



# Celecoxib-loaded liposomes: effect of cholesterol on encapsulation and *in vitro* release characteristics

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## Synopsis

CLX (celecoxib) is a highly hydrophobic non-steroidal anti-inflammatory drug with high plasma protein binding. We describe here the encapsulation of CLX in MLVs (multilamellar vesicles) composed of DSPC (1,2-distearoyl-*sn*-glycero-3-phosphocholine) and variable amounts of cholesterol. The effects of cholesterol content on liposome size, percentage drug loading and *in vitro* drug release profiles were investigated. Differential scanning calorimetry and FTIR (Fourier-transform infrared) spectroscopy were used to determine molecular interactions between CLX, cholesterol and DSPC. The phase transition temperature ( $T_m$ ) of vesicles was reduced in a synergistic manner in the presence of both CLX and cholesterol. Encapsulation efficiency, loading and release of CLX decreased with increasing cholesterol content. FTIR results indicated that this decrease was due to a competition between CLX and cholesterol for the co-operativity region of the phospholipids. In the presence of cholesterol, CLX was pushed further into the hydrophobic core of the bilayer. However, MLVs prepared with DSPC only (without cholesterol) exhibited the lowest ability for drug retention after 72 h. Our results indicated that CLX, without the requirement of modifications to enhance solubilization, can be encapsulated and released from liposomal formulations. This method of drug delivery may be used to circumvent the low bioavailability and systemic side effects of oral CLX formulations.

**Key words:** celecoxib, cholesterol, 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), drug release, Fourier-transform infrared (FTIR), liposome

## INTRODUCTION

COX-2 (cyclo-oxygenase-2) overexpression is a frequent event in carcinogenesis, leading to increased prostaglandin production, which plays a role in the regulation of tumour-associated angiogenesis, cell migration and invasion, modulation of the immune system and inhibition of apoptosis [1]. Long-term regular use of the COX-inhibiting NSAIDs (non-steroidal anti-inflammatory drugs) has been shown to reduce the risk of several types of cancers in preclinical and clinical studies [2]. Recently developed selective coxibs (COX-2 inhibitors) were shown to be safer than conventional NSAIDs in terms of gastrointestinal side effects in two clinical trials: CLASS (Celecoxib Long-Term Arthritis Safety Study) and VIGOR (Vioxx Gastrointestinal Outcomes Research) [3,4].

CLX {celecoxib; 4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl] benzene sulfonamide} is a highly hydrophobic bioactive agent with a calculated log *P* of 3.68 and water solubility of 3–7 µg/ml [5] and it can selectively inhibit COX-2 activity. The coxibs, however, have been associated with adverse cardiovascular side effects in several clinical trials, possibly owing to an imbalance in the production of the vasodilatory compound prostacyclin and the vasoconstrictor thromboxane [6]. CLX has a wide tissue distribution of 455 ± 166 litres, indicating extensive penetration in various organs [7,8]. In addition, owing to the reported 97% plasma protein binding [9], oral CLX formulations are prescribed at high daily doses, thereby increasing concerns about cardiovascular risks [10]. To avoid possible systemic side effects and attain enhanced therapeutic activity, numerous delivery systems have been designed for CLX, such as solid lipid nanoparticles [11], CLX-PLGA [poly(lactic-co-glycolic acid)]

**Abbreviations used:** CLX, celecoxib; COX, cyclo-oxygenase; coxib, COX-2 inhibitor; DSC, differential scanning calorimetry; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; EE, encapsulation efficiency; FTIR, Fourier-transform infrared; LUV, large unilamellar vesicle; MLV, multilamellar vesicle; NSAID, non-steroidal anti-inflammatory drug.

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microparticles [12], chitosan microspheres [13],  $\beta$ -cyclodextrin complexes [14, 15] and niosomal gels [16].

Liposomes, which are spontaneously formed phospholipid bilayers, have been useful tools as model membranes and drug-delivery systems for hydrophilic, hydrophobic and amphiphilic agents [17]. MLVs (multilamellar vesicles) are concentric spherical phospholipid bilayers of more than 1  $\mu\text{m}$  in size. They can be used as slow-release systems, due to multiple membrane layers serving as barriers to diffusion and drug release [18,19]. Taking into account the high volume of tissue distribution and plasma protein binding of CLX, a liposomal delivery system will provide a protective shell for the molecules, enabling longer circulation half-life. Considering possible parenteral or local site-specific delivery systems for a proposed liposomal formulation, the amount of CLX oxidized to form inactive metabolites mainly by liver cytochrome P450 enzymes is anticipated to decrease. These indications all lead to the possibility of lowering the initial treatment dose of CLX when encapsulated in liposomes, serving to reduce any systemic adverse effects.

Optimization of liposome composition *in vitro* is the initial step in developing a liposomal formulation for delivery of bio-active agents. The fundamental properties important in such systems include size, permeability and stability of the liposome, all of which are dependent on the amount and structure of the membrane constituents used [20]. Therefore a proper understanding of the molecular interactions as a function of the liposomal membrane composition is a prerequisite for efficient encapsulation and optimum release outcomes [21]. Cholesterol is a common component of liposomes, providing rigidity to the membrane, controlling permeability and improving plasma stability in a dose-dependent manner [22]. Despite these well-recognized effects of cholesterol in conventional liposomal formulations, recent research also focuses on low cholesterol or cholesterol-free liposomes for entrapment of hydrophobic molecules such as idarubicin [23], paclitaxel [24], nystatin [25], tamoxifen [26] and triamcinolone acetate [27].

