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Characterization and In-vivo Ocular Absorption of Liposome-encapsulated Acyclovir

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Abstract

The potential of liposomes as an in-vivo ophthalmic drug delivery system for acyclovir was investigated.

The drug–membrane interaction was evaluated by means of differential scanning calorimetry analysis. These experiments showed that acyclovir is able to interact with both positively and negatively charged membranes via electrostatic or hydrogen bonds. No interaction was observed with neutral membranes made up of dipalmitoylphosphatidylcholine.

Different liposome preparation procedures were carried out to encapsulate acyclovir. The drug encapsulation mainly depends on the amount of water which the liposome system is able to entrap. In the case of multilamellar vesicles, charged systems showed the highest encapsulation efficiency. No particular difference in the encapsulation efficiency was observed for oligolamellar vesicles prepared with the reverse-phase evaporation technique. Oligolamellar liposomes showed the highest acyclovir encapsulation parameters and had release profiles similar to those of multilamellar liposomes. In-vivo experiments using male New Zealand albino rabbits were carried out to evaluate the aqueous humour concentration of acyclovir bioavailability.

The most suitable ophthalmic drug delivery system was oligolamellar systems made up of dipalmitoylphosphatidylcholine–cholesterol–dimethyldioctadecyl glycerole bromide (7 : 4 : 1 molar ratio), which presented the highest encapsulation capacity and were able to deliver greater amounts of the drug into the aqueous humour than a saline acyclovir solution or a physical liposome/drug blend.

Acyclovir is a nucleoside analogue with a significant and highly specific antiviral activity against herpes viruses, particularly herpes simplex virus and varicella zoster virus (Elion 1982; Laskin et al 1982; Fletcher & Bean 1985). The topical application of the drug is largely used in the treatment of recurrent labial and genital herpes simplex infections (Hayden 1996). Some ocular pathologies such as keratoconjunctivitis, herpes simplex retinitis and herpes simplex keratitis are usually treated by acycloguanosine therapy (Fisher & Prusoff 1984).

Herpes simplex keratitis represents a severe disease with about half a million cases per year in the US alone (Pavan-Langston 1994). In the worst cases, damage to the stromal cells may result in irreversible morphological changes leading to corneal clouding and visual loss. Herpes simplex keratitis is characterized by recurrences because the virus remains latent in the trigeminal ganglion nerve body. Therapy for this form of the disease consists of strategies to inhibit virus growth, such as topical application of antiviral agents (Fisher & Prusoff 1984). Idoxuridine, vidarabine and trifluorothymidine are widely used for the treatment of herpes simplex keratitis (Fisher & Prusoff 1984). Unfortunately, these compounds are only weakly effective or are poorly tolerated (Naito et al 1987; Mauger 1994).

Acyclovir is the drug of choice for herpes simplex keratitis and acts by inhibiting viral DNA

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polymerase after phosphorylation by viral thymidine kinase. Because of its poor solubility in water, acyclovir is administered in an ointment form, but this tends to cause visual disturbance for about 30 min and reduces compliance.

Phospholipid vesicles (liposomes) have also been used to formulate a variety of hydrophobic, poorly soluble drugs (Van Bloois et al 1987; Janknecht et al 1992). In particular, encapsulation of chemotherapeutic agents in liposome formulations may result in an improvement of the biological activity both in-vitro and in-vivo, leading to a reduction in side effects, an increase in the cellular and bacterial penetration and may ensure a more suitable biodistribution (Magallanes et al 1993; Fresta et al 1995a; Puglisi et al 1995).

It is well known (Fitzgerald et al 1987a; Davies et al 1993) that liposomes, like other colloidal systems, have a great potential in ocular drug delivery, with the advantage of being completely biodegradable and relatively non toxic. The use of liposomes as an ophthalmic drug delivery system was first considered in the animal studies of Smolin et al (1981), who reported that in the treatment of herpetic keratitis in rabbits, idoxuridine entrapped within liposomes was more effective than the unentrapped drug. In many other applications liposomes have shown the possibility to enhance ocular drug absorption (Schaeffer & Krohn 1982; Meisner et al 1989) and to prolong the precorneal retention time (Fitzgerald et al 1987a, b), thus increasing drug effectiveness.

The aim of this study was the preparation and characterization of acyclovir-loaded liposome formulations in order to improve corneal permeability and therapeutic effectiveness. In-vivo corneal penetration studies were also carried out on rabbit eyes.

Materials and Methods

Chemicals

1, 2-Dipalmitoyl - sn-glycerol-3-phosphocholine mono-hydrate (DPPC) was purchased from Fluka Chemicals Co. (Buchs, Switzerland). Cholesterol (Chol), stearylamine (SA), 1,2-dipalmitoyl-sn-glycerol-3-phosphatidic acid (DPPA) and dimethyldioctadecyl glycerole bromide (DDAB) were provided by Sigma Chemicals Co. (St Louis, MO). Dipalmitoylphosphatidyl glycerole (DPPG) was obtained from Genzyme Pharmaceuticals and Fine Chemicals (Chem. Progress s.r.l., Milano, Italy). The phospholipid purity (>99%) was assayed by two-dimensional thin-layer chromatography (TLC) on silica-gel plates (E. Merck, Darmstadt, Germany) loaded with a solution of the lipid in chloroform–methanol (3 : 1 v/v). TLC was performed by eluting the plate first with a solvent system consisting of chloroform–methanol–5 M ammonium hydroxide (60 : 3 : 5 v/v/v) and then with chloroform–methanol–acetic acid–water (12 : 60 : 8 : 2·5 v/v/v/v). The phosphorus content of the phospholipid was determined as inorganic phosphate (Bartlett 1959). Acyclovir was provided by Sigma Chemical Co. (St Louis, MO). Distilled and apyrogenic water was used. All other materials and solvents were of analytical grade.

