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Solubilisation of drugs within liposomal bilayers: alternatives to cholesterol as a membrane stabilising agent

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Abstract

Objectives The aim of this work was to investigate the effect of cholesterol on the bilayer loading of drugs and their subsequent release and to investigate fatty alcohols as an alternative bilayer stabiliser to cholesterol.

Methods The loading and release rates of four low solubility drugs (diazepam, ibuprofen, midazolam and propofol) incorporated within the bilayer of multilamellar liposomes which contained a range of cholesterol (0–33 mol/mol%) or a fatty alcohol (tetradecanol, hexadecanol and octadecanol) were investigated. The molecular packing of these various systems was also investigated in Langmuir monolayer studies.

Key findings Loading and release of drugs within the liposome bilayer was shown to be influenced by their cholesterol content: increasing cholesterol content was shown to reduce drug incorporation and inclusion of cholesterol in the bilayer changed the release profile of propofol from zero-order, for phosphatidyl choline only liposomes, to a first-order model when 11 to 33 total molar % of cholesterol was present in the formulation. At higher bilayer concentrations substitution of cholesterol with tetradecanol was shown to have less of a detrimental impact on bilayer drug loading. However, the presence of cholesterol within the liposome bilayer was shown to reduce drug release compared with fatty alcohols. Monolayer studies undertaken showed that effective mean area per molecule for a 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) : cholesterol mixture deviated by 9% from the predicted area compared with 5% with a similar DSPC : tetradecanol mixture. This evidence, combined with cholesterol being a much more bulky structure, indicated that the condensing influence of tetradecanol was less compared with cholesterol, thus supporting the reduced impact of tetradecanol on drug loading and drug retention.

Conclusions Liposomes can be effectively formulated using fatty alcohols as an alternative bilayer stabiliser to cholesterol. The general similarities in the characteristics of liposomes containing fatty alcohols or cholesterol suggest a common behavioural influence for both compounds within the bilayer.

Keywords cholesterol; fatty alcohols; liposomes; low soluble drugs; monolayer studies

Introduction

Despite sustained interest in the field, the formulation and delivery of low solubility drugs remains an unresolved issue. There are a wide range of systems being considered to address this problem including molecular, colloidal and particulate strategies.[1] Amongst these various options, liposomes are potential candidates for enhancing the delivery and targeting of low solubility drugs.

The delivery of drugs by liposomes is not new: first proposed by Gregoriadis[2] in the early 1970s, thousands of studies have been undertaken investigating the application of these systems for the delivery of a range of moieties, from small molecules to delicate proteins through to large nucleic acid therapeutics. Their ability to deliver such a range of drugs is due to their rather unique structure, which is composed of lipid bilayer membranes surrounding an inner aqueous core, allowing both water soluble and hydrophobic drug candidates to be carried within the structure. By loading drug within liposomes, not only can the drug be protected from degradation in the body, the pharmacokinetic and tissue distribution patterns of the drug can be modified and delivery of the drug to the desired site of action may be improved. Therefore liposomes have the advantage of promoting both drug targeting and drug solubilisation if required. There are already several liposomal systems commercially

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available for the delivery of chemotherapeutic and antimicrobial agents including amphotericin B, cytarabine, daunorubicin and doxorubicin. Of these liposome products, the majority entrap their drug payload within the aqueous compartment of the liposomes and the liposomes are exploited to control the pharmacokinetic properties of the drug. Yet despite this attribute, combined with their biphasic nature and ability to solubilise drugs within their bilayers, both the systematic development and clinical application of liposomes as solubilisation agents is limited.

Studies from our laboratory have shown that both the incorporation and release of a sparingly soluble drug (ibuprofen) was influenced by the lipid composition of the liposomes.[3] Phosphatidyl choline (or its derivates) is the main lipid excipient of several commercially available liposome products due to its nontoxic biodegradable profile. In addition to phosphatidyl choline, cholesterol is the main membrane-stabilising material extensively investigated and is present in the majority of liposome products on the market. Cholesterol is widely accepted to improve bilayer stability, with early developmental studies demonstrating that an optimum of 50% mol/mol cholesterol within a liposome formulation increased the stability and reduced the permeability of liposomal bilayers.[4,5] The inclusion of cholesterol has also been shown to broaden and eventually eliminate the cooperative gel-to-liquid phase transition temperature of the phospholipid bilayer.[6] In addition, in some cases vesicles cannot be formed without it. For example, Gopinath *et al*.[7] discovered bilayer vesicles from ascorbyl palmitate could only be formed in the presence of cholesterol. In another example, bilayer vesicles formation from sorbitan esters (nonionic surfactants) was not possible without the inclusion of cholesterol.[8]

