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Comparative Study of Separation of Non-encapsulated Drug from Unilamellar Liposomes by Various Methods

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Abstract

The purpose of this study was to compare the various methods available to separate non-encapsulated drug from large unilamellar liposomes (LUV). Multilamellar liposomes (MLV) were prepared by thin film hydration using distearoylphosphatidylcholine:cholesterol (2 : 1 molar ratio). MLVs were passed through a 0·2-μm polycarbonate membrane using an extruder to prepare LUVs. Particle size of liposome preparations was characterized using a submicron particle-size analyser. The non-encapsulated drug was separated by: filtering through Centrifree tubes; passing through gel (Sepharose-4B and Sephadex G-25M); passing through minicolumn; ficoll density gradient; protamine aggregation; or dialysis.

The dialysis method was found to be unsuitable for separation of non-encapsulated drug due to equilibration of encapsulated drug as the free drug was dialyzed. The upper limit for lipid concentration was 5 mg mL^{-1} using the Centrifree method. Separation using gel chromatography led to dilution of liposome preparation. Minicolumn and density gradient techniques did not lead to sample dilution, however the minicolumn method was tedious. The time required for separation of liposomes by protamine aggregation was longer for neutral liposomes.

Thus it was concluded that the Centrifree was the fastest method to estimate encapsulation; the density gradient method was ideal to separate non-encapsulated drug; and protamine aggregation was the least expensive method to estimate encapsulation efficiency.

Liposomes have been researched as a drug delivery system since the early 1970s and offer a powerful tool to improve therapeutic efficacy and reduce toxic effects of drugs. Liposomes may be large or small and are composed of one or several concentric bilayers. Depending on their size and number of lamellae, they are distinguished as multilamellar vesicles (MLVs), and large and small unilamellar vesicles (LUVs and SUVs respectively).

It is important to estimate the amount of drug encapsulated within liposomes; this is easier for MLVs compared with LUVs and SUVs. MLVs, because of their large size, settle at the bottom and form a pellet when centrifuged at high speeds, and the non-encapsulated drug remains in the supernatant. However, LUVs and SUVs do not settle upon centrifugation. It is important to accurately estimate the encapsulation of drug in LUVs and SUVs using a convenient, rapid and economical process. While several reported methods are effective they have several important limitations. In the present study we have compared various methods to separate non-encapsulated drug from LUVs, with a separation technique based on filtration through prefabricated Centrifree tubes (Amicon) developed in our laboratory.

Materials and Methods

Chemicals

Didanosine was a gift from Bristol-Myers Squibb Co (Princeton, NJ). Distearoylphosphatidylcholine (DSPC) was purchased from Avanti Polar Lipids (Alabaster, AL). Cholesterol, protamine and ficoll were obtained from Sigma Chemical Co (St Louis, MO). [^{14}C]Cholesterol was purchased from Amersham (Arlington Heights, IL) and [^{3}H]didanosine from Moravek Biochemicals Inc. (Brea, CA). Centrifree units were from Amicon Inc. (Beverly, MA). Sepharose 4B and Sephadex G-25M from Pharmacia (Sweden). Dialysis membrane from Spectrum Medical Industries Inc. (Houston, TX), and polycarbonate membrane filters from Nucleopore Corp. (Cambridge, MA). Solvents used include chloroform, tertiary butanol, and methanol, and were purchased from Fisher Scientific (Fairlawn, NJ). Scintillation fluid (Ultima Gold) was purchased from Packard (Meriden, CT)

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Preparation and characterization of liposomes

The liposome preparation was composed of distearoylphosphatidylcholine : cholesterol (2 : 1 molar ratio) at a lipid concentration of 100 mg mL^{-1}. [^{14}C]Cholesterol (0·5 μCi mL^{-1}) and [^{3}H]didanosine (0·5 μCi mL^{-1}) were used as radioactive tracers. For the preparation of the liposomes the phospholipid and cholesterol were dissolved in a mixture of 10 : 4 tertiary butanol : chloroform and the organic solvent was removed under reduced pressure using a rotary evaporator. The thin film left behind was stored in a vacuum desiccator overnight to remove any traces of organic solvent. MLVs were prepared by the classical procedure of Bangham & Cohen (1972). The MLVs were extruded through two stacked 0·2-μm polycarbonate membranes to form LUVs using an extruder (Lipex Biomembrane, Vancouver, Canada). The submicron particle size distribution of the liposomal formulation was determined using large-angle dynamic light scattering (Brookhaven Instruments, model BI-90). The liposome preparations were diluted with filtered saline. The measurements were obtained at a temperature of 25°C, assuming a medium viscosity of 0·01 P and a medium refractive index of 1. The

mean particle size of liposomes was found to be in the range of 210 nm, with a standard deviation of about 15 nm.

