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Drug encapsulation and release from multilamellar and unilamellar liposomes

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Summary

The concept of using liposomes as carriers for the delivery of drugs is well established, and the liposomal incorporation of various molecules, including peptides and proteins, has been described. In this study, propranolol (PPL) and atenolol (ATL) were used as model drugs to measure the encapsulation efficiency and release characteristics from multilamellar (MLV) and small unilamellar (SUV) liposomes. MLVs were prepared by hydration of thin lipid films and agitation using PPL and ATL solutions in phosphate-buffered saline (pH 7.4). Unilamellar liposomes were prepared by sonicating these MLVs. The non-encapsulated drug was separated either by centrifugation (MLV) or by size exclusion chromatography (SUV). The encapsulation of ATL and PPL was higher in small unilamellar liposomes. The encapsulation of PPL was higher than ATL in multi- as well as in unilamellar liposomes. The rate of drug efflux from liposomes was determined in vitro at 37°C and pH 7.4. The maximum release of both ATL and PPL was found with DSPC MLVs and DMPC:CHOL:DCP SUVs. The liposomal encapsulation and release of drug molecules are governed by the lipophilicity of drug molecules, type of liposomes and lipid composition.

Introduction

The use of liposomes for the delivery of therapeutic agents, such as enzymes, hormones and anti-cancer drugs, is well established. Various lipids have been used to prepare liposomes, and the method of preparation can be altered to control the liposome size distribution. Multilamellar liposomes (MLVs) are readily prepared by hydration of thin lipid films and subsequent agitation (Bangham et al., 1965). The size distribution and entrapment of aqueous phase are governed by the hydration time, method of lipid dispersion, thickness of the lipid film, and the concentration and composition of the lipid phase (Olson et al., 1979). Sonication of MLVs produces a more homogeneous population of small unilamellar vesicles (SUVs). Liposomes of intermediate size between MLVs and SUVs and entrapping a high percentage of aqueous phase can be produced by the techniques of ether infusion (Deamer and Bangham, 1976) or reverse-phase evaporation (Szoka and Papahadjopoulos, 1978).

Several investigators have studied the efflux of drugs from liposomes. Multilamellar (Ahmed et al., 1980; Arrowsmith et al., 1983; Taylor et al.,

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1990) and unilamellar (Juliano and Stamp, 1979; Muranishi et al., 1980; Hermetter and Paltuf, 1981) systems have been examined from two main points of interest. First, phospholipid bilayers may simulate some fundamental properties of biological membranes and constitute a model system to investigate passive drug transport. Second, there is the possible application of phospholipid vesicles as a drug delivery system. Until recently, both objectives faced the serious problem that a method for the preparation of a homogeneous and well-characterized population of unilamellar vesicles with a sufficiently large internal volume was not available. Yet, knowledge of these properties is necessary for a quantitative approach to transport phenomena if water-soluble compounds are to be encapsulated and released.

To investigate the in vivo behavior of liposomes it is necessary to study the in vitro release rate of an entrapped drug. The rate of release of a molecule from liposomes is governed by the material's physico-chemical properties. Liposomes are freely permeable to water, but cations are released at a slower rate than anions (Bangham et al., 1965), whereas aqueous hydrogen bonding may determine the efflux rate of non-electrolytes (Cohen, 1975).