Investigating liposomes as solubilising agents, our studies have shown that the presence of cholesterol could reduce drug incorporation efficiency within liposomal bilayers, thereby presenting a counter argument to cholesterol use in liposomal solubilising agents.[3] Therefore, possible alternatives to cholesterol, which could improve drug retention profiles whilst not inhibiting bilayer drug loading, would be advantageous. This study has looked at the effect of cholesterol on drug bilayer loading and aimed to model the release profiles of the drugs from the liposomes. Fatty alcohols as alternatives to cholesterol were considered and the molecular packaging of the various molecules investigated.

Materials and methods

Materials

Ibuprofen, midazolam, diazepam, tetradecanol and octadecanol were purchased from Sigma-Aldrich, Poole, Dorset, UK. Propofol was supplied by MP Biomedicals, LLC, Eschwege, Germany. Egg phosphatidyl choline (PC; grade I), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), and 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), were obtained from Lipid Products, Epsom, Surrey, UK. Hexadecanol was supplied by Fluka Chemie, Bucks, UK. All the chemicals used were of analytical grade and were used without further modification.

Table 1 Summary of key features of the drugs loaded

Drug	Molecular weight (Da)	Water solubility (μg/ml)	log P	pK_a
Ibuprofen	206	60	3.48	4.9
Midazolam	326	240	3.87	6.2
Propofol	178	153	3.84	11.1
Diazepam	285	50	2.99	3.4

Preparation of multilamellar vesicles

Multilamellar vesicles (MLVs) were generated using a technique based on the established film method and modified for low solubility drugs.[3,9] Briefly the lipid entities were dissolved in chloroform : methanol (9 : 1) and the solvent evaporated on a rotary evaporator to yield a dry film as per the standard lipid film hydration method. To entrap drugs within the bilayer, the required amount of drug (1.00 mg; Table 1) was added to the solvent mixture and subsequently hydrated as per the normal hydration method. In all cases, the film was hydrated with 2 ml double distilled water to give a final lipid concentration of 16–24 μmol/ml dependent on formulation. We have previously reported on the formulation of liposomes prepared using this modified method and have shown that liposomes could be effectively prepared with a range of lipid formulations.[3]

Determination of drug loading in liposomes

The drug loading of liposomes was determined by measuring the nonincorporated drug present in the hydration and wash media after separation of liposomes by centrifugation (Beckman J2 centrifuge) at 27 200*g* for 30 min. All samples were diluted enough (with respect to solubility values) to avoid drug precipitation. The drug content of the supernatant was analysed by UV spectroscopy (Unicam Helios) at the appropriate wavelength for each compound (ibuprofen 221 nm; midazolam 258 nm; propofol 268 nm; diazepam 228 nm). This protocol was validated by a direct method established by Fatouros and Antimisiaris,[10] analysing propofol concentration within a randomly selected set of liposome preparations, whereby the resulting pellet was mixed with isopropanol in a 50/50 v/v mixture and solubilised by vortexing, with analysis performed using UV. Similarly, ibuprofen content was validated by HPLC analysis within a random selection of washed liposome preparations.

Determination of vesicle volume distribution and zeta potential

The MLV mean volume distribution was determined by laser diffraction spectroscopy using a Malvern Mastersizer.[3,4] Zeta potential was measured by photon correlation spectroscopy using a Zetaplus (Brookhaven Instruments) in 0.001 M phosphate buffered saline (PBS) at 25°C.

Drug release studies

The release rate of drug was determined by incubating drug-loaded vesicles (after separation of nonincorporated

drug) in 30 ml PBS at 37°C in a shaking (constant; 150 oscillations/min) water bath. Initially, three independent samples each containing 56 μmol liposomes were prepared. Each homogenous suspension was divided into seven batches each containing 8 μmol of the preparation. At time intervals of 0, 2, 4, 8, 24, 48 and 72 h, the medium was centrifuged at 27 200*g* for 30 min. The supernatant was analysed spectrophotometrically at the appropriate wavelength and the amount of drug released was assayed by comparison with a calibration curve for drug in PBS.