Separation of non-encapsulated drug

Centrifree filtration. Each unit consists of a sample reservoir underneath which is a filter membrane (molecular weight cutoff of 30 000) followed by a support base and a filter cup. A liposome sample (200 μL) after suitable dilution was placed in the sample reservoir and the unit was centrifuged at 2000 rev min^{-1} for 30 min., followed by 2 washings with buffer, and centrifugation for 10 min after each wash. The liposomes along with the encapsulated drug remained on top of the membrane and the free drug associated with supernatant was separated in the bottom filter cup. The concentration of drug was estimated using associated radioactivity in a liquid scintillation counter.

Gel filtration (Jederstrom & Russell 1981). A glass column (1 cm × 15 cm) packed with either sepharose-4B or sephadex G-25M was used. After allowing the gel to equilibrate with the buffer, 200 μL of liposome suspension was placed gently on top of the column, after the liposomes entered into the gel the mobile phase was allowed to flow at a rate of 1 mL min^{-1}, which was maintained using a peristaltic pump, and 0·5-min fractions (60) were collected using an LKB 2211 fraction collector. Scintillation cocktail was added to each fraction and the radioactivity associated with the lipid as well as drug was estimated by liquid scintillation counting.

Minicolumn centrifugation (Fry et al 1978). Disposable syringes (1 mL) were first stoppered with glass wool then packed with hydrated sephadex G-25M gel. These syringes were then placed in test tubes and the whole assembly centrifuged to remove excess buffer. To the dried bed, liposome suspension was applied and the assembly centrifuged at 2000 rev min^{-1} for 15 min., and the liposomes were expelled in the void volume. This process was repeated three times using fresh syringes packed with gel each time, to ensure complete removal of all non-entrapped material. The encapsulated drug in liposomes was estimated by measuring the radioactivity associated with lipid and the drug.

Ficoll density gradient (Fraley et al 1980). Briefly, 0·5 mL of liposome suspension was placed in the bottom of polycarbonate centrifuge tubes followed by 1·5 mL of 40% ficoll, 2·5 mL of 20% ficoll and 0·5 mL of buffer. Each layer being placed on top of the previous layer gently without disturbing the previous layer. Then it was centrifuged for 30 min. at 35 000 rev min^{-1}. After centrifugation, 0·5-mL samples were taken from the top for analysis.

Protamine aggregation. To 100 μL of the liposome suspension in an Eppendorf tube was added 100 μL of a serial concentration of protamine; the mixture was incubated overnight at room temperature and centrifuged at 12 000 rev min^{-1} for 30 min in a microfuge. After washing the pellet formed twice with buffer the amount of drug in the supernatant was estimated.

Dialysis. In a 1 cm × 5 cm long dialysis bag with a molecular weight cut off of 3000, 0·5 mL of liposome suspension was placed. The dialysis bag was then suspended in 1 L of buffer with constant stirring for 8 h., the amount of drug remaining in the liposomes within the bag was estimated.

Table 1. Encapsulation of didanosine in large unilamellar (LUV) liposomes determined by various methods. The initial lipid concentration was 100 mg mL^{-1} and didanosine concentration was 1 mg mL^{-1}.

Method	Encapsulation (%)
Centrifree	31
Gel chromatography	
Sepharose 4B	29
Sephadex G-25M	28
Protamine aggregation	34
Density gradient	34
Minicolumn	34
Dialysis	26

Table 2. Effect of lipid concentration on estimation of encapsulation of didanosine by Centrifree method.

Dilution	Lipid concn (mg mL^{-1})	Encapsulation (%)
None	100	73
1 : 10	10	44
1 : 20	5	33
1 : 40	2·5	31

Results and Discussion

The encapsulation of didanosine determined by filtration through Centrifree tubes was 31% (Table 1). The principle of separation of non-encapsulated drug by Centrifree is by ultrafiltration through a membrane filter. Larger particles such as liposomes are retained on the filter, whereas aqueous phase containing non-encapsulated drug passes through the filter. Higher concentration of lipid blocks the membrane which prevents the transfer of free drug through the membrane resulting in overestimation of encapsulation, hence it is necessary to dilute the liposome sample before placing in the Centrifree unit. Table 2 shows the decrease in encapsulation (%) with increasing dilution of liposome sample up to 5 mg mL^{-1}. These results suggest that the upper limit of lipid concentration should not exceed 5 mg mL^{-1} in order to obtain accurate encapsulation efficiency. Although the cost of the Centrifree units may be discouraging, this method was found to be very convenient and to be a rapid procedure for estimation of encapsulation in LUVs. However, this method was not useful for recovering liposome sample.

