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Liposomes obtained by the ethanol injection method

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Summary

One of the most simple methods of obtaining liposomes is the so-called ethanol injection. This is a mild procedure which affords a reasonably homogeneous vesicle population, although rather diluted. We have observed the influence of lipid concentration, osmolality and pH of medium on the size distribution of liposomes. On the other hand, eight substances have been encapsulated in order to establish the relation between efficiency of encapsulation and chemical characteristics. Results obtained show that a 40 mg/ml lipid concentration produces vesicles below 100 nm average diameter, this population being the most homogeneous. The effect of osmolality and pH is less important. With respect to drug encapsulation, drugs dissolved in the same ethanol as the lipid are relatively well encapsulated, whereas hydrophilic substances such as carboxyfluorescein show lower entrapment. Other kinds of drugs like the quinolones cyprofloxacin and enrofloxacin are associated with high encapsulation efficiencies but the final yield is low.

Introduction

Liposomes can be obtained by means of several different methods, each of which affords vesicles with special characteristics. One of the most simple methods is the so-called ethanol injection (Batzri and Korn, 1973). Briefly, an ethanol solution containing the lipids is injected rapidly into an excess of saline or other aqueous medium, through a fine needle. The force of the injection is usually sufficient to achieve complete mixing, so that the ethanol is diluted almost instantaneously in water, and phospholipid molecules are dispersed evenly throughout the medium. This procedure has no degradatory effect on the phospholipid detectable by thin-layer chromatography, and the absorption spectra of liposomes after redissolving them in ethanol shows no evidence of oxidation. Sizing techniques such as electron microscopy or photon correlation spectroscopy of unfractionated liposomes reveal a reasonably homogeneous preparation. Its major shortcoming is the limitation due to the solubility of lipids in ethanol (for instance, 40 mM for phosphatidylcholine), and the volume of ethanol that can be introduced into the medium (7.5% v/v maximum), which in turn limits the

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amount of lipid dispersed, so that the resulting liposome suspension is usually rather dilute. The percentage encapsulation is thus extremely low if the materials to be entrapped are dissolved in the aqueous phase. The presence of ethanol can also be a disadvantage, but this can readily be removed by dialysis. Moreover, characterization of the vesicles by light scattering shows no differences between dialyzed and nondialyzed samples (Kremer et al., 1977).

The aim of this work was to observe the influence of lipid concentration, osmolality and pH of medium on size distribution of liposomes. Furthermore, the kinetics of vesicle precipitation was followed in order to determine the liposome stability in solution. The parameters chosen to measure the influence of physicochemical factors pointed out above were size and size distribution as values of diameter (z-average mean and n-diameter) and polydispersity of vesicles, respectively. Eventually, attempts were make to encapsulate some drugs by means of this method. These drugs ranged from the most useful aqueous marker, namely, carboxyfluorescein, to lipophilic substances, such as triamcinolone, as well as some amphiphilic ones.

Materials and Methods

The lipid used was Lipoid S-100 (Lipoid KG, Ludwigshafen, Germany), a mixture of lipids, whose main component is phosphatidylcholine. Enrofloxacin and cyprofloxacin were a gift of Cenavisa S.A. (Reus, Catalonia), thioguanine and mercaptopurine were purchased from Fluka (Germany), and carboxyfluorescein, indomethacin, flurbiprofen, and triamcinolone were obtained from Sigma (U.S.A.).

Liposome preparation

After dissolving an amount of lipid in absolute ethanol (in encapsulation studies a concentration of 40 mg lipid/ml was used), 0.75 ml of such a solution were rapidly injected into 10 ml of a magnetically stirred buffer solution. The drug to encapsulate was present in the ethanol solution or in the buffer, according its solubility. Vesicle formation becomes evident on the appearance of the characteristic opalescence of colloidal dispersions.

Liposome purification

Liposomes, except those containing carboxifluorescein, were purified by the minicolumn centrifugation method (Fry et al., 1978): a 2 ml disposable plastic syringe, of which the barrel was plugged with two circles of filter paper, was filled with Sephadex G-50 in saline (Pharmacia, Sweden), then placed in a suitable centrifuge tube. After placing the tubes containing the columns in a bench centrifuge, they were spun at 2000 rpm for 4 min at room temperature to remove excess saline solution. After spinning, the gel column was dry and had separated from the side of the barrel. The saline eluted from the tub was collected and employed as a reference solution. Carefully, 0.4 ml of liposome suspension were applied dropwise to the top of the gel bed and the column was spun as above to expel the void volume containing the liposomes into the centrifuge tube. After removing the eluate, 0.2 ml of the corresponding buffer was applied and the same conditions of centrifugation were used. This step was repeated but now applying 0.4 ml of the last eluate into a new dry column. The third eluate can contain a few liposomes and some untrapped solute, whereas only liposomes are present in the second one.