Liposome stability studies

Liposomal size and drug retention were used as parameters to preliminarily indicate the physical stability of liposomes. The protocol was adapted from du Plessis *et al*.[11] and Vangala *et al*.[12] The stability of formulations, with respect to retention of the entrapped drug and changes in the size distribution, was determined by incubating vesicles (after separation of the free drug) in 10 ml PBS at 4 and 25°C. Initially, three independent samples of 40 ml MLV containing 36 μmol liposomes were prepared. Each homogenous suspension was divided into four batches each containing 10 ml (9 μmol) of preparation. At time intervals of 0 (immediately after preparation), 7, 14 and 28 day samples were centrifuged to separate loaded from 'free' drug, and supernatants analysed spectrophotometrically at the appropriate wavelength. The amount of drug released was assayed by comparison with a calibration curve for drug in PBS.

Langmuir studies

An automated controlled film balance apparatus (KSV Langmuir Mini-trough, KSV Instruments Ltd, Helsinki, Finland) equipped with a platinum Wilhemy plate and placed on a vibration-free table was used to collect the surface pressure–area isotherms as previously reported.[13] The size of the trough was 24 225.0 mm^2 enclosing a total volume of approximately 220 ml; the subphase was filtered double-distilled water. The compounds (at fixed total concentration of 1 mg/ml) were dissolved in chloroform and 20 μl of each solution was spread onto the air/water interface with a Hamilton microsyringe, precise to $\pm$ 0.2 μl. After spreading, the monolayers were left for 10 min for the chloroform to evaporate. In these studies DSPC was used rather than phosphatidyl choline, as a pure sample of DSPC can be obtained whilst phosphatidyl choline is a mixture of compounds. Thereafter, the molecules underwent constant compression (10 mm/s) until the required surface pressure was attained. Optimisation work revealed that a routine compression speed of 10 mm/min yielded the best isotherms, therefore this speed was maintained throughout all monolayer studies. The temperature of the subphase was kept constant at 20 $\pm$ 1°C by means of an external water bath circulation system. Each experiment was only compressed once and performed at least three times with monolayers prepared from different solutions. KSV software (KSV Instruments Ltd, Helsinki, Finland) was used for data analysis.

Statistical analysis

The treatment of the experimental results was based on the analysis average and the analysis of variance. Thereafter, Bonferroni's multiple *t*-test and Student–Newmann–Keuls test were used to compare the difference between encapsulation efficiency, MLV size and zeta potential of the formulations. Differences were considered significant when $P < 0.05$. Each value was obtained from at least triplicate samples from each case and expressed as mean $\pm$ SD.

Results

Drug loading studies: the effect of cholesterol content

Previously, work from our laboratories had shown that inclusion of cholesterol within the liposomal bilayers had a competitive action on the incorporation of ibuprofen.[3] Therefore, initially, the effect of cholesterol content on ibuprofen was compared with three different drugs (diazepam, midazolam and propofol; chosen as they offer a range of basic and acid drugs, with low solubility and a range of molecular weights to compare with ibuprofen). Various concentrations of cholesterol, ranging from 0 to 33 total molar %, were investigated, whilst keeping the concentration of the phospholipid constant (16 μmol). A drug quantity of 1.00 mg was added to each formulation and encapsulation figures determined as relative percentage to the total stated.

Results in Figure 1 show that the incorporation efficiency varied between the four drugs studied, with drug incorporation being influenced by the drug employed such that in terms of drug loading propofol > ibuprofen > diazepam > midazolam. However, the main purpose of this study was to investigate the effect of cholesterol on drug loading. From Figure 1 it can be seen that there was a trend of decreasing drug loading with increasing cholesterol content. This was most notable for propofol. Of the cholesterol concentrations tested (0, 11, 20, 33 total molar %), liposomes formulated without cholesterol incorporated the highest quantity of propofol (23.9 $\pm$ 1.5% mol/mol; Figure 1). A parallel trend for formulations encapsulating midazolam and diazepam could

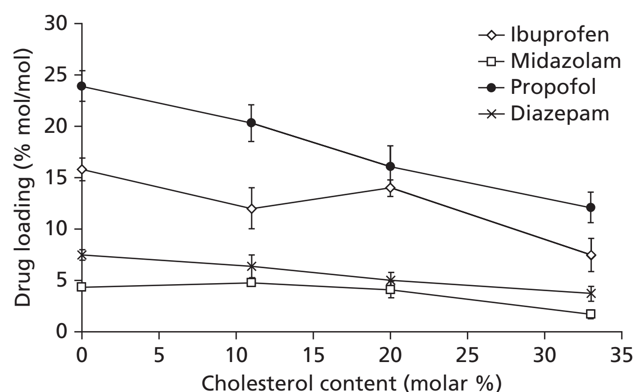

Figure 1 The influence of cholesterol content on entrapment. Liposomes encapsulating 1.00 mg drug were formulated from phosphatidyl choline and varying levels of cholesterol inclusion (0–33 total molar %). Drug encapsulation efficiency within multilamellar vesicles was determined as stated in Materials and Methods. The drug content was analysed by UV spectroscopy at appropriate wavelengths for the given drug. Results are expressed as the means of at least four experiments $\pm$ SD.