In the present study, we report a new liposomal delivery system for CLX where the NSAID was encapsulated in MLVs composed of DSPC (1,2-distearoyl-*sn*-glycero-3-phosphocholine) and various amounts of cholesterol. We have investigated the effect of cholesterol content on vesicle size, percentage drug loading and *in vitro* release profiles. In order to determine the molecular interactions between CLX, cholesterol and DSPC, we used two non-invasive techniques, DSC (differential scanning calorimetry) and FTIR (Fourier-transform infrared) spectrophotometry, and these allowed us to analyse the structural or dynamical changes in the membranes.

## MATERIALS AND METHODS

### Materials

18:0 PC (DSPC) and cholesterol (from ovine wool, >98%) were purchased from Avanti Polar Lipids (Alabaster, AL, U.S.A.) and

stored at  $-20^{\circ}\text{C}$ . CLX was obtained from Ranbaxy Laboratories Limited (Mumbai, India). HPLC-grade chloroform and all other reagents of analytical grade were purchased from Applichem (Darmstadt, Germany).

### Preparation and characterization of liposomes

#### *Preparation of liposome formulations with CLX*

MLVs were prepared by the thin lipid film hydration method [17]. Briefly, DSPC and cholesterol (at DSPC/cholesterol mole ratios of 2:1, 5:1 and 10:1) were mixed with CLX (18 mol%) and dissolved in chloroform in round-bottomed test tubes. Chloroform was then evaporated under a gentle argon stream to form thin lipid films, which were kept under vacuum overnight at 100 mbar (HETO, Allerod, Denmark). The tubes were flushed with argon to remove any residual solvent and stored at  $4^{\circ}\text{C}$  in dry form until use. The films were hydrated with 1 ml of PBS (0.1 M, pH 7.4) by sequential heating at  $70^{\circ}\text{C}$  and vortex-mixing in 2 min cycles for a total duration of 1 h. Upon sonication in a bath type sonicator (Bandelin Sonorex, Berlin, Germany) at  $70^{\circ}\text{C}$  for 15 min, MLVs were allowed to re-anneal at room temperature ( $25^{\circ}\text{C}$ ) for at least 2 h.

MLVs were separated from untrapped CLX molecules by two subsequent 15 min centrifugations at 12000 g with washing the pellet with PBS in between. After centrifugation, the supernatants were analysed for their lipid content to ensure that most of the lipids in the system were incorporated into the MLVs.

#### *Determination of liposome size*

The particle size distributions of MLVs were determined by dynamic light scattering (Malvern Mastersizer 2000; Malvern Instruments, METU Central Laboratory). To assess the stability of MLVs during release experiments, size analyses were repeated after a 72 h release period.

#### *DSC measurements*

MLVs (2:1, 5:1 and 10:1 DSPC/cholesterol molar ratio) for DSC studies were prepared according to the above-mentioned procedure with slight modifications. DSPC (2 mg) and the corresponding amounts of cholesterol were dissolved in chloroform and dried under vacuum for 2 h. The lipid films formed were then hydrated by adding 50  $\mu\text{l}$  of PBS (0.1 M, pH 7.4) and vortex-mixing the mixture at  $70$ – $75^{\circ}\text{C}$  for 20 min. The DSC experiments were carried out with a Universal TA DSC Q 100 instrument (TA Instruments, New Castle, DE, U.S.A.). The samples were placed in hermetically sealed standard aluminium DSC pans and scanned over a temperature range of  $20$ – $75^{\circ}\text{C}$  at a heating rate of  $1^{\circ}\text{C} \cdot \text{min}^{-1}$ . An empty pan was used as a reference to exclude the calorimetric effect of the pan.

#### *FTIR measurements and data analysis*

Thin lipid films were prepared as above, except that 5 mg of DSPC and corresponding amounts of cholesterol were used to

give the 2:1, 5:1 and 10:1 DSPC/cholesterol molar ratio. The thin films were hydrated in 35  $\mu\text{l}$  of PBS, and 20  $\mu\text{l}$  of sample suspensions were placed between  $\text{CaF}_2$  windows using a spacer of 12  $\mu\text{m}$  thickness. Spectra were recorded on a Spectrum One FTIR spectrometer (PerkinElmer, Norwalk, CT, U.S.A.) equipped with a DTGS (deuterated triglycine sulfate) detector. Interferograms were averaged for 50 scans at  $2 \cdot \text{cm}^{-1}$  resolution. The samples were incubated at  $35^\circ\text{C}$ , close to the physiological temperature of  $37^\circ\text{C}$  at which drug-release studies were conducted for 10 min and scanned at that temperature for data acquisition.

The spectra were analysed using Spectrum v5.0.1 software (PerkinElmer). In order to provide a better resolution of the infrared bands, the buffer spectrum at  $35^\circ\text{C}$  was digitally subtracted by flattening the water band located at approx.  $2125 \cdot \text{cm}^{-1}$ . The band positions were measured according to the centre of weight. The subtracted original spectra were used to determine spectral parameters.

