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Drug entrapment and release from multilamellar and reverse-phase evaporation liposomes

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Summary

The entrapment of sodium cromoglycate was determined in multilamellar liposomes prepared from natural and synthetic phospholipids. Drug entrapment was low, but was increased by inclusion of cholesterol or stearylamine into vesicle bilayers. Reverse-phase evaporation vesicles entrapped greater amounts of drug than multilamellar liposomes of the same composition. The rate of drug efflux from liposomes was determined in vitro and was dependent upon bilayer composition and the method of preparation.

Introduction

The concept of using liposomes as carriers for the delivery of drugs is well established, and the liposomal incorporation of therapeutic agents, such as enzymes, hormones and anti-tumour drugs has been described. Multilamellar liposomes (MLVs) are readily prepared by hydration of thin lipid films and subsequent agitation (Bangham et al., 1965). The size distribution and captured aqueous volume 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). A more homogeneous population of smaller vesicle size is produced when MLVs are sonicated to produce small MLVs or small unilamellar vesicles (SUVs).

Liposomes of size intermediate 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).

A knowledge of the in vitro release rate of an entrapped drug is a necessary prerequisite to investigation of the in vivo behaviour of a liposomal drug delivery system. The rate of release of a material from liposomes is governed by its physico-chemical properties; liposomes are freely permeable to water, but cations are released at a slowed rate than anions (Bangham et al., 1965), whilst aqueous hydrogen bonding may determine the efflux rate of non-electrolytes (Cohen, 1975).

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Liposome bilayer permeability is determined by the degree of disorder of the bilayer. Consequently phospholipids in the liquid crystalline state are more permeable to entrapped materials than when 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), when rapid loss of material has been attributed to loss 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, such that 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). Incorporation of cholesterol into liposomes having phospholipids above their T_c decreases the release rate of hydrophilic materials (Ganapathi et al., 1980; Senior and Gregoriadis, 1982), whilst producing a much smaller effect on the loss of lipophilic materials (Ganapathi and Krishan, 1984).

Materials and Methods

Materials

Unless otherwise stated materials in this study were AnalaR grade and obtained from BDH (U.K.). Water was glass distilled. Sodium cromoglycate (SCG; micronised) was a gift from Fisons (U.K.). Egg phosphatidylcholine (EPC; about 90%, Sigma, U.K.) was subsequently purified as described by Bangham et al. (1974). Dimyristoyl-L-α-phosphatidylcholine (DMPC), dipalmitoyl-L-α-phosphatidylcholine (DPPC) cholesterol (Chol; 99 + %) and stearylamine (SA; about 90% were all obtained from Sigma (U.K.).

Methods

Preparation of liposomes All liposome preparations had a total lipid concentration of 10 $mg \cdot ml^{-1}$ and a concentration of SCG in the aqueous phase of 10 $mg \cdot ml^{-1}$.

The required amount of phospholipid, with stearylamine and/or cholesterol if required, was weighed into a quickfit round bottom flask and dissolved in a small volume of chloroform. Organic solvent was slowly removed at reduced pressure, on a rotary evaporator, at 40 °C, such that a thin film of dry lipid was deposited on the inner wall of the flask. Aqueous phase (SCG in 0.9% saline) was added at 40 °C for EPC and at a temperature 15 °C in excess of the lipid T_c for synthetic lipids (i.e. DMPC = 38 °C; DPPC = 56 °C). The flask was maintained at that temperature for 1 h, then shaken on a mechanical agitator for 2 min to produce MLVs.

Small MLVs were produced from MLVs by probe sonication (150 W sonicator with 10 mm probe diameter) under nitrogen. Ten 1-min periods of sonication (16 μm amplitude peak to peak) were alternated with 1 min cooling periods to maintain liposomes at the temperature of film hydration.

Reverse-phase evaporation liposomes (REVs) were prepared by the method of Szoka and Papahadjopoulos (1978). Lipid components were weighed into a long-necked 200 ml quickfit round-bottom flask and dissolved in chloroform/diethyl ether (1 ; 1). SCG in 0.9% saline was added such that the organic to aqueous phase ratio was 6 : 1. The flask was sealed under nitrogen and the mixture sonicated for 4–6 min at 40 °C (EPC) or 50 °C (DPPC) in an ultrasonic bath. An emulsion was produced from which organic solvent was slowly removed at 45 °C with a rotary evaporator to produce the liposomes. The flask remained on the evaporator until organic solvent could not be detected by olfactory means and was then flushed with nitrogen.