Liposome preparation

An aqueous solution of acyclovir (400 μg mL^{-1} per sample) was prepared immediately before liposome preparation. Four different preparation procedures were carried out to trap acyclovir in liposomes. For all procedures, films of neutral and charged phospholipids were prepared. The desired amount of lipids was weighed in a round-bottomed flask and dissolved in chloroform in the presence of 40 g of glass beads (2–3 mm mean size) (Carlo Erba, Milano, Italy). The use of chloroform–methanol was avoided because phase separation of cholesterol frequently occurs during evaporation. The organic solvent was then removed at 30°C on a rotating evaporator under nitrogen stream and stored overnight under high vacuum (Edwards high vacuum pump mod. Serial E 2M8 42810). A thin film of dry lipids was deposited on the inner wall of the flask and on the glass bead surface.

Large multilamellar vesicles (MLV) were prepared following the thin-layer evaporation technique (TLE) under an atmosphere of nitrogen by hydrating (vortex mixing) lipid films with acyclovir solution, maintaining a temperature of 55°C throughout the process (Fresta et al 1994). The frozen and thawed multilamellar vesicles (FATMLV) were obtained by freezing the MLVs in liquid nitrogen and thawing the samples in a warmed water bath. Ten freeze–thaw cycles were performed (Fresta et al 1995b). For REV (reverse phase evaporation) preparation, the lipidic film was dissolved in diethyl ether and acyclovir solution was then added. The water–lipid mixture was emulsified at 10°C for 20 min under a nitrogen atmosphere in a Bransonic model 2200 bath sonicator. Any residual organic solvent present in the emulsion was removed by rotary evaporation under reduced pressure at room temperature (20°C). The resulting viscous gel was resuspended in an isotonic pH 7·4 phosphate buffer (Fresta et al 1994). For DRV (dehydrated-rehydrated vesicles) preparation, the lipidic film was dissolved in acyclovir

solution and sonicated (Bransonic model 2200 bath sonicator), thus obtaining small unilamellar vesicles (SUV). The suspension was then freeze-dried overnight (Edwards freeze-dryer Modulayo equipped with an Edwards 8 two-stage vacuum pump). The powder was rehydrated with isotonic solution (0·9%) by vortex, until a homogeneous suspension was obtained (Gregoriadis et al 1990). Empty liposomes were prepared in the same way except that isotonic phosphate buffer was used instead of drug solution.

To evaluate the loading capacity of the various liposomal systems, untrapped acyclovir was removed from the liposomal suspension by centrifugation at 20 000 rev min^{-1} for 30 min, at 5°C, using a Beckman mod. J2-21 centrifuge, equipped with a Beckman JA-20·01 fixed-angle rotor. The supernatant was assayed for free acyclovir at 251 nm using an UV spectrophotometer (Uvikon 860, Kontron Instruments, Zurich, Switzerland). The amount of drug loaded in the liposomes was calculated as the difference between the initial amount of drug added to the suspensions (400 μg mL^{-1}) and the amount detected in the supernatant. Results are expressed as encapsulation yield (EY) and encapsulation capacity (EC), as reported elsewhere (Benita et al 1984).

Morphological characterization

Photon correlation spectroscopy (PCS) was used to determine the vesicle size (Zetamaster, Malvern Instruments Ltd, Sparing Lane South, Worcs, England). The experiments were carried out using a solid-state laser as light source. This laser is a nominal 4·5 mW laser diode with a maximum output of 5 mW at 670 nm. The PCS measurements were carried out at a scattering angle of 90°. The correlation functions were performed by a Malvern PCS sub-micron particle analyser and a third-order cumulant fitting (Chu 1974; Berne & Pecora 1976) with a dilation of 1·20 to obtain the mean diameter and polydispersity. The real and imaginary refractive indices were set at 1·59 and 0·00, respectively. The following parameters were used for experiments: medium refractive index 1·330, medium viscosity 1·0 and a dielectric constant of 79. The samples were suitably diluted with filtered water (Sartorius membrane filters 0·22 μm) to avoid multi-scattering phenomena and placed in a quartz cuvette. Thirty measurements per sample were performed.

Electrophoretic mobility and zeta potential distribution were measured with the Zetamaster particle electrophoresis analyser set-up equipped with a 5 mW HeNe laser (633 nm). Zeta limits ranged from −120 to 120 V. Strobing parameters were set as follows: strobe delay −1·00, on time 200·00, off time 1·00. A Smoluchowsky constant F (Ka) of 1·5 was used to achieve zeta potential values from electrophoretic mobility.

The morphological characterization was carried out by freeze-fracture electron microscopy, using the propane-jet technique (Müller et al 1980). The samples were fractured at −165°C and platinum/carbon replicas were observed in a Philips EM 301 electron microscope at 100 kV. ^{31}P-NMR was employed to provide the liposome lamellarity (Fresta et al 1995c).

Differential scanning calorimetry (DSC) analysis

The various samples (40 μL) were sealed in an aluminium pan and submitted to DSC analysis. A Mettler DSC 12E differential scanning calorimeter equipped with a Haake D8-G was used. Indium was used to calibrate the instrument. The detection system was a Mettler Pt100 sensor. The sensor presented a thermometric sensitivity of 56 μV K^{-1}, a calorimetric sensitivity of about 3 μV mW^{-1}, and a noise level lower than 60 nV ($< 20\,\mu$W) peak to peak. The baseline reproducibility was $< 3\,\mu$V ($< 1\,\mu$W). Each DSC scan had an accuracy of ± 0·4 K with a reproducibility and a resolution of 0·1 K. The reference was an aluminium pan containing 40 μL isotonic phosphate buffer. The samples were submitted to 3 cycles of heating and cooling, in the temperature range 293–333 K at a scanning rate of 1 K min^{-1}. Enthalpy changes (ΔH) were calculated from peak areas with Mettler TA89E and FP89 system software (version 2·0). After each DSC experiment the DPPC amount was determined spectrophotometrically by means of the formation of a red-coloured complex with isothiocyanate (Stewart 1980).