As carboxyfluorescein liposomes are susceptible to leakage in high gravitational fields, the use of the minicolumn centrifugation method for purification may be inadvisable (New, 1990). Consequently, we have used the classical technique of purification through Sephadex G-50 employing a 30×1.5 cm column. The flux rate was 10 ml h^{-1} cm^{-1} and the eluates were collected at 1-ml fractions. Void volume was determined with Blue Dextran (Pharmacia, Sweden).

Once the liposomes had been purified, the following step was the separation of drug from the lipid. This was achieved by means of cartridges for solid-phase extraction (Sep-Pack C18, Millipore, U.S.A.). The recovery of drug as well as the lipid is quantitative.

Size distribution determination

Diameter and polydispersity of vesicles were determined by photon correlation spectroscopy (Phillies 1990) which analyses the fluctuations in scattered light intensity generated by diffusion of vesicles in solution. The apparatus employed was an Autosizer IIc (Malvern Instruments, U.K.) consisting of a 5 mW, 632.8 nm, helium-neon laser irradiating the scattering cell placed inside a temperature-regulated enclosure. Data acquisition was via a Malvern 7032-N, 72-channel multibit correlator. Experimental conditions were: temperature, 25.0°C; reference angle, 90°; viscosity, 0.899×10^{-3} Pa s; refractive index, 1.330. Two principal methods can be used for data analysis according to the sample distribution: assuming a log-normal size distribution of particles, the method of cumulant analysis is available, while for broad unimodal distributions (polydisperse systems) the exponential sampling method is used.

Chemical analysis

Lipid was determined by the assay for phospholipids reported by Steward-Marshall (1980). Since quinolones interfere with this determination, the drug must be separated from the lipid if this method is to be used.

After breaking up the vesicles into the cartridge the drug was obtained first, by elution with MeOH:HCl (2:1, v/v), and then, after elution with chloroform, the lipid. The encapsulated drug was determined spectrophotometrically by fluorescence measurements. Table 1 shows the analytical conditions of the individual determinations.

TABLE 1

Analytical conditions for determination of encapsulating drugs

Drug	Technique used
Enrofloxacin	absorption at 318 nm
Ciprofloxacin	absorption at 318 nm
Thioguanine	fluorescence after oxidation (Finkel, 1975)
Mercaptopurine	absorption at 324 nm
Indomethacin	absorption at 322 nm
Flurbiprofen	fluorescence at 270/312 nm
Triamcinolone	absorption at 525 nm (USP XXII)
Carboxyfluorescein	absorption at 492 nm

Results

Influence of lipid concentration

Lipid concentrations ranged from 8 to 72 mg/ml ethanol. The buffer solution was Tris-HCl, pH 7.4, ionic strength 0.16 (imOsm = 310 ± 10).

Fig. 1 shows the variation in vesicle diameter as a function of lipid concentration. As can be observed, below 40 mg/ml lipid concentration, the diameter mean value (the *z*-average mean as well as the number diameter) does not exceed 100 nm, the size being barely dependent on lipid concentration. Above 64 mg/ml, a large increase in size is produced. As far as polydispersity is concerned, at 40 mg/ml, fairly monodisperse vesicle are obtained (Fig. 2). This concentration results therefore in the most homogeneous vesicle population.

From the above results, it is reasonable to consider that the optimal lipid concentration is that of 40 mg/ml, since it produces a rather homogeneous vesicle population and the size never exceeds a diameter of 100 nm. For this reason, this concentration was chosen henceforth as the basis of the following assays.

Influence of osmolality

Solutions of different osmolality were obtained by dissolving NaCl in bidistilled water. The final

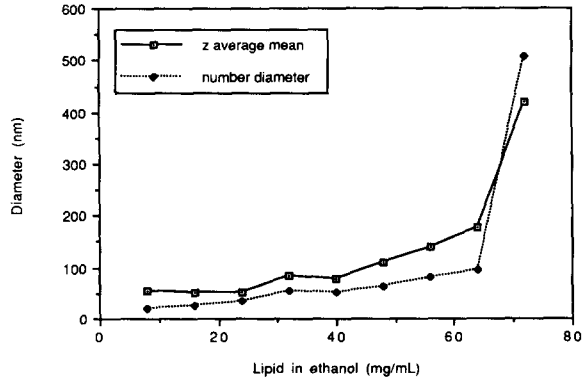

Fig. 1. Vesicle diameters (expressed in nm) as a function of lipid concentration of ethanolic solution.