Following production, all liposomes were maintained for 1 h at a temperature exceeding the phospholipid T_c (MLVs: EPC = 37 °C, DMPC = 38 °C, DPPC = 56 °C and REVs: EPC = 40 °C, DPPC = 45 °C) to anneal the liposome structure.

Determination of SCG entrapment by liposomes. Five ml samples were centrifuged at 200 000 × *g* and 37 °C for 30 min in a temperature pre-equilibrated head. The concentration of free SCG was calculated from the UV absorbance of the supernatant at 326 nm. A knowledge of the total drug in the preparation allowed the amount of drug associated with the liposomes to be calculated by

difference. Entrapment was expressed as mg SCG entrapped per 100 mg lipid.

Determination of liposome size. Vesicle size was determined using a Coulter Counter, TAII, with a 50 μm aperture tube and using 0.9% saline, filtered through a 0.05 μm pore membrane filter, as a conductive medium. Photon correlation spectroscopy (Malvern RR144) was used when Coulter analysis indicated a mean particle size less than 3 μm. Measurements were carried out at a scattering angle of 90° at 37°C in filtered 0.9% saline.

Assessment of SCG leakage rates from liposomes. Efflux of drug was assessed by periodic centrifugation of samples following a 1 in 200 dilution of the initial preparation with 0.9% saline. Diluted preparations were shaken in a water bath at 37°C. Duplicate 5-ml samples were centrifuged at 200000 × g for 30 min and the supernatant assayed for SCG at 326 nm. Total release of drug was determined from the concentration of SCG in diluted preparations estimated in the presence of Triton X-100 (1% final concentration).

TABLE 1

Entrapment of sodium cromoglycate in liposomes

Each result is a mean for 3 preparations after 20 h shaking at 37°C.

Lipid composition (mol ratio)	Entrapment (±S.E.) ($mg \cdot ml^{-1}$ %)	Size [a] (μm)	(σg)
MLVs			
EPC	3.27 (0.10)	3.57	2.25
EPC/Chol (1:0.5)	4.42 (0.17)	3.70	2.22
EPC/Chol (1:1)	6.25 (0.20)	5.25	2.40
EPC/Chol/SA (1:1:0.2)	8.35 (0.13)	5.33	2.21
DMPC	2.97 (0.05)	4.55	2.37
DMPC/Chol (1:1)	6.55 (0.57)	5.81	2.33
DPPC	3.75 (0.13)	5.14	2.18
DPPC/Chol (1:0.5)	4.85 (0.05)	5.72	2.06
DPPC/Chol (1:1)	5.35 (0.22)	5.96	2.03
Sonicated MLVs			
EPC/Chol (1:1)	1.07 (0.07)	0.111 [b]	0.18 [c]
REVs			
EPC/Chol (1:1)	11.17 (0.90)	3.25	1.83
DPPC/Chol (1:1)	10.54 (0.94)	3.34	1.77

[a] Mean diameter was determined by Coulter analysis, except [b] by photon correlation spectroscopy.
[c] Polydispersity index.

Results and Discussion

Entrapment of SCG in liposomes

The data in Table 1 show the dependence of liposomal SCG entrapment on the composition of the lipid bilayers and method of preparation.

The low entrapment of 3.3% SCG in EPC MLVs, is typical for a hydrophilic molecule since entrapment is dependent upon the volume of aqueous phase encapsulated during liposome formation. Inclusion of 33 and 50 mol% cholesterol resulted in respective increases in mean vesicle diameter of 3.0 and 45.2%. This corresponded to increased SCG entrapments of 35.0 and 90.3%.

A cholesterol-induced increase in mean vesicle size was also found for synthetic lipids (Table 1). Addition of 50 mol% cholesterol to DMPC liposomes increased mean vesicle diameter by 30.7% and entrapment by 112.7%. Similarly diameters increased by 12.7 and 20.8% and entrapment by 31.4 and 41.2% when 33 and 50 mol% cholesterol, respectively was included in DPPC MLVs. X-ray diffraction methods have demonstrated that cholesterol increases the width of bilayers of saturated phosphatidylcholines containing 12–16 carbon atoms per chain by removing the hydrocarbon chain tilt from gel-state lipids or by increasing the trans conformations in the chains of liquid crystalline lipids (McIntosh, 1978). Thus the increased SCG entrapment when cholesterol was included in these synthetic lipid liposomes was the result of the increased vesicle size, whilst an increased bilayer thickness was probably responsible for the lack of direct proportionality between vesicle volume and entrapment.