Liposome release

After the separation of the untrapped drug by centrifugation, the liposome pellet was made up to 10 mL with isotonic solution. The final phospholipid concentration was 1·1 mg mL^{-1}. The release experiments were carried out at 37°C in a thermostatic bath. At predetermined time intervals, 0·5-mL samples were taken from the suspension and transferred into Whatman VectaSpin3 ultrafiltration membrane cones (molecular-weight cut-off 20 000; Whatman International Ltd, Milano, Italy) and centrifuged at 500 *g* for 5 min. The supernatant was assayed for acyclovir at 254 nm by using a UV spectrophotometer (Uvikon 860, Kontron Instruments, Zurich, Switzerland). UV calibration straight-line ($y = x4{\cdot}623 \times 10^{-2} + 0{\cdot}036$; where y is UV absorbance and x is drug concentration) presented an r^2 value of 0·99936. As each sample was taken, it was replaced by an equal volume of

the isotonic solution. The amount of drug released was calculated according to the following equation:

$$\% \text{ Drug released} = 100 \times (Cr - Ci)$$

where Cr and Ci are released drug amount and initial drug amount, respectively.

In-vivo ocular absorption

Male New Zealand albino rabbits (Charles River, Calco, Italy) 1·8–2·0 kg, free of any sign of ocular or gross abnormality, were used. Animal procedures conformed to the ARVO (Association for Research in Vision and Ophthalmology) resolution on the use of animals in research. The animals were allowed to move their heads freely and their eye movements were not restricted.

Various formulations (50 μL) containing acyclovir were instilled only once in the conjunctival sac of the rabbit eyes. After 30 min, the rabbits were killed by intravenous injection of 0·3 mL kg^{-1} euthanasia solution (Tanax, Hoechst AG, Frankfurt am Main, Germany) and aqueous humour samples were collected by a 26-gauge needle, attached to a tubercolin syringe, introduced into the anterior chamber through the cornea. Aqueous humour levels of acyclovir were detected by HPLC analysis. Samples were denaturated by the addition of an equivalent volume of 2% $ZnSO_4$. $7H_2O$ solution, centrifuged and the supernatants were filtered through a 0·2-μm Teflon membrane.

Samples (20 μL) were analysed by an HPLC Hewlett Packard model 1050 equipped with an UV detector. A Hypersil C18 cartridge column (125 mm × 4·6 mm i.d.; 5 μm) obtained from Alltech (Milan, Italy) equipped with a direct-connect guard column was used. The mobile phase was ammonium acetate (10 mM; pH 6·8)–acetonitrile (99 : 1) at a flow rate of 1 mL min^{-1}. The UV-detector was positioned at 254 nm. Aqueous acyclovir levels were calculated from linear regression of external standards of acyclovir, relating peak area and concentration. Standards in a range of 1·0–55·5 μg mL^{-1} were used for the calibration curve. The straight-line equation was $y = -1{\cdot}0013x + 0{\cdot}00046$ (y = drug concentration; x = peak area) with an $r^2 = 0{\cdot}9995$. The method was found to be reproducible with a relative standard deviation of 1·80%. The sensitivity was 0·61 μg mL^{-1}. No interfering peak was observed in the blank aqueous humour chromatograms.

Results and Discussion

Acyclovir-membrane interaction

It has been shown that a variety of chemotherapeutic agents strongly interact with the phospholipid components of cellular bilayer membranes, demonstrating that drug–membrane interactions seem to have a role in the biological action of several drugs (Deliconstantonos et al 1987; Canaves et al 1991; Fresta et al 1996). DPPC vesicles have been used as suitable models to investigate molecule–membrane interaction (Abrams & Yager 1993; Puglisi et al 1996). Furthermore, this kind of study may be of interest for the preparation of liposome carriers with high drug-entrapment capacity and with suitable drug-release characteristics. The interaction was studied by DSC, which represents a non-invasive analytical technique.

DPPC vesicles were used as model membranes since this phospholipid is able to mimic many aspects of biological membranes, being one of their most abundant constituents. When submitted to DSC analysis, DPPC bilayers show three phase transitions: the subtransition at ~293 K; the pretransition at ~310 K; and the main transition at ~315 K (Chapman et al 1967; Jain 1988). The pretransition ($L_\beta \rightarrow P_\beta$) is related to a structural transformation of the glycerol backbone of the DPPC bilayers triggering the formation of the ripple phase (Berleur et al 1985). The main transition ($P_\beta \rightarrow L_\alpha$) arises from the transformation of an all-*trans* configuration of the acyl chains in the ordered gel state to a more fluid state in which the population of gauche conformers of the acyl chains noticeably increases (Berleur et al 1985). The presence of a compound in the DPPC membranes could interact and influence the thermotropic parameters of the vesicle transition as a function of its own physicochemical properties.

DSC findings showed that acyclovir was mainly placed at the level of the aqueous compartments of DPPC membranes. In fact, as shown in Figure 1, the presence of the drug did not cause any change of the thermotropic parameters of the DPPC main transition. No variation of the DSC scan profiles with respect to the pure DPPC was observed for any of the samples prepared in the presence of acyclovir up to a drug molar fraction of 0·18. Namely, DSC scans were still characterized by the $P_\beta \rightarrow L_\alpha$ transition and the $L_\beta \rightarrow P_\beta$ ripple-phase pretransition, which is very sensitive to the presence of foreign molecules (it suddenly disappears when even slight interaction occurs with the bilayer). These findings showed that the drug was unable to interact with either the hydrophobic zone of the bilayer or the hydrophilic zone of the phospholipid headgroups, thus showing it was simply solubilized in the aqueous compartments of liposomes.

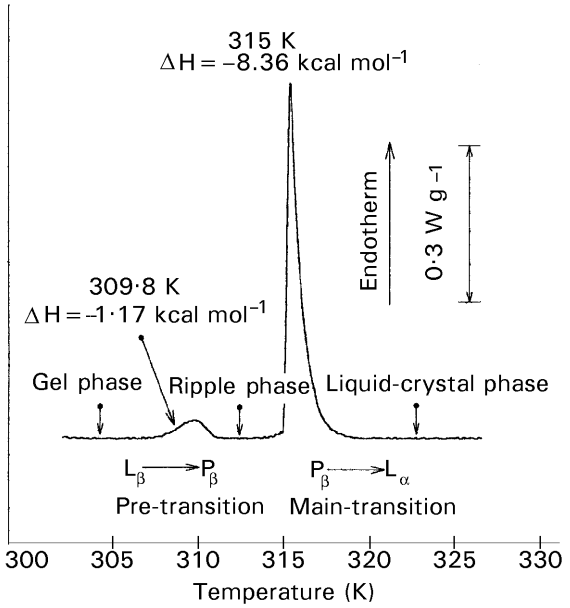

Figure 1. DSC curve of DPPC membranes prepared in the presence of acyclovir (0·09 molar fraction). The curve refers to the second scan in heating mode at a temperature scanning rate of 1 K min^{-1}. Isotonic phosphate buffer (pH 7·4) was used as reference. The presence of the drug did not influence the thermotropic characteristics of DPPC membranes.