The encapsulation of didanosine was 29 and 28%, respectively, when sepharose 4B and sephadex G-25M were used. Gel filtration using sepharose-4B has been widely used for the estimation of amount of drug encapsulated in unilamellar liposomes. There was no significant difference between the two gels used in this study. Liposomes, being larger particles, pass through the void volume and elute in the first peak and free drug is eluted in the second peak as shown in Fig. 1. This

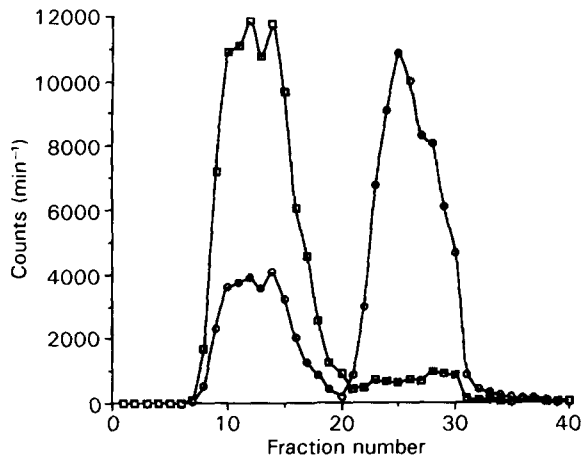

FIG. 1. Elution of fractions following gel filtration separation of non-encapsulated drug from liposomes. ○ [^{3}H]Didanosine; □ [^{14}C]cholesterol.

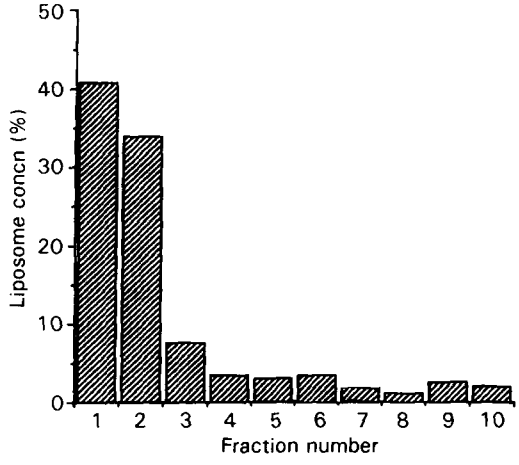

FIG. 2. Elution of fractions following gel filtration separation of non-encapsulated drug from liposomes. Ficoll solution (40%; 1·5 mL) was placed on 0·5-mL liposome sample. This was followed by 1·5 mL of 20% ficoll solution and 0·5 mL of phosphate-buffered saline, then centrifuged at 143 000 *g* for 30 min. Fractions (0·5 mL) starting from the top layer were placed in scintillation vial and the radioactivity of [^{3}H]didanosine and [^{14}C]cholesterol was measured by liquid scintillation counting.

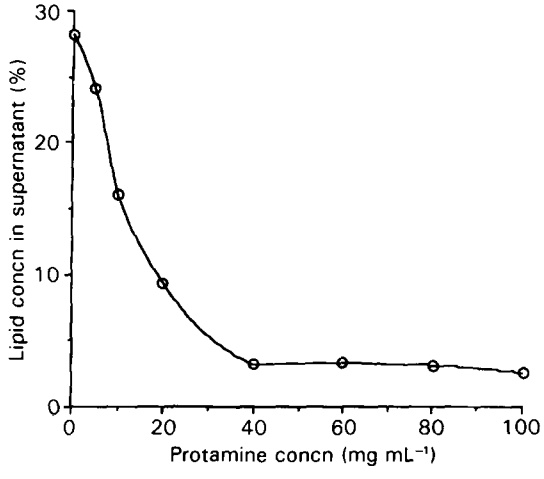

FIG. 3. Effect of protamine concentration on sedimentation of liposomes.

method is slow and may be difficult to carry out on a large number of formulations as may be required during initial screening of various lipid compositions. One advantage is, however, that liposome samples can be recovered for further use. Nevertheless, this method causes several-fold dilution of liposome sample. Chen & Schullery (1979) pointed out that Bio-Gel causes aggregation and/or fusion of all sonicated SUVs. Sepharose is similar to Bio-Gel in that both are agarose-based gels, the only difference being the smaller effective pore size of sepharose 4B and if Bio-Gel can cause aggregation of liposomes so can sepharose or any other gel. Chen & Schullery (1979) also indicated that other gel media may perturb the vesicles, but only to a lesser extent which may not be as prominent as in the case of Bio-Gel.