### Drug loading and EE (encapsulation efficiency)

MLVs were prepared as described above and a 100  $\mu\text{l}$  portion of them was dried completely under vacuum using a HETO spin vac system (HETO) and redissolved in chloroform. After vortex-mixing, the absorbance was measured in a scanning UV-visible spectrophotometer (Hitachi U-2800A) at 260 nm, corresponding to the wavelength of maximum absorption ( $\lambda_{\text{max}}$ ) of CLX. This  $\lambda_{\text{max}}$  was obtained by scanning the absorbance of the drug between 200 and 900 nm. A calibration curve constructed for CLX in chloroform (range: 10–100  $\mu\text{g}/\text{ml}$ , with the linear equation  $y = 0.0154x + 0.1942$  and  $R^2 = 0.963$ ) was used to quantify CLX in the liposomal preparation to calculate the percentage EE and loading.

The percentage EE was calculated as:

$$\text{EE (\%)} = \frac{(\text{mg of CLX in MLVs})}{(\text{mg of CLX initially added})} \times 100$$

The percentage loading was calculated as:

$$\text{Loading (\%)} = \frac{(\text{mol of CLX})}{(\text{mol of DSPC} + \text{mol of cholesterol in MLV aliquot})} \times 100$$

### Quantification of DSPC in MLVs

The amount of DSPC in MLVs was determined by the Stewart method [28]. Aliquots from disrupted MLV suspensions were mixed with ammonium ferrothiocyanate solution (1:1, v/v) and the absorbance at 485 nm was measured. DSPC was quantified using a standard DSPC calibration curve (range: 5–50  $\mu\text{g}/\text{ml}$ , with the linear equation  $y = 4.5793x + 0.0121$  and  $R^2 = 0.985$ ).

### In vitro drug-release profile

The drug-release profile of MLVs encapsulating CLX was studied by directly mixing 1 vol. of the MLV suspension (125  $\mu\text{l}$ )

with 10 vol. of PBS (1250  $\mu\text{l}$ ). The samples were incubated at  $37^\circ\text{C}$  with gentle agitation and 100  $\mu\text{l}$  aliquots were withdrawn at 6, 12, 24, 48 and 72 h to calculate the CLX and lipid contents. For quantification of CLX, the aliquots were centrifuged at 12000 g for 15 min and their supernatant, containing the released CLX, was removed. The pellets were dried completely under vacuum using a maxi dry lyo system (HETO), and redissolved in chloroform. CLX was quantified by UV spectrophotometry as described above. DSPC was also quantified from the same sample using the Stewart method as described above.

### Statistical analysis

Data analysis and graph preparation were performed using the GraphPad Prism 5 software package (GraphPad Software, La Jolla, CA, U.S.A.). In FTIR studies, unless otherwise mentioned, the mean for at least five experiments was plotted together with the S.E.M. Statistical significance was assessed using the Mann–Whitney non-parametric test. In percentage encapsulation calculations, one-way ANOVA was performed using Dunnett's multiple comparison test with the 'DSPC only' group as a control. In release profiles, one-way ANOVA using Tukey's multiple comparison test was conducted separately for each time point.  $P \leq 0.05$  was considered statistically significant.

## RESULTS AND DISCUSSION

### MLV preparation

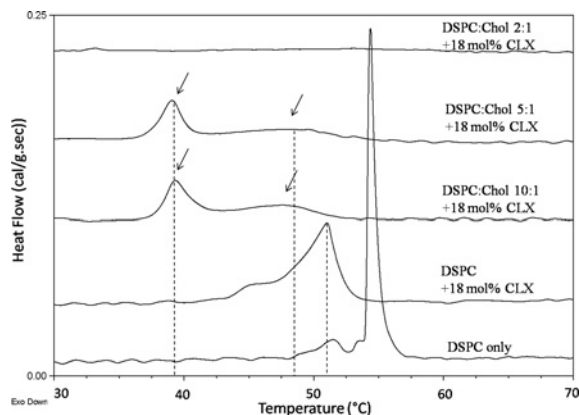
In the current system, lipid analyses indicated that more than 99.5% of the lipids were pelleted after centrifugation of the hydrated films and were therefore incorporated into the MLVs (results not shown).

### Vesicle size

The mean vesicle sizes varied between  $5.45 \pm 0.24$  and  $6.23 \pm 0.13 \mu\text{m}$  (Table 2). Addition of cholesterol at a low ratio to DSPC (1:10) resulted in larger MLVs, but did not have any effect at the highest ratio (1:2). However, the mean sizes were very similar for all groups and the differences were not at significant levels (Table 2).

Vigorous vortex-mixing and brief sonication steps are known to result in the formation of smaller vesicles with more uniform size distribution, although standardizing sonication power and duration is not easy [20]. Therefore it is likely that the small changes in mean diameters may have resulted from the experimental procedures employed in the present study.

As an indication of vesicle stability during release, MLVs were also subjected to size distribution analysis after 72 h of incubation in PBS. The mean vesicle sizes were constant or decreased slightly, but no major difference was observed between the four groups (results not shown).