The degree of disorder of the lipid bilayer determines the permeability of liposomes. Phospholipids in the liquid crystalline state are more permeable to entrapped material than when they are in the gel state. Thus, loss of entrapped material is temperature dependent, generally being greatest around the phospholipid phase transition temperature (T_c) (Papahadjopoulos et al., 1973). At T_c rapid efflux of material has been attributed to passage through regions of high bilayer disorder, where gel and liquid crystalline states temporarily coexist. The incorporation of cholesterol into liposomal bilayers decreases the rotational freedom of the phospholipid hydrocarbon chains. At 50 mol% cholesterol the phase transition is lost, the efflux rate of cations is decreased, and the release rate exhibits little temperature dependence (De Gier et al., 1968). At temperatures below the T_c of the phospholipids incorporation of cholesterol into liposomes decreases the release rate of hydrophilic materials (Ganapathi et al., 1980; Senior and Gregoriadis 1982), while producing a much smaller effect on the loss of lipophilic materials (Ganapathi and Krishnan, 1984). The ability of β-blockers to interact with liposomal bilayers has been previously studied (Sasaki et al., 1984; Betageri and Rogers 1987; Zachowski and Durand 1988; Rogers et al., 1986, 1990; Betageri et al., 1990). The encapsulation of propranolol, a lipophilic drug, and atenolol, a hydrophilic drug, was measured in liposomes of various lipid composition. In addition, release characteristics from multilamellar and unilamellar liposomes were studied.

Materials and Methods

Materials

Dimyristoyl-L-α-phosphatidylcholine (DMPC) dipalmitoyl-L-α-phosphatidylcholine (DPPC), and distearoyl-L-α-phosphatidylcholine (DSPC) were obtained from Avanti Polar Lipids, Birmingham, AL. Cholesterol (CHOL), dicetyl phosphate (DCP), propranolol (PPL) and atenolol (ATL) were purchased from Sigma Chemical Co., St. Louis, MO. Glass triple-distilled water was used in the preparation of all aqueous solutions. All other chemicals and solvents were reagent grade.

Methods

Preparation of liposomes All liposome preparations had a total lipid concentration of 10 mg/ml. The concentrations of PPL and ATL were 1 and 10 mg/ml, respectively.

The required amount of phospholipid, with dicetyl phosphate and/or cholesterol if required, was weighed into a 50 ml round bottom flask and dissolved in a small volume of chloroform. Chloroform was slowly removed under reduced pressure using a rotary evaporator at 40°C to deposit a thin film of dry lipid on the inner wall of the flask. Aqueous phase (5 ml of either PPL or ATL in phosphate-buffered saline (PBS), pH 7.4) was added at room temperature for DMPC and at a temperature of 5°C above the T_c for DPPC and DSPC (i.e., DPPC at 46°C; DSPC at 60°C). The flask was agitated on a vortex mixer until all of the lipid was dispersed to produce MLVs. Small unilamellar vesicles (SUVs) were produced from

MLVs by sonication for 5 min using probe sonication (Heat systems, Ultrasonic Inc.). This process turned samples into clear solutions.

Particle size analysis Light scattering measurements were performed with a Coulter submicron particle size analyzer (Model N4MD). The liposome preparations were diluted 1:20 with filtered PBS (pH 7.4). The instrument settings used were as follows: temperature, 20°C; viscosity, 0.01 P; refractive index, 1.333; scattering angle, 90°; run time, 300 s; range, 0–3000 nm.

Determination of drug encapsulation efficiency in MLV 5-ml samples of MLV were centrifuged at 143 000 × *g* and 37°C for 30 min in a temperature pre-equilibrated head (Beckman L3-50 ultracentrifuge). The concentrations of free ATL and PPL were determined from the UV absorbance of the supernatant at 273 and 290 nm, respectively (Beckman DU-65 spectrophotometer). A knowledge of the total drug content in the preparation allowed the amount of drug associated with the liposomes to be calculated by mass balance. Encapsulation was expressed as mol of drug per mol of lipid.

Determination of drug encapsulation efficiency in SUV 5-ml samples of SUVs were added to a Sepharose-4B column and eluted using PBS, pH 7.4. This procedure separates free drug from liposomes based on size exclusion. The drug concentration and encapsulation efficiency were determined as explained above.

Measurement of release of drug from liposomes 1-ml samples of either MLVs or SUVs were placed inside a dialysis membrane and transferred to a 100 ml graduated cylinder containing 50 ml PBS. The assembly was stirred on a magnetic stirrer in a temperature controlled room at 37°C. 3-ml samples were withdrawn at fixed time intervals and replaced with equal volumes of PBS. Samples were analyzed for ATL and PPL concentration by measuring absorbance at 273 and 290 nm, respectively.