Considering that corneal epithelium, in common with other biological epithelia, is negatively charged, the interaction of acyclovir with charged membranes was also studied. Negatively charged membranes were made up of DPPC–DPPA (8 : 2 molar ratio), whereas positively charged membranes were made up of DPPC–SA (8 : 2 molar ratio). With regard to the DPPC–DPPA membranes, DSC data showed that acyclovir was able to interact with the negatively charged component of the bilayers (Table 1). In particular, no pretransition peak was observed for the system prepared in the absence of the drug, due to the presence of DPPA in the phospholipid matrix of DPPC. When the DPPC–DPPA membranes were prepared in the presence of acyclovir, a shift of the main transition peak towards higher temperatures than those observed with the pure membranes occurred as a function of drug molar fraction. The ΔH values of the main transition peak were also influenced by the presence of acyclovir, that is, the higher the molar fraction of the drug, the greater the reduction of the enthalpy variation (Table 1). These findings were probably due to an interaction of acyclovir with DPPA headgroups via hydrogen bonds or electrostatic attraction. In fact, the presence of acyclovir reduced the repulsion among the negatively charged DPPA headgroups, thus leading to an increase of the transition temperature of the vesicular system. Due to interaction with the DPPA component, a loss of bilayer co-operativity was observed as a function of the acyclovir molar fraction (Figure 2). In fact, besides the reduction of ΔH values, a broadening of the main transition peak as a function of the drug molar fraction was also observed (see $\Delta T_{1/2}$ and T10–95% K values, Table 1). In particular, the broadening of the main transition peak is much more evident at higher drug molar fractions (Figure 2) probably due to a bilayer phase segregation. In fact, the deconvolution of the main transition peak of DPPC–DPPA (8 : 2 molar ratio) vesicles prepared in the presence of acyclovir (0·09 molar fraction) generated three peaks centred at 311·6, 318·2 and 322·3 K, respectively (Figure 3). The peak component at 322·3°K may be attributed to a DPPA/acyclovir-rich phase domain; the component centred at 318·2 K may arise from the transition of the DPPA–DPPC domain, in which the presence of the drug is really poor, since the presence of even a small amount of acyclovir (0·005 molar fraction, see Table 1) shifted the peak transition temperature by about two degrees. The last component may be due to mixed and heterogeneous domains with a very poor co-operativity and, hence, a lower transition temperature.

The interaction of acyclovir with positively charged vesicles made up of DPPC–SA (8 : 2 molar

Table 1. Thermotropic parameters of the interaction between acyclovir and DPPC–DPPA (8 : 2 molar ratio) biomembranes with regard to endothermic peak of the phase transition from gel to liquid-crystal state as a function of the drug molar fraction.[a]

Drug molar fraction	Tm (K)[b]	ΔH (kcal mol^{-1})	ΔS (cal mol^{-1} K^{-1})	T10% (K)[c]	T95% (K)[d]	$\Delta T_{1/2}$ (K)[e]
0·000	317·1	9·90	31·22	315·6	319·0	2·3
0·005	319·2	9·94	31·14	317·0	323·8	3·9
0·015	319·5	9·18	28·73	317·1	323·9	6·1
0·030	319·8	8·97	28·05	317·3	324·0	6·8
0·060	319·9	8·52	26·63	316·6	324·4	7·2
0·090	320·1	7·51	23·46	308·9	326·1	8·7

[a]Each value is the average of three different experiments. [b]Transition peak temperature. [c]Melting range temperature: 10% of the sample underwent phase transition. [d]Melting range temperature: 95% of the sample underwent phase transition. [e]Width half-height of the transition peak.

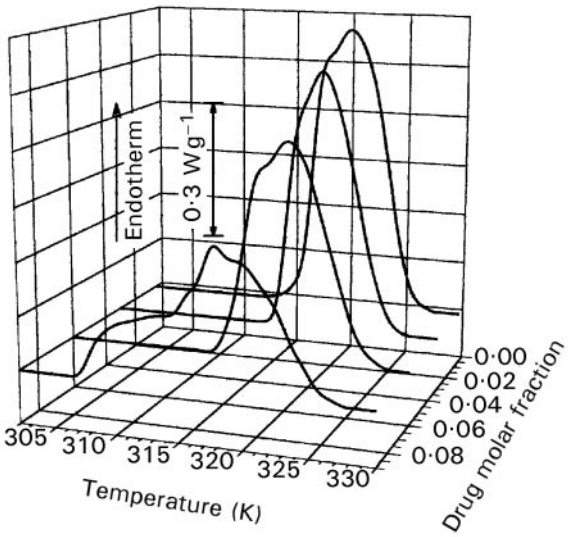

Figure 2. Three-dimensional DSC scans in heating mode of DPPC–DPPA (8 : 2 molar ratio) MLVs prepared in the presence of isotonic phosphate buffer (pH 7·4) containing acyclovir at different concentrations. The furnace heating rate was 1 K min^{-1}. The samples were submitted to DSC analysis 6 h after their preparation. Aqueous buffer was used as reference.