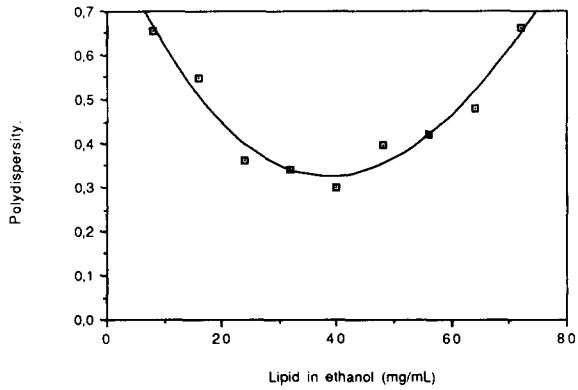

Fig. 2. Polydispersity of vesicle population as a function of lipid concentration of ethanolic solution. A polydispersity value of unity indicates a polydisperse vesicle population.

pH was kept constant at 6.2 ± 0.3. The osmolality, expressed as imOsm/kg, was determined in a Fiske One-Ten Osmometer.

In Table 2 it can be seen that the osmolality, apart from extreme values appears to have a considerable effect on neither size nor polydispersity. Nevertheless, it is clear that the different ionic strengths should affect the efflux of encapsulated material from the interior of the liposome.

Influence of pH

Five buffer solutions at pH 1 (HCl/KCl), 4.7 (acetic acid/acetate solution), 7.4 (Tris-HCl), 9.7 (Hepes) and 12 (NaOH/glycine) were employed. All solutions were iso-osmotic.

TABLE 2

Influence of osmolality on size and size distribution

mOsm/kg	*z*-diameter (nm)	Polydispersity	*n*-diameter (nm)	pH
1500	100.6	0.375	61.7	6.5
750	85.4	0.341	54.3	6.5
310	81.7	0.332	60.8	6.4
154	71.1	0.304	48.8	5.9
77	67.2	0.313	40.5	6.0
0	79.0	0.318	47.0	6.4

z-diameter: mean diameter expressed in intensity; *n*-diameter: mean diameter of the vesicles expressed in number. Values are the means of three determinations.

TABLE 3

Influence of pH on size and size distribution

Solution	pH	*z*-diameter (nm)	Polydispersity	*n*-diameter (nm)
HCl/KCl	1.2	62.7	0.279	43.9
AcOH/AcH	4.7	80.5	0.353	54.0
Tris-HCl	7.4	72.9	0.354	47.6
Hepes	9.7	75.4	0.346	50.0
NaOH/glycine	12.0	80.3	0.339	52.8

z-diameter: mean diameter expressed in intensity; *n*-diameter: mean diameter of the vesicles expressed in number.

Table 3 shows that the lowest pH (1.20 resulted in fewer vesicles than neutral, basic or moderately acidic solutions. This is probably due to steric and electrical effects produced by the negative charge of the phosphate group of phospholipid present at low pH.

Precipitation kinetics

Liposomes were obtained from three different lipid concentrations in ethanol (24, 48 and 64 mg/ml, respectively) after injecting the solution into 10 mM Tris-HCl buffer solution, pH 7.4. Assay was performed at room temperature and took 4 days. The precipitation kinetics was established by checking the size variation in the supernatant of the vesicle suspension. If precipitating occurs, owing to a process of aggregation or fusion, vesicles remaining suspended would show a smaller size. Precipitation was following by measurements of size and polydispersity, or of absorbance at 400 nm.

Fig. 3 shows the variation of the *z*-average mean as a function of time. As can be observed, this parameter remained unchanged when the lowest concentrations were injected. Concerning the 64 mg/ml concentration, the size decreased until a constant value was achieved. A similar result can be obtained when the dispersed light at 400 nm is measured (Fig. 4).

Encapsulation efficiency

Table 4 lists the results obtained with several drugs. Entrapment efficiency is the parameter chosen in order to compare the different encapsulation abilities as a function of the chemical

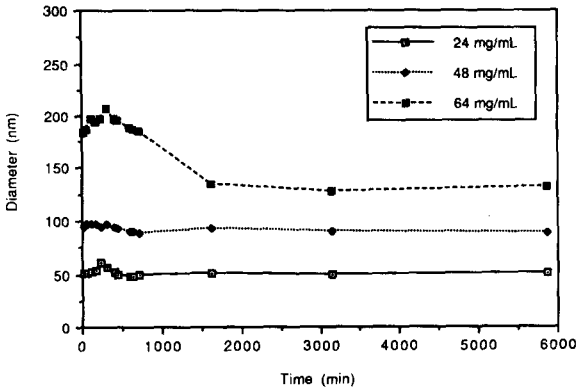

Fig. 3. Precipitation kinetics of liposomes at different lipid concentrations. The parameter monitored is the *z*-average mean.