Results and Discussion

Encapsulation of ATL and PPL in liposomes

The data in Tables 1 and 2 show the dependence of liposomal encapsulation on the composition of the lipid bilayers and the size of the liposomes. The low encapsulation of ATL in MLVs is possibly due to its hydrophilic character. In this case, encapsulation is completely dependent upon the volume of aqueous phase encapsulated during liposome formation. An increase in chain length of fatty acid and inclusion of cholesterol resulted in an increase in the encapsulation efficiency. This could be explained by the increase in the mean diameter of these liposomes and greater encapsulation of the aqueous phase as shown in Table 3. Similar results have been reported in the literature (Taylor et al., 1990). The relative increase in the encapsulation of ATL in DMPC:CHOL:DCP liposomes (MLVs) may be due to an electrostatic interaction between the negatively charged liposomes and positively charged atenolol at pH 7.4. However, this effect was not observed in SUVs. It may be due to

TABLE 1

Encapsulation of atenolol in multilamellar and unilamellar liposomes

Lipid composition	MLV (mol drug/mol lipid)	SUV (mol drug/mol lipid)
DMPC	0.04	0.89
DPPC	0.08	0.83
DSPC	0.10	0.75
DMPC:CHOL (1:1 mol ratio)	0.15	0.63
DMPC:CHOL:DCP (7:2:1 mol ratio)	0.19	0.70

DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; CHOL, cholesterol; DCP, dicetyl phosphate.

TABLE 2

Encapsulation of propranolol in multilamellar and unilamellar liposomes

Lipid composition	MLV (mol drug/mol lipid)	SUV (mol drug/mol lipid)
DMPC	0.86	1.52
DPPC	0.74	1.33
DSPC	0.94	1.23
DMPC:CHOL (1:1 mol ratio)	0.44	1.67
DMPC:CHOL:DCP (7:2:1 mol ratio)	0.99	1.84

TABLE 3

Phase transition temperature (T_c) and mean particle size of liposomes of various lipid compositions

Lipid composition	T_c (°C) [a]	Particle size	
		MLV	SUV
DMPC	23	788 ± 28	30 ± 5
DPPC	41	1370 ± 37	32 ± 6
DSPC	58	1670 ± 41	35 ± 6
DMPC:CHOL (1:1 mol ratio)	-	820 ± 29	31 ± 6
DMPC:CHOL:DCP (7:2:1 mol ratio)	< 23	795 ± 28	30 ± 5

[a] From Chapman et al. (1967).

saturation of binding sites on liposome surface and also due to change in radius of curvature.

The encapsulation efficiencies of ATL were higher in SUVs compared to MLVs. The maximum encapsulation of ATL was observed with SUVs prepared using DMPC. Higher encapsulation efficiency in SUVs can be explained by greater surface area of lipid bilayer structure. Since both ATL and PPL interact and distribute into lipid bilayers, the increased surface area with reduced particle size provides a greater extent of interaction of ATL and PPL with the bilayer. Relative to ATL, encapsulation of PPL was considerable in MLVs. This is probably the result of the much greater lipophilicity of PPL. Encapsulation of PPL was much lower in MLVs prepared with DMPC:CHOL. This may be due to a competitive incorporation of PPL and CHOL between DMPC molecules during the formation of liposomes. As with ATL, encapsulation efficiencies of PPL were greater in SUVs than in MLVs. Again, this could be due to the greater surface area of the lipid bilayer structure of SUVs. Encapsulation of PPL was maximum in MLVs and SUVs prepared with DMPC:CHOL:DCP. This implied a significant electrostatic interaction between PPL and DCP. Similar results have been reported for propranolol (Schlieper and Steiver, 1983; Kubo et al., 1986). These data indicate the importance of molecular structure, lipophilicity and charge of drug molecules with respect to