**Figure 1** DSC thermograms of MLVs containing 18 mol% CLX with different DSPC/cholesterol mole ratios

### DSC studies

The thermal phase-transition behaviour of CLX-loaded MLVs in response to increasing concentrations of cholesterol was monitored with DSC. The phase-transition temperature ( $T_m$ ) of DSPC MLVs in the absence of cholesterol showed a shift from 55 to 51°C with CLX addition (Figure 1). Cholesterol incorporation exerted strong perturbing effects on CLX-loaded liposomes with a gradual decrease in the  $T_m$  of the liposomes with increasing cholesterol concentrations. In addition, an asymmetric broadening of the main phase-transition peak could easily be observed in low-cholesterol-containing groups (DSPC/cholesterol; 10:1 and 5:1). In these groups, the peak split into two signals (indicated by arrows in Figure 1), which could be interpreted as an indication of phase separation and presence of more than one domain in the membrane system [29,30].

It is worth noting that neither CLX nor cholesterol showed this effect individually (Figure 1 and Supplementary Figure S1 at <http://www.bioscirep.org/bsr/030/bsr0300365add.htm>); therefore the changes observed in the endotherms may have resulted from a combined action of both compounds. When comparing the thermal effects of similar amounts of CLX and cholesterol, the differences in the endotherms of DSPC-only liposomes containing 18 mol% CLX and 5:1 DSPC/cholesterol empty liposomes (containing approx. 18 mol% cholesterol) arise from the fact that cholesterol is located in the co-operativity region ( $C_1$ – $C_8$ ), making H-bonds with the carbonyl groups of phospholipids and spanning almost the entire length of the acyl chains [31–33]. CLX, on the other hand, is a much smaller molecule that again locates itself in the co-operativity region (at 18 mol%), but without any H-bond formation with the carbonyl groups [34]. As reported in other studies, hydrophobic interactions between molecules and the aliphatic chains of phospholipids are important in determining the physical properties of a membrane. Many hydrophobic molecules have been shown to induce phase separation, and affect the fluidity (permeability) and order of membranes by their interactions with the aliphatic tails of phospholipids [35–37]. Our

**Table 1** Total transition enthalpies ( $\Delta H_{cal}$ ) of ternary mixtures of DSPC/CLX/cholesterol MLVs containing 18 mol% CLX

Liposome formulation	$\Delta H_{cal}$ (cal/g)
DSPC only	10.96
DSPC/cholesterol 10:1	7.83
DSPC/cholesterol 5:1	6.25
DSPC/cholesterol 2:1	–

previous study has indicated that 18 mol% CLX induces phase separation in DSPC MLVs [34].

The endotherm for the ternary mixture of 10:1 DSPC/cholesterol and 18 mol% CLX consists of a new, sharp and low  $T_m$  (approx. 40°C) component and a broad high- $T_m$  (approx. 50°C) component. The absence of the new 40°C peak in the binary mixtures of either CLX/DSPC or cholesterol/DSPC (Figure 1 and Supplementary Figure S1) implies the presence of a new domain formed by interactions between CLX and cholesterol, which in turn interact with DSPC. This kind of interaction was also suggested for Amphotericin B and ergosterol-containing DPPC vesicles [38]. The sharpness of the new peak at 40°C indicates that this domain is formed of relatively co-operative units [39,40]. Since cholesterol and CLX both prefer to locate themselves in the co-operativity region of the bilayer, the relative increase in the co-operativity of the new peak could be achieved by the deeper penetration of one of the molecules into the hydrophobic core of the membrane. As mentioned above, since CLX (18 mol%) does not make any H-bonds with the phospholipid molecules, it would be a better candidate for this localization.

The broad, higher- $T_m$  component (50°C) of the transition endotherm of the ternary mixture appears to be the summation of different kinds of domains formed by DSPC/CLX and DSPC/cholesterol units, since it encompasses a similar range of temperatures with the binary mixtures of DSPC/CLX and DSPC/cholesterol. The broad, less co-operative nature of this component implies that the interaction of CLX with DSPC is perturbed by the presence of cholesterol and that both molecules are located in the co-operativity region in these domains.

The transition enthalpy approaches zero at DSPC/cholesterol ratio of 2:1; thus the phase transition is almost eliminated (Table 1). At this cholesterol concentration the transition endotherm encompasses a very broad temperature range (39–60°C), which can be thought of as the merging of the sharp and broad components into a single, broader endotherm. It is known that low cholesterol concentrations induce the formation of large domains in membranes and that when the cholesterol concentration increases, the sizes of the domains are reduced and finally become indistinguishable [41]. Therefore, in our case, DSPC/cholesterol ratios of 10:1 and 5:1 create phases similar in nature and at the DSPC/cholesterol ratio of 2:1 a different phase is formed such that the separate domains are not observable. This is also supported by the decrease in the total transition enthalpy of the endotherms with an increase in the amounts of cholesterol (Table 1).