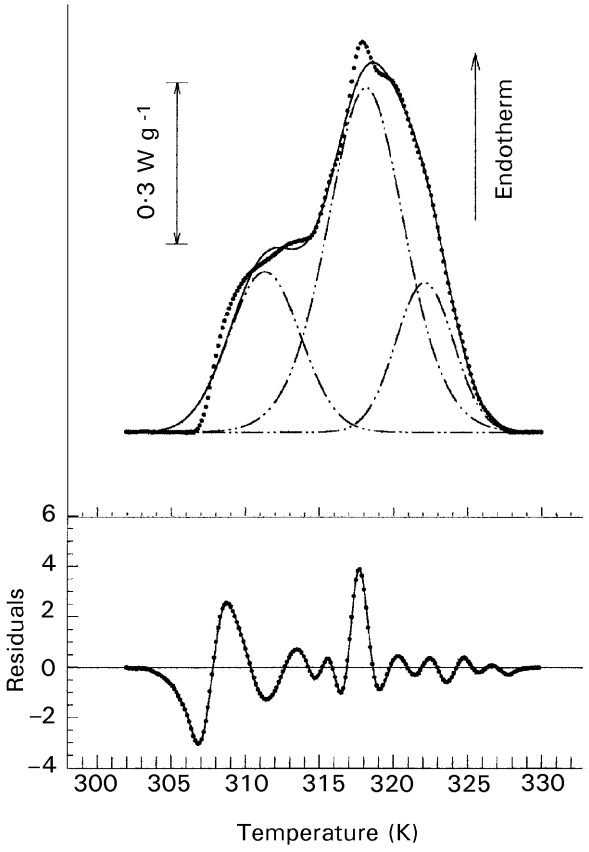

Figure 3. Computer fitting (solid line) of the experimental DSC curve (filled circles) of DPPC–DPPA (8 : 2 molar ratio) membranes prepared in the presence of acyclovir at 0·09 molar fraction. The deconvolution (dotted–dashed line) of the theoretical curve led to three peaks centred at 311·6 K, 318·2 K and 322·3 K. The residuals of the fitting procedure are shown in the lower plot. The regression value of the fitting curve with regard to the experimental curve is $r^2 = 0{\cdot}99417$.

ratio) was also studied. In fact, DSC experiments can also be useful as preformulation studies to achieve suitable liposomal drug-delivery devices. In the case of ophthalmic delivery, the possibility of obtaining positively charged delivery devices may have noticeable advantage in terms of electrostatic attraction and bioavailability. Similarly to negatively charged vesicular systems, acyclovir was also able to interact with positively charged vesicles mostly via hydrogen bonds.

As reported in Table 2, the transition peak temperature was only slightly influenced by the presence of acyclovir whereas the transition enthalpy was shifted towards lower values as a function of acyclovir molar fraction (i.e. the higher the drug concentration the lower the ΔH values). The presence of the drug also determined a broadening of the membrane transition peak (see T10–95% K, Table 2; Figure 4). These findings may be attributed to the formation of hydrogen bonds between acyclovir and the charged headgroups of stearylamine, thus eliciting a reduction of bilayer cooperativity. Similarly to negatively charged liposomes, the highest acyclovir molar fractions triggered a noticeable broadening of the transition peak due to a phospholipid phase segregation into stearylamine-acyclovir rich domains and stearylamine-acyclovir poor domains (Figure 4).

Acyclovir–liposome formulation

DSC findings showed that acyclovir was mainly placed at the level of the vesicle aqueous compartments and was also able to interact with the phospholipid headgroups of negatively and positively charged bilayers via hydrogen or electrostatic bonds. Therefore, the preparation method of liposome formulation was crucial for the encapsulation efficiency of various liposome carriers, namely, the greater the aqueous phase entrapped within liposomes, the higher the acyclovir encapsulation capacity. Various liposome formulations were prepared following four different methods: TLE, REV, FAT and DRV.

As reported in Table 3, the preparation methods TLE, FAT and DRV led to the formation of multilamellar vesicles with a mean size of $\sim 1{\cdot}2\ \mu$m and a wide size distribution (polydispersity index $\sim 0{\cdot}7$). The REV preparation procedure achieved oligolamellar vesicles (mean size ~ 350 nm) with a narrow size distribution (Figure 5). These findings were also confirmed by freeze–fracture electron microscopy (data not reported). The presence of DPPG/DPPA, or SA/DDAB in all the kinds of liposomes provided a negative or a positive charge along the surface of the vesicles bilayers, respectively (Table 3).

Table 2. Thermotropic parameters of the interaction between acyclovir and DPPC-SA (8 : 2 molar ratio) biomembranes with regards to the phase transition from gel to liquid-crystal state as a function of the drug molar fraction.[a]

Drug molar fraction	Tm (K)[b]	ΔH (kcal mol^{-1})	ΔS (cal mol^{-1} K^{-1})	T10% (K)[c]	T95% (K)[d]	$\Delta T_{1/2}$ K[e]
0·000	326·3	8·48	25·99	325·6	327·6	0·9
0·005	325·5	8·31	25·53	322·9	327·3	2·1
0·015	325·6	7·85	24·11	322·2	327·3	3·5
0·030	325·5	7·59	23·32	322·4	327·4	4·1
0·060	325·9	7·21	22·12	322·8	327·3	4·4
0·090	325·8	6·79	20·84	320·6	328·7	5·1

[a]Each value is the average of three different experiments. [b]Transition peak temperature. [c]Melting range temperature: 10% of the sample underwent phase transition. [d]Melting range temperature: 95% of the sample underwent phase transition. [e]Width half-height of the transition peak.

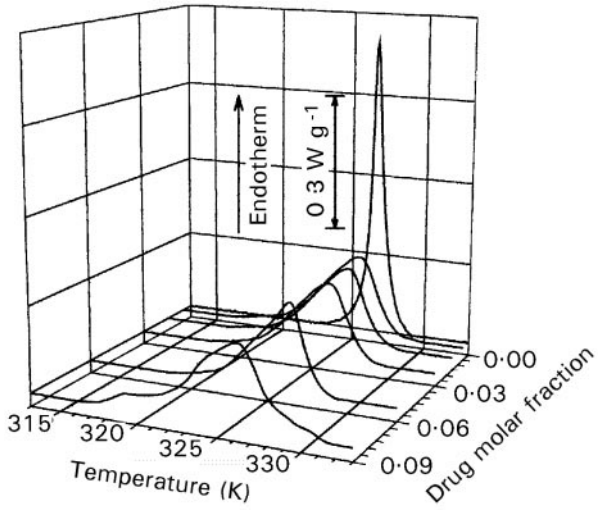

Figure 4. Three-dimensional DSC scans in heating mode of DPPC–SA (8 : 2 molar ratio) MLVs prepared in the presence of isotonic phosphate buffer (pH 7·4) containing acyclovir at different concentrations. The furnace heating rate was 1 K min^{-1}. The samples were submitted to DSC analysis 6 h after their preparation. Aqueous buffer was used as reference.

