reducing the DRV liposome size prior to spray drying resulted in the improvement of the retention of the water-soluble compounds upon spray drying and rehydration. The

Z-average diameter exhibited by the liposomes prior to spray-drying was found to be an important parameter contributing to improved retention of the encapsulated material upon drying and rehydration .

If the requirements for an improved stability upon spray drying and rehydration are the use of very small liposomes, the latter will be processed through homogenization and exhibit a substantially low percentage entrapment. But this approach is not worth considering.

In some cases the stabilization involved liposomes where the drug encapsulation is achieved with active loading methods, specifically the ammonium sulfate precipitation method. This requires the use of very small liposomes that do not require further sizing. Stabilizing such preparations will be advantageous. The application of this method to doxorubicin led to the formulation of a gel-like structure of the drug upon precipitation within the vesicles that enhanced its stabilization (ie. retention by the vesicles) upon freeze-drying in the presence of carbohydrates. Thus physicochemical state of the drug within the vesicles played an important role.

To sum up, adequate stabilization of preformed DRV liposomes required vesicle size reduction and a washing to remove the non-encapsulated material prior to spray drying. This approach does not seem to be very advantageous because of the many steps involved and of the low quantities of drug involved.

The use of these spray-dried liposomes for inhalation may require other approaches since the release of the drug after incubation with the lung surfactant was rapid. However, the methodology used to evaluate this phenomenon was not adequate as it does not reproduce the conditions involved when particles are inhaled and reconstituted within lung fluids. In vivo evaluation seems to be the most appropriate way for testing such preparations. Most of the lung fluid is constituted of lipid and of

surfactant proteins. It may be rather more appropriate to spray-dry the liposomes in the presence of polymers rather than sugars, as this will lead to polymeric particles able to constitute a shield against the surfactant thus allowing the reconstitution of liposomes more adequately. Furthermore, these particles could serve as a "reservoir" of reconstituted liposomes releasing them in a controlled manner. Thus the use of hydrophobic polymers may be a better alternative to sugars for inhalation applications.

7.3 The use of sucrose during the drying step of liposome preparations

The novel method presented here consisted of freeze-drying liposome SUVs with the drug in the presence of certain amounts of sucrose. This led to a high drug entrapment values and to a modestly larger vesicle size upon reconstitution with water, presumably because the use of sucrose permitted a certain degree of fusion during the controlled rehydration step. The preparations obtained did not need any further sizing as their Z-average diameters were as low as 120-160 nm. The liposomes prepared using this method were found to exhibit better characteristics (in terms of solute entrapment and size) than those prepared by extrusion of preformed drug containing DRV liposomes.

However, the size distribution of the extruded liposomes was narrower than that obtained using the novel method. Moreover, the use of high phase transition temperature lipids in preparing liposomes promoted a narrower size distribution. Spray drying as an alternative to freeze-drying was equally successful in the preparation of drug-loaded small liposomes by the novel method.

The mass ratio of sucrose to lipid appeared to be the most important parameter controlling the characteristics of liposomes upon rehydration. Moreover, the incorporation of anionic lipid into the liposome structure permitted a decrease of the mass ratio of sucrose to lipid leading to better encapsulation efficiencies. In the case of doxorubicin, it appeared to promote some kind of active incorporation of the drug into liposomes. The use of lipids which interact (eg. complex formation or electrostatic interaction) with the drug destined for encapsulation could be of a great importance in optimizing drug loading (complexation and entrapment) when applying this novel method.

In most of the cases the molarity of sucrose played a role in controlling the final characteristics of the reconstituted liposomes. For instance, in some cases, it allowed the optimization of entrapment values (eg. ^{14}C-penicillin). Dilution prior to freeze-drying was a better alternative to heat application upon rehydration as it allowed a better control of the final liposomal size.

The incorporation of the pegylated lipid DSPE-PEG in the lipid bilayer during liposome preparation requires a higher amount of sucrose.

Upon reconstitution, liposomes (prepared by the novel method) containing DSPE-PEG exhibited slightly larger Z-average diameters than those that did not contain DSPE-PEG. The use of high phase transition lipid and of a temperature lower than the Tc during rehydration led to improved results.

The substitution of sucrose by glucose using the same mass ratio of sugar to lipid, lead to the generation of liposomes exhibiting slightly larger Z-average diameters. The freeze-drying protocol used and the limitations of the freeze-drier did not allow further examination. Glucose solutions have a low collapse temperature in the frozen state. Freezing these preparations to even lower temperatures is possible using liquid

nitrogen. But this was not useful, as low temperature could not be maintained in the freeze-dryer chamber as the equipment used does not offer temperature control.

There is a need for a more elaborated freeze-drying protocol in order to be able to compare the efficiencies of different carbohydrates when performing the novel method.

7.4 Perspectives and suggestions for further work

7.4.1 Use of the mathematical modeling to optimize the preparation of empty SUVs by the microfluidizer

The mathematical modeling methodology should be used to evaluate the microfluidizer for the preparation of small unilamellar liposome to serve as a starting material in the novel method. The variable parameter of the formulation could be the lipid composition (e.g.: the amount of added cholesterol or of any anionic or pegylated lipid). The pressure should be set up at the highest value possible. The operational variable parameter could be the number of cycles. The measured response could be the final Z-average diameter (the smaller, the better) and also the zeta potential in the case of anionic or cationic lipids. These data will be useful when it comes to the scaling-up of the novel method.

7.4.2 Spray-dried liposomes for inhalation

Spray-dried liposomes for inhalation could be prepared in the presence of polymers instead of sugars which are more suitable for rehydration. These polymers could be insoluble in water or water-soluble. The liposomes could be formulated to contain lipophilic compounds inserted in the lipid bilayer, as well as water-soluble drugs.

Large molecular weight drugs such as proteins and DNA could be easily encapsulated with their leakage upon rehydration probably being less pronounced because of their size.

The spray drying of emulsified lipids in the presence of drugs and of polymers could also be an interesting approach to prepare suitable particles for inhalation. One of the problems in performing such experiments will be the use of organic solvents.

Possibly, other spray-drying conditions should be tested, for example, higher inlet temperature. This will have implications on the polymorphism and the physical state of the added thermoprotectant.

7.4.3 Long-term stability of liposomal products

The stability of amorphous products such as freeze-dried sugar solutions or drug formulations depends on their Tg.

A study investigating the stability of liposome-sugars mixtures during storage at a temperature range should be carried out. Physical and chemical degradation can occur below Tg of freeze-dried cakes. The choice of the sugar used as well as low residual water content after freeze-drying will help to optimize long-term stability. Different sugars such as lactose, trehalose should be tested. Tests involving the storage of the freeze-dried cake at different percentages of relative humidity (RH%) will help to measure the water uptake (by weight) by the solid and thus determine optimal storage conditions.

Conducting proper freeze-drying protocols can also help to optimize the outcome of the novel method as well as the long-term stability of the obtained cake.

7.4.4 Incorporation of lipophilic compounds into liposomes using the novel method

The novel method should be tested on its ability to incorporate into liposomes lipophilic compounds. The removal of water implies the intimate mixing of lipid with the other added compounds. The addition of lipophilic compounds prior to freeze-drying must be tested for their potential incorporation within the liposomes. The physicochemical properties of the molecules should be considered and the appropriate lipid composition chosen accordingly so that it facilitates their incorporation within the vesicles. The presence of sucrose or other sugars as already shown with water-soluble compounds, could help to maintain small sizes upon reconstitution.

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