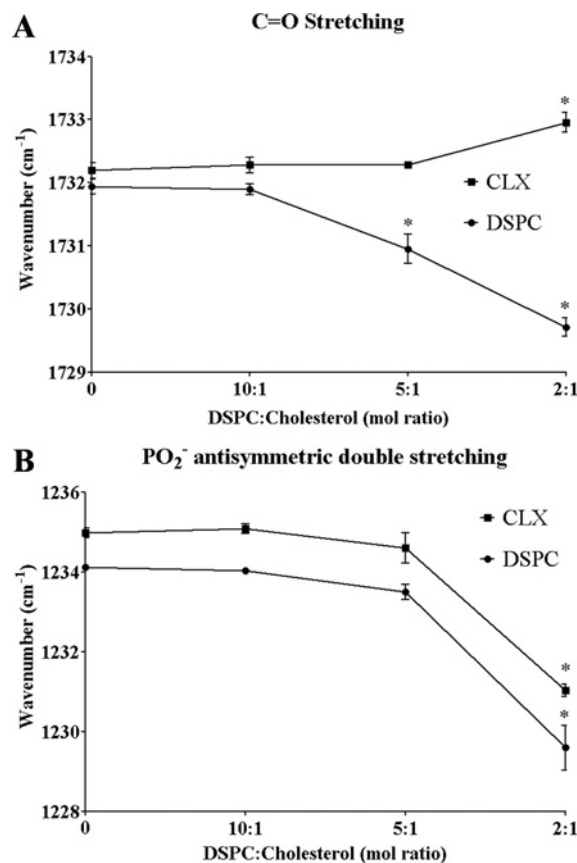
## FTIR studies

Lipophilic and amphipathic compounds that penetrate into the lipid bilayer are reported to be able to modify the physical characteristics of the membranes [29,35,42]. FTIR spectroscopy was therefore used to monitor changes in the liposomal membrane structure by analysing the frequency of different vibrational modes, which represent the headgroup and interfacial region of lipid molecules, as a function of cholesterol concentration [43,44]. Our DSC results showed that the temperature at which the release studies were conducted (37°C) corresponded to the gel phase of the MLVs.

Information about the glycerol backbone and the head group region of the lipids can be monitored by the respective analyses of the carbonyl absorption band at 1735  $\text{cm}^{-1}$  and the  $\text{PO}_2^-$  antisymmetric double stretching band at 1220–1240  $\text{cm}^{-1}$ , which are conformationally sensitive to the level of hydration at these regions [43,44]. In Figures 2(A) and 2(B), the frequency variations in the C=O stretching and  $\text{PO}_2^-$  antisymmetric double stretching bands of pure and CLX-containing DSPC MLVs as a function of cholesterol concentration are given. For DSPC MLVs without added CLX, cholesterol caused a remarkable decrease in the frequency of both regions, which indicates new hydrogen-bond formation around these groups. This may have resulted from an interaction of the C=O and  $\text{PO}_2^-$  groups with either water molecules or the hydroxy group of cholesterol. The possibility of H-bonding between cholesterol and the carbonyl groups is more likely than with the headgroups [31–33,45,46]. According to these results and the existing literature, we propose that the hydroxy group of cholesterol interacts with the carbonyl group of phospholipids and that it increases the amount of water molecules around the headgroup probably by increasing the distance between two headgroups. For CLX-containing liposomes, increasing cholesterol incorporation increased the frequency of the C=O stretching band, indicating the presence of free carbonyl groups in the system (Figure 2A). The frequency of the  $\text{PO}_2^-$  stretching band was higher for DSPC/CLX and DSPC/cholesterol (10:1 and 5:1) compared with pure DSPC (Figure 2B). However, the values started to decrease after DSPC/cholesterol 10:1, reaching a value significantly lower than pure DSPC for DSPC/cholesterol 2:1. This indicates that DSPC/cholesterol 10:1 induces dehydration around the  $\text{PO}_2^-$  groups, the hydration levels start to increase with DSPC/cholesterol 5:1 and maximum hydration occurs at DSPC/cholesterol 2:1. The effect for DSPC/cholesterol 10:1 may result from the displacement of cholesterol with water molecules around the  $\text{PO}_2^-$  group, and the abrupt increase in hydration at DSPC/cholesterol 2:1 may reflect an increase in hydrogen-bond formation between the headgroups and cholesterol or water.

## CLX encapsulation and loading

Increasing the amount of cholesterol in MLVs to 2:1 (DSPC/cholesterol) molar ratio caused a significant reduction in CLX EE (Table 2). The values above 100% were thought to be mainly due to the evaporation of chloroform during spectrophotometric measurements. In accordance with EE results, CLX loading to DSPC/cholesterol liposomes also decreased with an



**Figure 2** Average frequency changes in the C=O and  $\text{PO}_2^-$  stretching modes of DSPC MLVs

Average frequency changes in the (A) C=O stretching mode and (B)  $\text{PO}_2^-$  antisymmetric double stretching mode of DSPC MLVs as a function of cholesterol concentration, in the presence and absence of 18 mol% CLX at 30°C are shown. Each point represents the mean  $\pm$  S.E.M. ( $n = 5$ ). \* $P < 0.05$  compared with controls (no cholesterol).

increase in cholesterol concentrations (Table 2). Thus MLVs prepared with DSPC only had the highest loading of CLX, whereas MLVs having the DSPC/cholesterol molar ratio of 2:1 had the lowest amount of loaded CLX. The theoretical percentage loading where all components are considered to be retained in the MLV structure was 22% (% mol/mol lipid). Hence, the EE of liposomes was found to be dependent on the amount of cholesterol in their structure. In order to compare whether similar EE and loading characteristics were maintained when the liposomes were in the nanometre range, CLX-loaded LUVs (large unilamellar vesicles) in the size range of 116–153 nm were prepared by extrusion through 100-nm-pore size filters at DSPC/cholesterol molar ratios of 5:1 and 10:1 and without cholesterol (results not shown). The percentage loading values were found to be between 23.63 and 25.86%. Statistical analyses performed with percentage EE and percentage loading values of LUVs with varying cholesterol contents showed no significant difference between the LUV groups (one-way ANOVA, Tukey's multiple comparison test,  $P < 0.05$ ).