With regard to liposome encapsulation efficiency, multilamellar systems obtained with both the FAT and the DRV method showed a higher degree of acyclovir encapsulation than MLVs (Table 4). This was probably due to the capacity of FATMLV and DRV to incorporate greater amounts of aqueous phase than MLV, thus leading to higher EC and EY values (Table 4). It is worth noting that a difference in the encapsulation parameters of the various multilamellar systems was observed as a function of the phospholipid mixtures. The neutral phospholipid mixture (DPPC–Chol 7 : 4 molar ratio) showed the lowest encapsulation efficiency, probably because no interaction occurred between the drug and the lipid components as demonstrated by DSC experiments. In this case, acyclovir encapsulation only depended on the amount of aqueous phase encapsulated.

Negatively charged liposomes (DPPC–Chol–DPPG 7 : 4 : 1 molar ratio) showed the highest encapsulation efficiency (Table 4). The presence of a charge along the bilayer surface ensured a repulsion among the various liposome lamellae

Table 3. Dimensional and morphological characterization of various oligolamellar and multilamellar liposomes.

Liposome composition	MLVs[a] size (nm)	PI[b]	N[c]	ζ–Potential (mV)	Mobility[d] (μm s^{-1} V^{-1} cm^{-1})
DPPC–Chol	2701·5	0·753	13·4	−9·1	−1·0
DPPC–Chol–DPPA	1195·3	0·845	8·5	−27·3	−1·9
DPPC–Chol–DPPG	1264·6	0·907	9·4	−12·9	−0·9
DPPC–Chol–SA	851·9	0·461	8·3	13·5	0·9
DPPC–Chol–DDAB	1266·6	0·129	9·7	7·6	0·7
	REVs				
DPPC–Chol	383·4	0·101	4·1	−3·6	−0·7
DPPC–Chol–DPPA	323·7	0·034	3·5	−28·1	−2·1
DPPC–Chol–DPPG	322·5	0·029	3·4	−14·7	−1·3
DPPC–Chol–SA	318·4	0·074	3·7	18·3	1·7
DPPC–Chol–DDAB	327·2	0·051	3·1	12·8	0·9

[a]Multilamellar systems obtained with the FATMLV or DRV preparation procedure presented morphological and colloidal properties similar to those of MLV. [b]Polidispersity Index. [c]Number of lamellae determined by ^{31}P–NMR. [d]This value represents velocity divided by electric field.

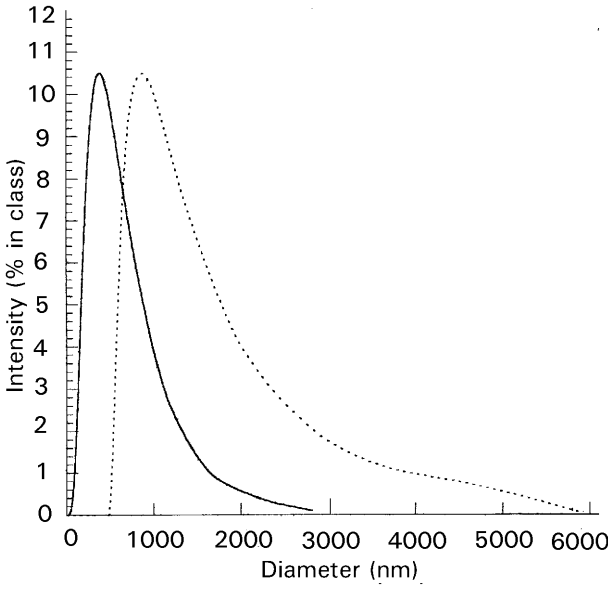

Figure 5. Size distribution of REVs (solid line) and FATMLVs (broken line) (made up of DPPC–Chol–DDAB, 7 : 4 : 1 molar ratio) estimated from light-scattering analysis by Laplace inversion transform. The various samples were diluted to achieve the most suitable optical density for the analysis.

Table 4. Acyclovir encapsulation efficiency parameters of various liposome formulations.[a]

Liposome composition	MLVs EC[b]	EY[c]
DPPC–Chol	9·77 ± 0·41	18·88
DPPC–Chol–DPPA	13·37 ± 0·27	20·57
DPPC–Chol–DPPG	14·06 ± 0·31	21·83
DPPC–Chol–SA	11·63 ± 0·72	20·16
DPPC–Chol–DDAB	11·07 ± 0·53	19·65
	FATMLVs	
DPPC–Chol	10·22 ± 0·33	19·70
DPPC–Chol–DPPA	15·71 ± 0·47	27·63
DPPC–Chol–DPPG	15·80 ± 0·39	28·00
DPPC–Chol–SA	11·87 ± 0·61	22·73
DPPC–Chol–DDAB	12·13 ± 0·69	23·40
	DRVs	
DPPC–Chol	10·09 ± 0·74	19·50
DPPC–Chol–DPPA	13·53 ± 0·29	21·07
DPPC–Chol–DPPG	13·40 ± 0·45	20·80
DPPC–Chol–SA	11·08 ± 0·66	19·49
DPPC–Chol–DDAB	11·48 ± 0·81	20·40
	REVs	
DPPC–Chol	45·56 ± 1·09	78·20
DPPC–Chol–DPPA	47·34 ± 0·97	79·34
DPPC–Chol–DPPG	47·16 ± 0·86	79·56
DPPC–Chol–SA	46·20 ± 1·28	78·91
DPPC–Chol–DDAB	45·98 ± 1·39	78·50

[a]Each value is the average of five experiments ± s.d. [b]Encapsulation capacity in $\mu L\,mol^{-1}$. [c]Encapsulation yield as percentage of acyclovir amount which is entrapped within liposomes.

leading to a higher aqueous phase entrapment. In fact, a lower number of bilayers was observed for charged systems than neutral systems, even though both MLVs showed similar mean size (Table 3). For negatively charged liposomes, acyclovir may have also been adsorbed along bilayers via electrostatic or hydrogen bonds, as shown by DSC experiments. Positively charged liposomes showed slightly lower encapsulation efficiency values than negatively charged liposomes. These findings were probably due to the fact that acyclovir was able to interact with the DDAB and stearylamine component to a less extent than with DPPG or DPPA. As shown by DSC experiments, only hydrogen-bond interactions were possible with stearylamine. No electrostatic interaction occurred between DDAB and acyclovir, which may present electropositive regions (amino groups) as a function of the pH environment. This hypothesis was confirmed by zeta-potential experiments (Figure 6). The negatively charged vesicles showed a reduction of the zeta-potential values as a function of the drug concentration, probably due to an electrostatic interaction between the polar phospholipid headgroups and acyclovir. The zeta-potential of positively charged systems were not influenced by the presence of acyclovir. Both the phospholipid headgroups and the drug may present a positive charge.