Sonication of EPC/Chol (1:1) MLVs produced a homogeneous population of smaller liposomes having mean diameter 111 nm (Table 1). A vesicle diameter greater than 100 nm suggested that the liposomes produced after 10 min sonication were small MLVs rather than SUVs. The trapping efficiency of drug was low, corresponding to an apparent captured volume of approximately 1 $\mu l \cdot mg^{-1}$ lipid.

Incorporation of stearylamine into EPC/Chol (1:1) MLVs increased SCG entrapment with an

insignificant effect on vesicle size ($P < 0.05$, analysis of variance test) (Table 1). Stearylamine is commonly incorporated into bilayers to confer a positive charge (Gregoriadis, 1973). Electrostatic repulsion of adjacent bilayers increases liposome size and the size of the internal aqueous compartments (Johnson, 1973). Since the mean size of EPC/Chol (1 : 1) MI Vs was not increased when stearylamine was present in the bilayer at 9.1% it is likely that the improved entrapment of SCG was due to the formation of an ion-pair between SCG and stearylamine which partitions into the bilayer.

Producing liposomes by the reverse-phase evaporation technique resulted in an almost 2-fold increase in SCG entrapment compared to MLVs (Table 1). Calculation of the apparent captured aqueous volume per mg lipid gives 11.2 $\mu l \cdot mg^{-1}$ and 10.5 $\mu l \cdot mg^{-1}$ for EPC/Chol (1 : 1) and DPPC/Chol (1 : 1) REVs, respectively.

Particle size analysis of REVs by Coulter Counter indicated a mean diameter of 3–3.5 μm. With a 50 μm aperture tube the lower limit of determination was 0.8 μm. The resolution of photon correlation spectroscopy is determined by laser wavelength allowing particles of 10 nm and greater to be measured accurately. Analysis by photon correlation spectroscopy suggested a bimodal distribution with modes ($\pm < 10\%$) of 232 nm and 3342 nm for EPC/Chol (1 : 1) REVs and 244 nm and 3410 nm for DPPC/Chol (1 : 1) REVs.

Negative stain transmission electron microscopy indicated that the REVs produced in this study comprised at least 2 liposome populations: Large unilamellar liposomes having diameters up to 300 nm and large vesicles of diameters up to 4 μm (Fig. 1). These latter vesicles were oligolamellar or multilamellar. Each type of liposome has a large internal aqueous core relative to its diameter and this was responsible for the more efficient entrapment of aqueous volume than MLVs.

REVs are generally in the size range 0.1–1.0 μm with the majority of liposomes being unilamellar (Szoka and Papahadjopoulos, 1978; Szoka et al., 1980). The present method differs from previous studies in the large volumes of aqueous and organic phases employed. Incomplete emulsification during sonication of the flask or the rapid

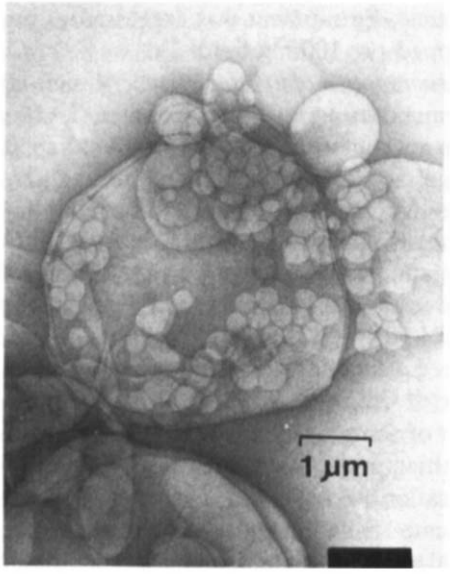

Fig. 1. Transmission electron micrograph of EPC/Chol (1 : 1) REVs.

loss of diethylether at 45 °C followed by slower removal of chloroform may have been responsible for the presence of large vesicles having more than one bilayer in the final preparation. The SCG molecule may influence liposome formation by an ability to bind water molecules and form liquid crystal structures in aqueous solutions (Attiga et al., 1979).