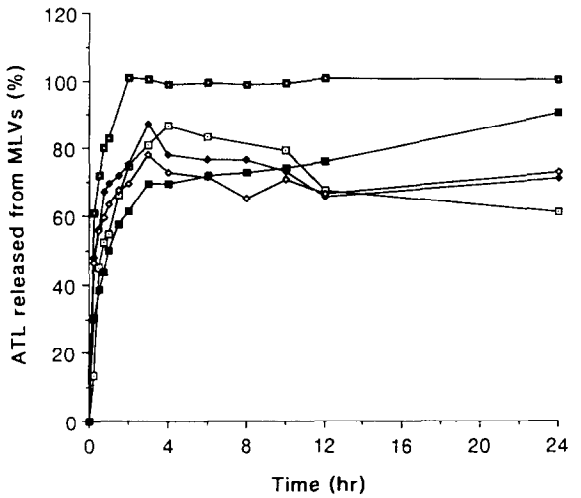

Fig. 1. Release of atenolol from multilamellar (⊡) DMPC, (◆) DPPC, (■) DSPC, (◇) DMPC:CHOL, (■) DMPC:CHOL:DCP liposomes at 37°C and pH 7.4. Each point is the mean of duplicate preparations.

their encapsulation in liposomes. The drug molecule may also influence liposome formation by an ability to bind water molecules and form liquid crystal structures in aqueous solutions (Attiga et al., 1979).

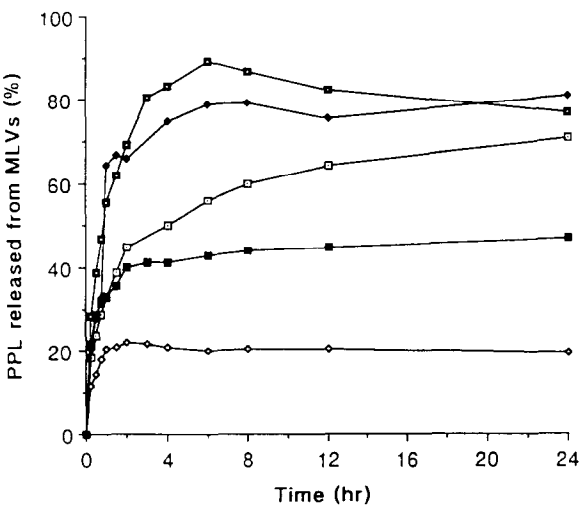

Fig. 2. Release of propranolol from multilamellar (⊡) DMPC, (◆) DPPC, (■) DSPC, (◇) DMPC:CHOL, (■) DMPC:CHOL:DCP liposomes at 37°C and pH 7.4. Each point is the mean of duplicate preparations.

Release of ATL and PPL from MLVs

The release profiles of ATL and PPL from MLVs are shown in Figs 1 and 2, respectively. The release of PPL was more dependent upon the liposome composition than that of ATL. After 24 h, only 20% PPL was released from DMPC:CHOL (1:1 mol ratio) liposomes and 45% from those of DMPC:CHOL:DCP (7:2:1 mol ratio). The release rates of ATL and PPL decreased with increase in T_c of liposomes prepared from single phospholipid. Cholesterol in bilayers above the phospholipid T_c modulates membrane fluidity by restricting the movement of the relatively mobile hydrocarbon chains. This reduces the liposome bilayer permeability. Similar results due to the effect of PPL on the T_c of PC liposomes have been reported (Kursch et al., 1983). The maximum release (90%) of PPL and ATL (100%) was found with DSPC liposomes. Maximum release was achieved in 2 h for ATL and in 6 h for PPL. In general, the release of ATL was greater than that of PPL. This is due to the hydrophilic character of ATL which is a major determinant for leakage. However, the release of PPL is controlled by liposome structure due to the lipophilic property of this drug.

Release of ATL and PPL from SUVs

The release of ATL is shown in Fig. 3. In contrast to MLVs, the release of ATL was slower in SUVs prepared with DSPC. Increases in fatty acid chain length of DSPC and the gel state of DSPC liposomes are the factors responsible for reduced leakage. Release of ATL is greater from DPPC than from DMPC liposomes. This is due to the increased permeability of DPPC liposomes near the T_c. The inclusion of CHOL and/or DCP resulted in greater release of ATL from SUVs. A phase of rapid release has previously been described for release of hydrophilic drugs from charged liposomes (Alper et al., 1981). Hence, this effect may be the result of an inherent property of the liposome bilayer structure or may reflect loss of surface-associated material.