The oligolamellar systems obtained with the REV method showed the highest encapsulation efficiency. These findings were probably due to the greater amount of water that may be entrapped

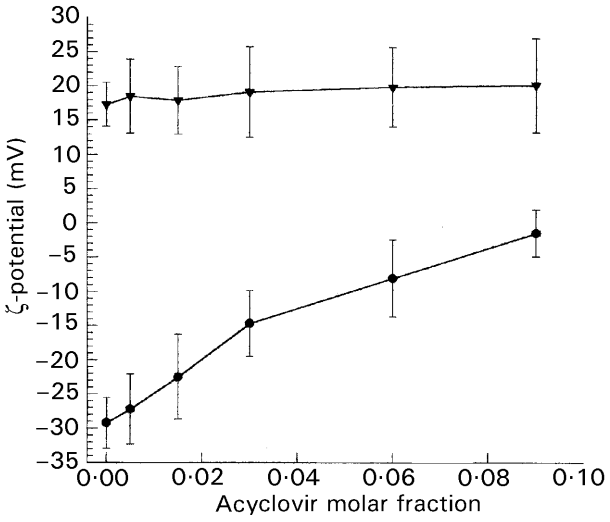

Figure 6. Zeta-potential values of positively (DPPC–Chol–SA, 7 : 4 : 1 molar ratio; ▼) and negatively (DPPC–Chol–DPPA, 7 : 4 : 1 molar ratio; ●) charged REVs prepared in the presence of different molar fractions of acyclovir. The experiments were carried out 6 h after liposome preparation. The various samples were diluted to achieve the most suitable optical density for the analysis.

within REVs compared with multilamellar systems. In the case of oligolamellar systems, no difference was observed among the various phospholipid mixtures regarding encapsulation efficiency. This phenomenon may be attributed to the oligolamellar nature of this system. In fact, the repulsion between the various bilayers was of minor importance to the encapsulation of the aqueous phase and, hence, of the drug, whereas the bilayer repulsion played an important role in acyclovir encapsulation for multilamellar systems.

Another important parameter to evaluate the carrier capacity of a drug-delivery device was the drug-release properties. As shown in Figure 7, the release of acyclovir from the positively and negatively charged REVs was characterized by a fast release for the first hour followed by a more gradual drug release. This initial burst effect may have been due to a rapid desorption of acyclovir from the liposome bilayers. In the case of neutral REVs, the release of acyclovir presented no particular burst effect and showed a more gradual acyclovir release. REVs made up of DPPC–Chol–DDAB showed the lowest acyclovir release, probably because the electrostatic repulsion between acyclovir and charged phospholipid headgroups hampered a free passage of acyclovir through bilayers triggering the release of the lowest drug amount. Similar release profiles were observed for multilamellar systems (Figure 8), which were characterized by the release

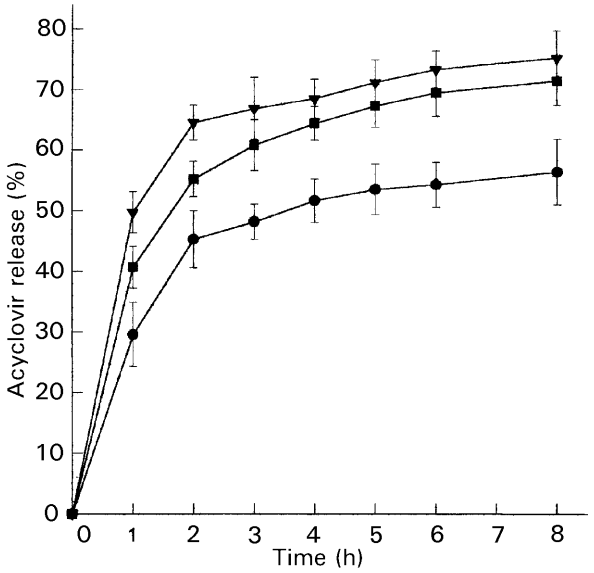

Figure 7. Acyclovir leakage from oligolamellar liposomes (REVs) suspended in pH 7·4 phosphate buffer. The release experiments were carried out at 37 ± 0·2°C, immediately after sample preparation. Unloaded drug was removed by means of gel-permeation chromatography. Each point is the average of five experiments ± s.d. ●, DPPC–Chol–DDAB (7 : 4 : 1 molar ratio); ■, DPPC–Chol–DPPG (7 : 4 : 1 molar ratio); ▼, DPPC–Chol (7 : 4 molar ratio).