The encapsulation of didanosine obtained by the mini-column method was 34% (Table 1). Minicolumn centrifugation works on the principle of adsorption of external aqueous phase of liposome sample containing non-encapsulated drug. This method is slow, tedious and not suitable for routine analysis. An advantage of this method compared with gel chromatography is that there is no dilution of liposomes, since the liposomes are loaded on to a dry gel bed. Loading the liposome sample on to the dry column followed by centrifugation, causes the adsorption of external aqueous phase during the passage of liposome sample through the gel bed. Another limitation of this method is the sample volume that can be processed (100 μL). Repeating this process three times ensures complete removal of non-entrapped drug.

The encapsulation of didanosine obtained by the density gradient method was 34% (Table 1). Flotation in a discontinuous ficoll gradient is the principle behind this method. The liposomes float in the buffer layer on top of 20% ficoll layer, while the free drug remains in the bottom of the tube (Fig. 2). Relatively large sample volumes (500 μL) can be processed, liposome sample with encapsulated drug can be recovered and there is no dilution of the sample. Also, this method is rapid and an accurate estimation of encapsulation can be obtained.

The encapsulation of didanosine obtained by protamine aggregation method was 34% (Table 1). Protamine is a small protein with a molecular weight of less than 10 000; it is mainly composed of arginine and thus has a net positive charge. Protamine interacts electrostatically with liposomes and, because of its net positive charge, the interaction with negatively charged liposomes is very rapid. It is necessary to incubate the reaction mixture of neutral and positively charged liposomes overnight to ensure complete aggregation. This is a simple and inexpensive method. This method is rapid for negatively charged liposomes; however, overnight incubation is required for neutral and positively charged liposomes (Kulkarni et al 1995). Fig. 3 shows that at least 40 mg mL^{-1} protamine is necessary to precipitate 20 mg mL^{-1} of lipid. The advantage of this method is that large number of samples can be processed simultaneously without using any sophisticated equipment and also the method is economical. However, one disadvantage is that the liposome sample with encapsulated drug is contaminated with protamine, and if a particular

Table 3. Summary of advantages and disadvantages of various methods used to separate non-encapsulated drug from large unilamellar liposomes.

Method	Advantages	Disadvantages
Centrifree	Rapid; requires a small sample volume	Expensive; applicable only to unilamellar liposomes; lipid concentration cannot exceed 5 mg mL^{-1}
Gel chromatography	Sample recovery	Slow and tedious; dilution of samples
Protamine aggregation	Economical; applicable to MLVs and LUVs	Slow with neutral and positively charged liposomes; contamination of liposome sample
Density gradient	Economical; rapid; sample recovery	Sample volume (0·5 mL)
Minicolumn	Economical; sample recovery	Tedious, small sample volume (0·1 mL)
Dialysis	Sample recovery	Inaccurate

drug interacts with protamine then this method can not be used for estimation of encapsulation. Currently, we are developing a method to remove protamine from the liposome sample.

The encapsulation of didanosine obtained by the dialysis method was 26% (Table 1). Dialysis was found to be unsuitable for the estimation of drug encapsulated due to slow equilibration of encapsulated drug as the free drug is dialysed leading to either over or underestimation of encapsulation depending on the duration of dialysis. At the end of 8 h, 74% of the drug was dialysed. As noted by Bangham & Cohen (1972) small non-electrolyte molecules do diffuse out of the liposomes even in the presence of cholesterol in the liposomal bilayers. It is almost impossible to determine the critical time point at which the external free drug has equilibrated with the dialysate before the entrapped drug starts diffusing out. Also, this diffusion may be a spontaneous process in which case the entrapped drug starts diffusing out of the liposome as the concentration of drug in the external phase is depleted.

The Centrifree units although expensive were found to be the best method for the estimation of drug encapsulated as long as the concentration of lipid was < 5 mg mL^{-1}. In most cases this method requires dilution of the liposome sample. Minicolumn and gel chromatography methods are tedious. Gel filtration also causes dilution of the liposome sample after separating the non-encapsulated drug and is a slow process which may not be convenient for routine analysis. Protamine aggregation is the least expensive method, but requires prolonged incubation for neutral and positively charged liposomes and the liposome sample can not be recovered due to protamine contamination. Accurate estimation of encapsulation can be obtained except for by the dialysis method. The choice of method should take into consideration the purpose of the separation of non-encapsulated drug, availability of the equipment, the number of samples to be processed and the cost. A comparison of the advantages and disadvantages of the methods used to separate non-encapsulated drug in this study are summarized in Table 3.

Acknowledgement

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