**Table 2 Effect of cholesterol content on size, encapsulation efficiency (EE) and release of CLX-loaded DSPC/cholesterol liposomes**

MLVs incorporating CLX were prepared with pure DSPC (40  $\mu$ mol), various amounts of cholesterol (DSPC/cholesterol molar ratios of 2:1, 5:1 and 10:1) and CLX (18 mol%). d(0.5) value represents the volume median diameter where 50% of the distribution is above and 50% is below, calculated from the volume distribution. Values for EE and loading denote means  $\pm$  S.E.M. for three independent experiments.  $^{***}P < 0.01$  compared with DSPC-only MLVs.

Liposome formulation	MLV size ( $\mu$ m) d(0.5)	CLX EE (% mg/mg)	CLX loading (% mol/mol lipid)
DSPC only	5.49 $\pm$ 0.13	111.79 $\pm$ 5.24	27.56 $\pm$ 4.77
DSPC/cholesterol 10:1	6.23 $\pm$ 0.13	106.13 $\pm$ 4.74	26.82 $\pm$ 3.07
DSPC/cholesterol 5:1	5.59 $\pm$ 0.99	100.42 $\pm$ 4.34	24.19 $\pm$ 2.15
DSPC/cholesterol 2:1	5.45 $\pm$ 0.24	82.41 $\pm$ 3.96**	21.01 $\pm$ 0.79

At the MLV scale, the most pronounced effect of cholesterol on CLX encapsulation and loading was observed at the DSPC/cholesterol molar ratio of 2:1 (Table 2). However, interestingly, CLX-loaded vesicles with this high cholesterol content (although routinely used as the starting ratio regarding conventional liposome preparations) could not be reduced to nano-sized LUVs via extrusion using standard protocols. Approx. 5–20% of the vesicle population was composed of micrometre-sized liposomes. The resistance encountered during the extrusion process was noticeably higher than in the low-cholesterol groups. The main component of liposomes in the present study was a synthetic saturated lipid DSPC (C<sub>18,0</sub>/C<sub>18,0</sub>), which might have contributed to the rigidity of the membrane structure. However, when empty vesicles with the DSPC/cholesterol ratio of 2:1 (without CLX) were extruded and subjected to size distribution analysis, 96–100% of the LUVs were within the 117–120 nm size range. Therefore the observed inability to form stable LUVs containing CLX and high cholesterol were concluded to be due to the combined effect of the constituents on the membrane bilayer. LUVs of 100–150 nm size, having a higher radius of curvature than MLVs and single phospholipid bilayer to host CLX and cholesterol molecules, might have fused and/or aggregated. It is worth mentioning that, since CLX-loaded LUVs composed of DSPC and cholesterol have not been reported previously, a comparison with published results is not possible.

Several other studies using high cholesterol content have reported lower encapsulation percentages of hydrophobic molecules. MLVs prepared with egg PC (phosphatidylcholine) could encapsulate 29.5% of ibuprofen, while the EE decreased to 23.2% with 30% cholesterol and even to 17.1% with 50% cholesterol addition [19]. Similarly, inclusion of cholesterol in LUVs/small unilamellar vesicles caused lower entrapment of lipid-soluble drugs. For instance, in a study for developing Cremophor-EL free liposomal paclitaxel formulation, DOPC (dioleoyl/phosphatidylcholine)/cholesterol/cardiophilin liposomes were prepared where increasing cholesterol content (from 5 to 37 mol%) resulted in a dramatic decrease in drug loading efficiency from 99.3 to 6.2% respectively [24]. However, in some cases, increasing cholesterol amount in MLVs showed an opposite trend in terms of drug loading. Bhatia et al. [26] reported that 30% cholesterol addition increased the entrapment efficiency of tamoxifen in PC liposomes from 45.2 to 57.5%.

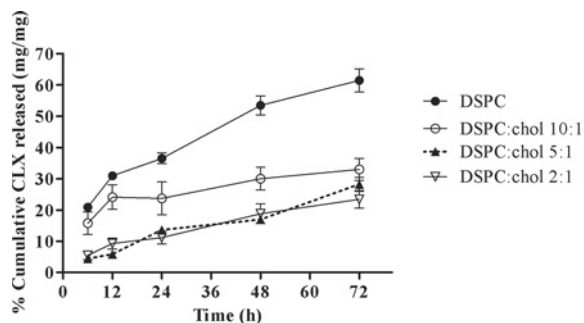
These differential effects of cholesterol on EE of hydrophobic molecules can be attributed to individual molecular interactions

between phospholipids, cholesterol and drug molecules. The fact that cholesterol increases the hydrophobicity in the central region of the membrane bilayer may favour the inclusion of hydrophobic molecules [47]. On the other hand, considering the fact that both cholesterol and the drug prefer to align themselves in the hydrophobic region of the membrane and there is limited space available for both, cholesterol and hydrophobic drug molecules might compete for this space between the acyl chains of phospholipids, resulting in lower encapsulation with increasing cholesterol content.