Release of SCG from liposomes

After an initial phase of rapid drug loss, SCG release from MLVs was an apparent first-order process. Incorporation of cholesterol into EPC MLVs progressively increased SCG retention within the diluted liposomes (Fig. 2). Total loss of encapsulated SCG occurred from EPC liposomes within 2 h. Efflux half-lives for preparations containing 33 and 50 mol% cholesterol were 13.3 h and 84.5 h, respectively. All encapsulated drug was lost from DMPC liposomes within 2 h, drug retention was prolonged by the inclusion of 50 mol% cholesterol, such that the efflux half-life for DMPC/Chol (1 : 1) MLVs was 101.9 h (Fig. 3). Cholesterol in bilayers above the phospholipid Tc modulates membrane fluidity by restricting the movement of the relatively mobile hydrocarbon

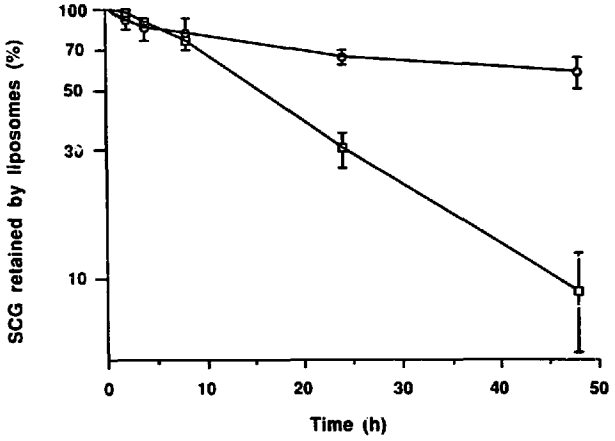

Fig. 2. Release of SCG from EPC/Chol (1:0.5) MLVs (□) and EPC/Chol (1:1) MLVs (○) into 0.9% saline at 37°C. Each point is the mean (±S.E.) for 3 preparations.

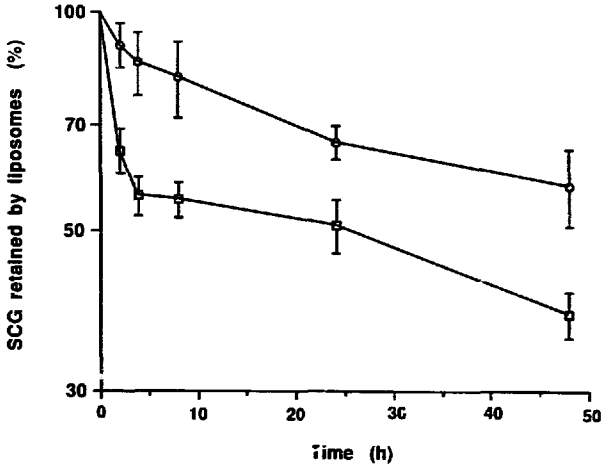

Fig. 4. Release of SCG from EPC/Chol (1:1) MLVs (○) and EPC/Chol/SA (1:1:0.2) MLVs (□) into 0.9% saline at 37°C. Each point is the mean (±S.E.) for 3 preparations.

chains, reducing bilayer permeability (Oldfield and Chapman, 1972).

Inclusion of cholesterol in DPPC MLVs at 33 and 50 mol% resulted in efflux half-lives of 53.7 h and 138.6 h, respectively (Fig. 3). At 37°C molecules of DPPC are in the ordered gel-state. Previous studies have shown that cholesterol increases the disorder of gel-state phospholipid bilayers by forcing apart the rigid hydrocarbon chains with a resultant increased vesicle permeability (Ladbrooke et al., 1968; Senior and Gregoriadis, 1982). SCG may have an inherent property inducing leakage in DPPC liposomes, which is inhibited by inclusion of cholesterol, and indeed it has been shown that certain anions can affect the hydrogen bonding of the phospholipid polar head group region of the bilayer resulting in perturbation of the hydrocarbon chain packing (Chapman et al., 1977). Incorporation of cholesterol into the bilayer might then be expected to decrease the ion-induced disorder, resulting in a more ordered bilayer and a decreased permeability.