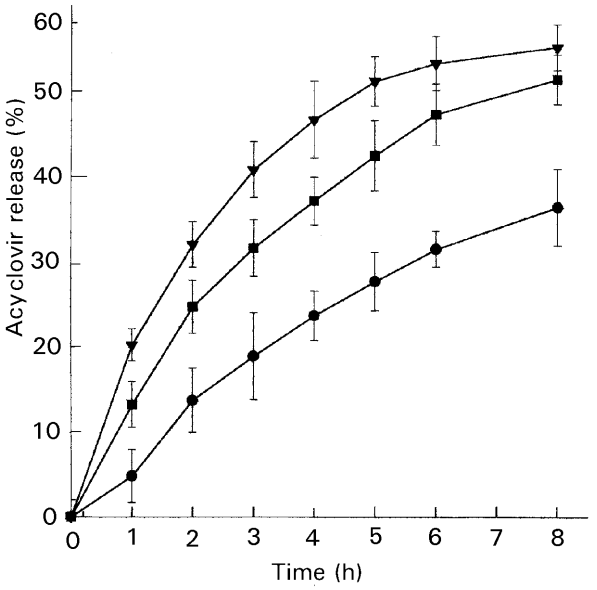

Figure 8. Acyclovir leakage from multilamellar liposomes (FATMLVs) suspended in pH 7·4 phosphate buffer. The release experiments were carried out at 37 ± 0·2°C, immediately after sample preparation. Unloaded drug was removed by means of gel-permeation chromatography. Each point is the average of five experiments ± s.d. ●, DPPC–Chol–DDAB (7 : 4 : 1 molar ratio); ▼, DPPC–Chol–DPPG (7 : 4 : 1 molar ratio); ■, DPPC–Chol (7 : 4 molar ratio).

of a lower amount of acyclovir than REVs. In the case of multilamellar systems, the drug had to pass through a number of bilayers to be released, thus a slower release than with REVs was observed.

In-vivo corneal penetration

Taking into account that positively charged delivery devices may ensure a suitable bioadhesivity with the corneal epithelium, positively charged small oligolamellar liposomes (DPPC–Chol–DDAB 7 : 4 : 1 molar ratio) were used for biological experiments. In addition, the high colloidal properties of REV systems may achieve a better interaction with the corneal epithelium (in terms of paracellular transport or passage) (Davies et al 1993), thus leading to a greater drug transport into the ocular tissues.

Acyclovir-loaded REVs were compared with both a formulation of empty liposomes physically blended with the drug and an aqueous solution of acyclovir. All three formulations tested contained the same drug concentration. Since the aqueous T_{max} of most topically administered drugs is around 30 min (Lee & Robinson 1986), the aqueous acyclovir levels of the three formulations were detected at 30 min (peak-time) after a single instillation (50 μL). In a preliminary experiment the ocular tolerability to liposomes and liposome-encapsulated acyclovir formulation was evaluated under the standard protocol (McDonald & Shedduck 1977) and no evidence of ocular inflammation or

discomfort were observed (data not shown). Studies showed (Davies et al 1993) that liposomes can enhance the ocular bioavailability of entrapped drugs compared with free drugs with similar peak-time.

The concentration of acyclovir in the aqueous humour after topical administration of the three formulations is shown in Table 5. The liposome-encapsulated acyclovir formulation showed a significant ($P < 0{\cdot}005$) increase of drug levels compared with the liposome–acyclovir physical mixture and the free drug. No significant difference was observed between the liposome-acyclovir blend and the free drug in terms of aqueous drug concentrations. A 42·3-fold and 28·2-fold increase in acyclovir in the aqueous humour was observed for acyclovir-loaded liposomes compared with the free drug and the liposome-acyclovir blend, respectively.

Several mechanisms have been proposed (New et al 1990) to elucidate the ocular effects of liposomes, but adsorption or lipid exchange seem to be most probably involved (Singh & Mezei 1984). It has been proposed (Singh & Mezei 1984) that liposomes can insert themselves into the corneal cell membrane or simply remain adsorbed on the cell surface, releasing their content directly into the cell. Since the corneal epithelium, at physiological pH, is negatively charged, adsorption may take place as a result of physical attractive forces. Hence, positively charged liposomes enhance the bioavailability of entrapped drugs over neutral or negatively charged vesicles. Studies on the interaction between different charged liposomes and corneal epithelium showed that the degree of association of liposomes with the corneal surface decreases in the order $MLVs^+ > SUVs^+ > MLVs^- > SUVs^- > MLVs$, SUVs (Schaeffer & Krohn 1982). With regard to incorporation of drugs, $SUVs^+$ were found to enhance bioavailability to a greater extent than $MLVs^+$. The superiority of the $SUVs^+$ in this respect is attributed to a smaller size allowing for a closer apposition to the corneal epithelium (Davies et al 1993). Alteration in the permeability of the cornea by liposomes themselves may be discarded as a plausible explanation for enhanced drug penetration, since the presence of empty lipid vesicles added to drug solutions does not enhance the availability of the drug (Stratford et al 1983). The advantages of liposomes in ocular drug delivery include their ability to control the rate of release of encapsulated drug, to protect the drug from the metabolic enzymes present at the tear–corneal epithelium interface and their ability to form intimate contact with the corneal and conjunctival surfaces, thereby increasing the potential for ocular drug absorption. The liposome-encapsulated acyclovir formulation tested in the rabbit eye was mostly based on small-size oligomellar vesicles as shown by light-scattering analysis (Table 3).

Table 5. Aqueous humour concentrations of acyclovir at 30 min following topical instillation (50 μL) of acyclovir–loaded oligolamellar liposomes (DPPC–Chol–DDAB 7 : 4 : 1 molar ratio), acyclovir–liposomes physical mixture and acyclovir in aqueous solution.

Acyclovir formulation	Aqueous humour concn of aciclovir ($\mu g\,mL^{-1}$)
Free drug	$2{\cdot}11 \pm 1{\cdot}80$
Drug–REVs physical mixture	$3{\cdot}10 \pm 2{\cdot}13$
Drug–loaded REVs	$88{\cdot}95 \pm 12{\cdot}31$

Values represent mean values ± s.d. of 4 experiments.

Acyclovir has been widely used against various manifestations of eye disease caused by herpes simplex. Secondary herpes simplex keratitis often appears as dendritic keratitis with a spread of the virus into the deeper corneal layers. In severe cases damage of the corneal stroma can lead to blindness. Therefore, treatment requires good permeation of the antiviral drug through the epithelium in order to reduce the virus load. It is well known that the level of a topically applied drug in the aqueous humour represents a good measure of the relative amount of drug reaching the stroma and other deeper corneal layers. We demonstrated that the acyclovir levels penetrating the corneal epithelium were significantly increased in the group treated with liposome-encapsulated acyclovir. The biological results described in this study indicate that the liposome-encapsulated acyclovir formulation may be useful in clinical practice for a better compliance and an improved outcome.

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