#### **In vitro CLX release**

Among the four compositions used in the present study, MLVs without cholesterol could release the highest amount of CLX, followed by DSPC/cholesterol groups of 10:1, 5:1 and 2:1 in decreasing order (Figure 3). The latter two groups showed almost equal rates of release. After 72 h of release, DSPC MLVs could retain 39% of their initial CLX, whereas DSPC/cholesterol ratios of 10:1, 5:1 and 2:1 were found to retain 67, 72 and 77% of their CLX content respectively. When the percentages of CLX retained in MLVs were normalized to the lipid content of each sample, DSPC only liposomes were seen to retain remarkably lower CLX (CLX/lipid molar ratio of 11%), whereas other MLVs could retain CLX at higher percentages, supporting the result that the highest extent of drug release occurred in cholesterol-free formulations. The drug release was not in a sudden burst in any of the four MLVs, with the cholesterol-containing MLVs providing a sustained release for even more than 72 h. This slow-release profile of CLX was not achieved in other delivery systems. For instance, a burst effect was observed for chitosan microspheres, releasing almost 50% of CLX in the first hour [13]. Similarly, solid lipid nanoparticles were shown to release approx. 70% of CLX after 72 h [11].

The effect of high cholesterol content on the release behaviour was apparent even at the sixth hour of release where DSPC only and DSPC/cholesterol 2:1 MLVs showed significant difference ( $P < 0.05$ ; see Supplementary Table S1 at <http://www.bioscirep.org/bsr/030/bsr0300365add.htm>). In the following time points, the differences in cumulative amounts of CLX released between DSPC only and the other three cholesterol containing formulations were also significant at different levels, the most noticeable difference ( $P < 0.0001$ ) being at the 72nd hour. Similarly, cholesterol-free liposomal formulations of



**Figure 3** Effect of cholesterol content on the release profile of CLX from liposomes

Drug release in MLVs prepared with 18 mol% CLX and DSPC only (40  $\mu$ mol, filled circles), DSPC/cholesterol (chol) 10:1 (open circles), DSPC/cholesterol 5:1 (filled triangles), and DSPC/cholesterol 2:1 (open inverse triangles) incubated in PBS at 37°C is shown. Values are means  $\pm$  S.E.M. for three independent experiments.

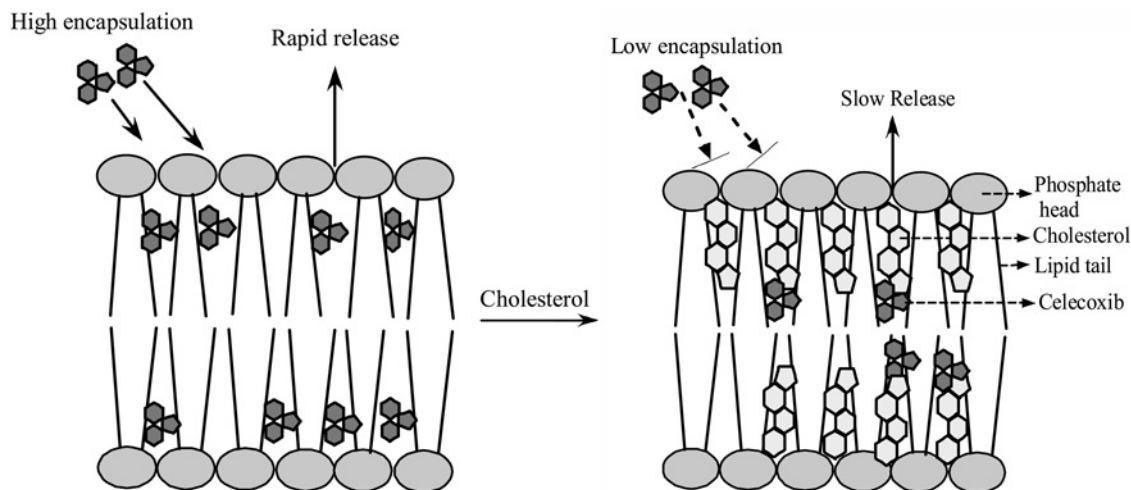
dexamethasone released higher amounts of the drug in 24 and 48 h [48]. CLX released from MLVs with different amounts of cholesterol, however, were not significantly different from each other at the 72 h time point. Cholesterol is normally incorporated at the lipid/cholesterol ratio of 2:1 in liposomal formulations [20]. We have shown here that the cumulative release profile of CLX is similar for MLVs with low cholesterol content as well. Considering the significant reduction in encapsulation at high cholesterol concentrations, a lipid/cholesterol ratio of 10:1 would be suitable for liposomal formulations of CLX.

When the release characteristics of MLVs were compared with those of LUVs containing similar ratios of DSPC and cholesterol, no significant difference was observed between the formulations at 24–72 h of release (results not shown). Evaluation of the overall results made apparent that all liposomes were capable of releasing comparable amounts of CLX during the 72 h

period. These results are an important indicator of the ability of DSPC/cholesterol liposomes to retain CLX, even in the form of LUVs.