be seen (Figure 1). For ibuprofen loading, whilst overall there was a similar trend apparent, there was no significant difference in ibuprofen entrapment between MLV formulated from phosphatidyl choline alone and the MLV containing 11 or 20 total molar % cholesterol. However, increasing the cholesterol content to 33 total molar % resulted in significant reduction ($P < 0.05$) in drug incorporation to 7.5 ± 1.6% mol/mol compared with phosphatidyl choline only MLV. Overall, the encapsulation rate of vesicles formulated without the addition of cholesterol was significantly higher than that of the formulation containing 33 total molar % cholesterol ($P < 0.05$) for each of the four drugs tested.

In terms of vesicle size and surface charge, neither the choice of drug incorporated in the MLV nor the cholesterol content of the MLV formulation made a significant difference (results not shown), with all vesicles being 5–7 μm in size. Similarly the zeta potentials were all within –6 to 6 mV with no significant difference between the formulations (results not shown). This was in line with our previous studies of MLV incorporating ibuprofen.[3]

The effect of liposomal cholesterol content on bilayer drug release kinetics

For therapeutic application, it is important that drugs are retained within liposomes for an appropriate time, and release kinetics may vary depending on the drug delivered, the site of action and the therapeutic application. Among the various factors that dictate the liposomal release of drug, the bilayer composition is an important factor.[14,15] As mentioned, inclusion of cholesterol was shown to reduce bilayer permeability in the case of small water soluble drugs.[16] Therefore, the effect of cholesterol concentration on the release of a bilayer incorporated drug was examined by preparing liposomes containing three concentrations (11, 20 and 33 total molar %) of cholesterol and comparing these to a formulation containing no cholesterol. Propofol was selected as the model drug candidate based on the studies in Figure 1, which showed that propofol had the highest loading efficacy, thereby offering the greatest insight into differing release profiles. Propofol release from the four formulations was studied in physiological buffer, pH = 7.4, at a constant temperature of 37°C.

The results showed a general correlation between release rates and cholesterol content of liposomes with retention values increasing with increasing cholesterol content (Figure 2a). The drug release profile of liposomes prepared from phosphatidyl choline only was characterised by an initial burst effect (more than 40% drug released in first 24 h). Addition of cholesterol to the formulations reduced this burst release, with liposomes containing 11% cholesterol releasing ~25% drug whilst formulations containing 20 and 33% cholesterol releasing intermediate amounts of propofol (between 15 and 20%) in the first 24 h (Figure 2a). After 72 h, approximately 80% of the incorporated drug had released from phosphatidyl choline only liposomes compared with only 40% of drug loaded within phosphatidyl choline : cholesterol (33 total molar %) formulations (Figure 2a).

In terms of modelling drug release, three of the most common kinetic profiles are zero-order, first-order and Higuchi.[17] To determine the mechanism of drug release from the four liposome formulations, the release data (Figure 2a) was treated according to zero-order (cumulative amount of drug released vs time) and first-order (log cumulative percentage of drug released vs time) models, with results presented in Figure 2b and c, respectively.[18] Results in Figure 2b suggested release of propofol from liposomes prepared from phosphatidyl choline only followed zero-order release kinetics (Figure 2b) with good correlation ($R^2 = 0.981$; Figure 2) between cumulative release and time. Increasing the cholesterol content of the