The release of PPL from SUVs is depicted in Fig. 4. The release of PPL is slower in SUVs compared to MLVs except for DMPC:CHOL:DCP liposomes. As with MLVs, the slowest release (< 10% after 24 h) of PPL was found with DMPC:CHOL liposomes. The maximum release was observed with DMPC:CHOL:DCP liposomes. Since SUVs are made of a single bilayer, a major portion of the PPL is associated with the surface of the liposome. A phase of rapid release

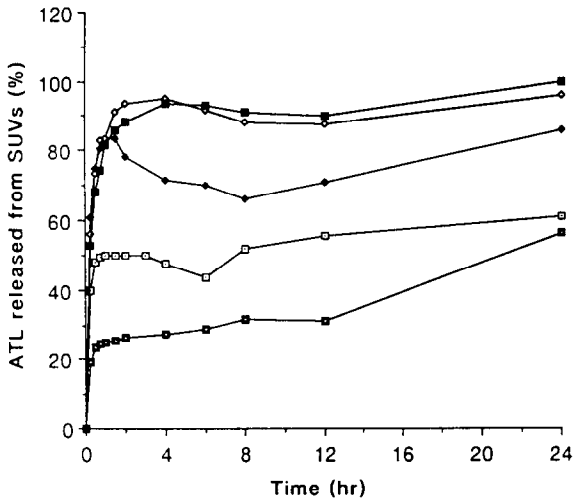

Fig. 3. Release of atenolol from unilamellar (⊡) DMPC, (◆) DPPC, (■) DSPC, (◇) DMPC:CHOL, (■) DMPC:CHOL:DCP liposomes at 37°C and pH 7.4. Each point is the mean of duplicate preparations.

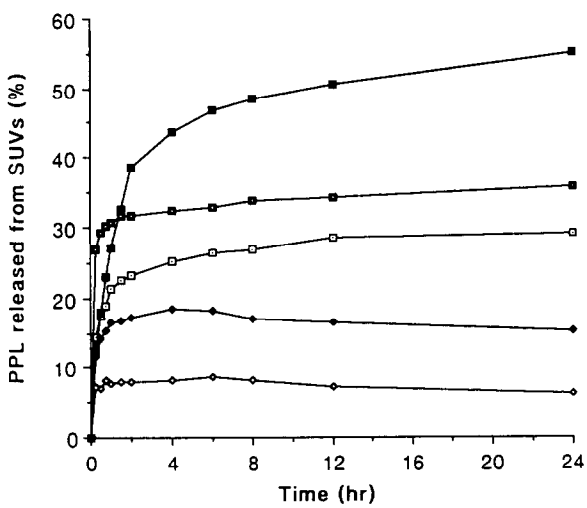

Fig. 4. Release of propranolol from unilamellar (⊡) DMPC, (◆) DPPC, (■) DSPC, (◇) DMPC:CHOL, (■) DMPC:CHOL:DCP liposomes at 37°C and pH 74. Each point is the mean of duplicate preparations.

for hydrophobic drugs from charged liposomes has previously been described (Juliano and Stamp, 1978). Loss of PPL from the liposomal surface explains the greater release from DMPC : CHOL : DCP liposomes.

This study has indicated that the physicochemical properties of drug molecules, the lipid composition of liposomes, liposomal size and number of bilayers influence the encapsulation of drug molecules. The encapsulation of a hydrophilic drug depends on the encapsulation volume of the aqueous phase, whereas encapsulation of a lipophilic drug depends on the lipid composition. The size of the liposomes affects encapsulation of both hydrophilic and lipophilic compounds. The rate of drug release can be altered by changing the lipid composition, size, and number of bilayers of the liposomes.

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