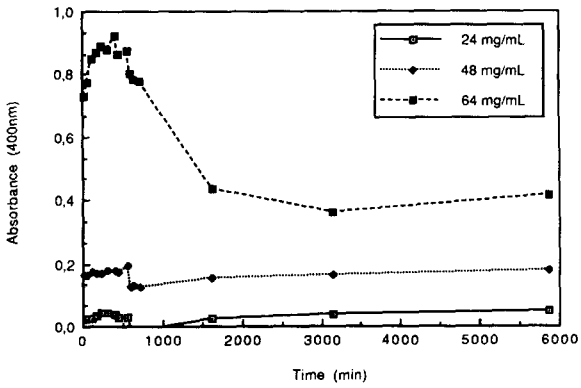

Fig. 4. Precipitation kinetics of liposomes at different lipid concentration. The parameter monitored is the absorbance at 400 nm.

characteristics of such drugs. Some drugs were present in the buffer, while others were solubilized in ethanol together with the lipid.

Discussion

The molecular weight and radius of the vesicles, produced by the injection method, may depend on the injection velocity, final alcohol concentration in the buffer, lipid concentration in the buffer and in the alcohol, and the pH and osmolality of the buffer. Also, the rate of stirring of the mixture during injection and the size of the vessel could be of importance. A study of the influence of some of these factors (Kremer et al., 1977) showed that the injection velocity did not influence the molecular weight or radius of the lipid particles. We maintained constant the final alcohol concentration in the buffer (7.5%), the rate of stirring and the size of the reaction vessel whilst varying the other factors.

According to our results, the lipid concentration is the main factor influencing the size of the lipid vesicles markedly. It has been stated (Hauser, 1982) that the injection of ethanolic phospholipid solutions affords vesicles belonging to the SUV type (with some aggregates of SUV), whose size range is 0.03–0.06 μm. Nevertheless, we have obtained not only SUVs, but also intermediate and large vesicles depending on the lipid concentration. It is very likely that the intermedi-

TABLE 4

Characteristics of encapsulation of several drugs by the injection method

Drug	Medium	Bulk concentration (M) ($\times 10^3$)	Entrapment efficiency (per mol lipid)	Entrapment percentage (%)
Carboxyfluorescein	phosphate buffer pH 7.4	1.95	0.26 μmol	0.10
Enrofloxacin	acetate buffer pH 4.7	52.04	810 mmol	11.17
Cyprofloxacin	acetate buffer pH 4.7	56.41	180 mmol	2.14
Thioguanine	acetate buffer pH 4.7	0.93	1.52 mmol	1.37
Mercaptopurine	acetate buffer pH 4.7	0.93	1.30 mmol	1.22
Indomethacin	ethanol	0.20	4.62 mmol	52.81
Flurbiprofen	ethanol	0.20	270 μmol	27.60
Triamcinolone	ethanol	0.15	291 μmol	23.72

Lipid content is referred to as phosphatidylcholine content. The entrapment percentage is the ratio between the amount of drug/lipid, after and before the purification process.

ate vesicles are unilamellar, due to the high curvature of the inner lamellae. However, the validity such a supposition has not been checked. At 40 mg/ml lipid, the most homogeneous population was obtained and, at this concentration, other factors tested such as osmolality and pH have a smaller effect, which is more evident only at unusually extreme values. Thereby, we may conclude that, by varying the lipid concentration, the method offers the possibility of making single bilayered vesicles of different radius in a reproducible and reliable way.

As far as the entrapment efficiency is concerned, this method presents an added advantage; it can encapsulate both hydrophilic and lipophilic substances. Lipophilic, amphipathic and a large number of hydrophilic substances can be dissolved in the same ethanol as for phospholipids. Our experiments indicate this procedure to be advisable for ethanol-soluble substances. However, this is not always possible, especially for a poorly ethanol-soluble drug, inasmuch as the lipid content influences the solvent ability of ethanol, decreasing the solubility product of the drug. Table 4 summarizes the encapsulation characteristics. The entrapment efficiency is expressed as the amount of drug in relation to the amount of lipid present in the vesicle population. In order to obtain a measure of the yield of the process, the entrapment percentage is also listed. This corresponds to the percentage ratio between the amounts of drug/lipid after and before the purification process, respectively. The medium denotes where the drug was (aqueous solution or ethanol) at the moment of injection.

The main disadvantage of this method is that the resulting lipid dispersion is very dilute. This problem can be resolved by concentrating the dispersion after injection. For this purpose, we have used two classical systems to concentrate macromolecular solutions, the Immersibles CX-30 (Millipore, USA) and the Bioram column (Schott, Germany). After a period of 30 min both methods concentrated the dispersion to a lipid content of double the initial value. This process affects neither the size and polydispersity of vesicles nor the stability. As in liposomes obtained by other methods, vesicle stability can be enhanced by adding a substance that increases the viscosity. On the other hand, it is possible to recycle unencapsulated drug and use it for further dispersions.

In conclusion, ethanol injection is a very easy method for obtaining liposomes. Its simplicity and reproducibility make it suitable for producing vesicles on a large scale.

Acknowledgements

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