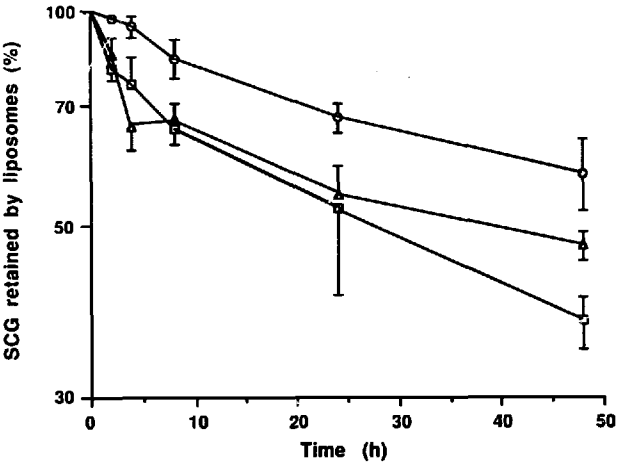

Fig. 3. Release of SCG from DMPC/Chol (1:1) MLVs (△), DPPC/Chol (1:0.5) MLVs (□) and DPPC/Chol (1:1) MLVs (○) into 0.9% saline at 37°C. Each point is the mean (±S.E.) for 3 preparations.

The release profile of SCG from stearylamine containing liposomes (Fig. 4 shows an apparent biphasic release process, with 44% of entrapped drug lost in 4 h from EPC/Chol/SA (1:1:0.2) MLVs compared to 14.7% from EPC/Chol (1:1) liposomes over the same period. The phase of rapid loss may be due to SCG partitioning out of the charged bilayers or due to desorption of SCG bound to the charged liposome surface. A phase of rapid release has previously been described for hydrophilic drug from charged liposomes (Alpar et al., 1981), for hydrophobic drugs from charged liposomes (Juliano and Stamp, 1978) and for hydrophobic materials from uncharged liposomes (Arrowsmith et al., 1983). Hence this effect may be the result of an inherent property of the bilayer or liposome structure or reflect loss of surface-associated material.

Dilution of REV preparations produced a biphasic release profile with a phase of rapid drug

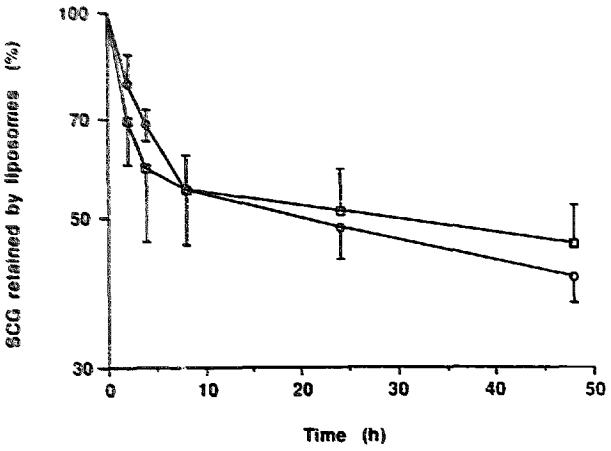

Fig. 5. Release of SCG from EPC/Chol (1 : 1) REVs (○) and DPPC/Chol (1 : 1) REVs (□) into 0.9% saline at 37 °C. Each point is the mean (± S.E.) for 3 preparations.

loss lasting approx. 8 h (Fig. 5). At 8 h post-dilution 45.1% and 45.2% of entrapped SCG was lost from EPC/Chol (1 : 1) and DPPC/Chol (1 : 1) REVs respectively, as compared to losses of 18.8% and 11.1% from EPC/Chol (1 : 1) and DPPC/Chol (1 : 1) MLVs. This phase of rapid loss may be due to the rapid release of drug from unilamellar vesicles present in REV formulations, since LUVs have a larger surface area to volume ratio than larger MLVs and oligolamellar vesicles and possess only a single lipid bilayer barrier to hydrophilic drug diffusion.

Post 8 h half-lives of drug release for EPC/Chol (1 : 1) formulations were 85 h (MLV) and 90 h (REV) and for DPPC/Chol (1 : 1) formulations half-lives were 139 h (MLV) and 151 h (REV); suggesting that after the initial phase of rapid release the SCG efflux rate was determined by bilayer composition rather than method of preparation.

This study has indicated that liposomal entrapment can markedly prolong the release of SCG under in vitro test conditions. Moreover, the rate of drug release can be modified by alteration of the liposomal lipid composition.

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