MLVs and LUVs capable of a considerable amount of CLX retention after 72 h are encouraging motives for designing PEGylated ‘stealth’ LUVs that can successfully evade the reticuloendothelial system for *in vivo* CLX delivery. The potential of high CLX retention in LUVs will enable delivery of this bioactive agent to the target site at effective concentrations with less liposomal carrier requirement, potentially less hepatotoxicity and with minimum exposure of healthy tissues to CLX during the circulation time of liposomes.

We hereby propose a mechanism for entrapment and release of CLX in MLVs with cholesterol (Scheme 1). Walter et al. [8] have shown, by small angle X-ray diffraction, that CLX (at 9 mol%) was located close to the phospholipids head group region in the upper hydrocarbon core, 5–20 Å from the centre of the membrane. This region is also where the polar part of the rigid head group of cholesterol is located. It is widely accepted that the -OH group in this rigid part of cholesterol is located close to the ester carbonyl group, with H-bonding between these moieties proposed by several investigators [32,45]. The steroid ring and the hydrophobic tail align in the upper hydrophobic part of the membrane parallel with the acyl chain of the phospholipid. This orientation of cholesterol in the membrane causes a remarkable broadening of the phase-transition profile (Figure 1 and Supplementary Figure S1). Recent FTIR studies performed by our group indicated that there is a dose-dependent effect of CLX on the hydration status of the carbonyl groups of DSPC model membranes [34]. In that study, even in the absence of cholesterol, up to 9 mol% CLX was suggested to form H-bonds with the carbonyl group of phospholipids, whereas at 18 mol% CLX was found to be located in the deep interior of the DSPC MLVs. Combining this finding with those of the present study lead us to conclude that cholesterol may locate itself close to the glycerol backbone



**Scheme 1** Effect of cholesterol on the encapsulation and release of CLX from MLVs



of the membrane, confining CLX to the inner core, which may explain the lower release rates with increasing concentrations of cholesterol (Scheme 1).

## CONCLUSIONS

Liposome-encapsulated CLX is a promising system for reducing the overall systemic toxicity, without altering the acute toxicity of the bioactive agent. Administration of liposomal CLX can be via either local site-specific injections or via a 'stealth' delivery system that can be passively targeted to the tumour site owing to enhanced permeability and retention effect [49]. In a slow-release system designed for the intravenous route, the bioactive agent is preferably retained within the carrier and remains in the bloodstream until delivered to a specific site or cleared with the carrier [50]. Therefore higher therapeutic doses can be attained at the desired site of delivery while maintaining minimum exposure of non-target tissues.

In the present study, a highly hydrophobic molecule, CLX, was encapsulated in MLVs composed of DSPC and cholesterol at different molar ratios. The effect of cholesterol content was investigated and increasing amounts of cholesterol were found to reduce EE, loading and drug released from the MLVs. On the other hand, MLVs prepared with DSPC only (without cholesterol) exhibited the most rapid release profile and the lowest drug retention after 72 h.

Since the addition of cholesterol at DSPC/cholesterol ratio of 2:1 provided no advantage in terms of CLX retention and yet resulted in the lowest EE among the four compositions, the present study has provided us with the vital knowledge that vesicles with low amounts of cholesterol are better candidates when designing a liposomal delivery system for CLX. Future studies in our attempts to design a delivery system for CLX will include loading nano-sized PEGylated LUVs with CLX and investigating functional characteristics of drug retention and release properties, cellular uptake kinetics and efficacy as therapeutic and chemopreventive agents *in vitro*.

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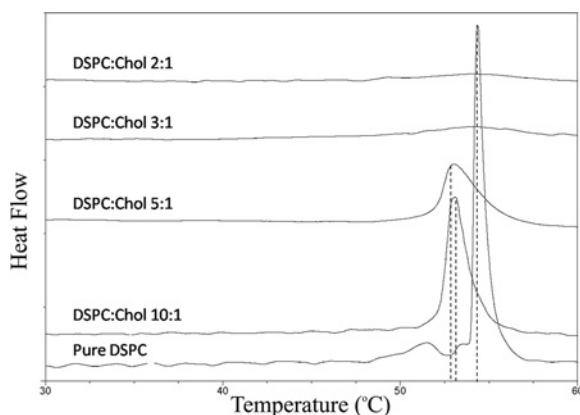
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**SUPPLEMENTARY ONLINE DATA**

# Celecoxib-loaded liposomes: effect of cholesterol on encapsulation and *in vitro* release characteristics

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**Figure S1 DSC thermograms of MLVs containing DSPC/cholesterol molar ratios of 10:1, 5:1, 3:1 and 2:1****Table S1 Statistical analysis by one-way ANOVA to indicate the differences in release of CLX by the different MLV preparations at the different time points**ns, not significant; \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.0001$ .

MLV composition	Statistical analysis				
	6 h	12 h	24 h	48 h	72 h
DSPC versus DSPC/cholesterol 10:1	ns	ns	*	***	***
DSPC versus DSPC/cholesterol 5:1	ns	***	***	***	***
DSPC versus DSPC/cholesterol 2:1	*	***	***	***	***
DSPC/cholesterol 10:1 versus DSPC/cholesterol 5:1	ns	***	ns	*	ns
DSPC/cholesterol 10:1 versus DSPC/cholesterol 2:1	ns	***	*	*	ns
DSPC/cholesterol 5:1 versus DSPC/cholesterol 2:1	ns	ns	ns	ns	ns

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