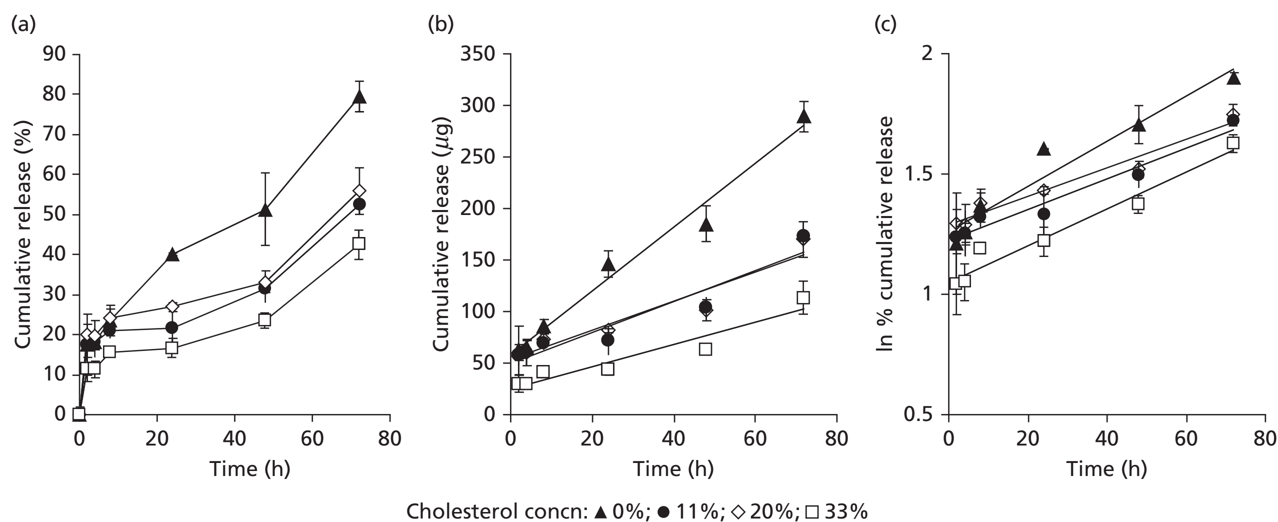

Figure 2 Effect of cholesterol concentration on the release of propofol from liposomes. The cumulative release profile of propofol under physiological conditions from formulations of varying cholesterol inclusion in aqueous buffer, pH = 7.4, at 37°C (a). The data are replotted according to zero-order (b) and first-order (c) models. Propofol was encapsulated and drug release was measured on the basis of un-entrapped propofol recovered in the suspension following centrifugation at 27 200*g* for 30 min at each time-point. Results represent percentage cumulative release of initially incorporated propofol and are expressed as the means of four experiments ± SD.

Table 2 Kinetic values of release of propofol from different liposomal formulations with increasing cholesterol content using the correlation coefficient parameter

Cholesterol content	R^2		
	Zero-order plot	First-order plot	Higuchi model
0	0.9806	0.9393	0.9518
11	0.9119	0.9593	0.8250
20	0.9137	0.9582	0.8091
33	0.914	0.9563	0.8255

Correlation coefficient parameter, R^2. Cholesterol content, total molar %.

liposomes resulted in reduced correlation values (R^2 ~ 0.91) suggesting propofol release from these formulations did not follow zero-order kinetics. However, when the data was plotted according to the first-order model (Figure 2c), liposomes containing cholesterol gave propofol release profiles with improved linearity with R^2 values between 0.956 and 0.959 (Figure 2c). Whilst not a very appropriate model for liposomal drug release, the data was also analysed using the Higuchi model; low correlation coefficients were obtained when the data was plotted as such (R^2 values shown in Table 2), indicating that none of the formulations followed a diffusion controlled matrix release model as previously reported for other hydrophobic drug-loaded liposomal preparations in the literature (acetazolamide and cinchocaine).[19,20]

Fatty alcohols as alternatives to cholesterol: drug loading studies

Given the competitive role of cholesterol in terms of drug loading and its ability to modulate drug release of bilayer loaded drugs, alternatives to cholesterol were investigated. Fatty alcohols are commonly used in lipid-based drug delivery systems including parenteral emulsions and solid lipid nanoparticles.[21] Devaraj *et al.*[14] demonstrated the formation of stable niosomes from fatty alcohols. Based on this previous work, we investigated three fatty alcohols (tetradecanol, hexadecanol, octadecanol; properties summarised in Table 3) in terms of their ability to form liposomes incorporating propofol and compared with liposomes formulated with cholesterol.

Initial investigations looked at the effect of alkyl chain length of the fatty alcohols on drug solubilisation within the bilayer using liposomes formulated from phosphatidyl choline and tetradecanol, hexadecanol or octadecanol, in comparison with previous results for MLVs containing cholesterol (all at a ratio of 89 : 11%; Figure 3a). Investigations of liposomes containing the various fatty alcohols tested revealed similar levels of propofol encapsulation, ranging between ~19 and ~21% mol/mol. There was no significance between the various fatty alcohols tested. Similarly, none of the fatty alcohol formulations had significantly different drug loading compared with their cholesterol containing counterparts (22% mol/mol) (Figure 3a).

The effect of fatty alcohol concentration on bilayer drug loading was then tested by varying tetradecanol concentration from 0 to 33 total molar % in combination with phosphatidyl choline (16 μmol) (Figure 3b). Whereas increasing the cholesterol content was already shown to significantly reduce propofol content, tetradecanol was shown to have a reduced impact on drug bilayer loading (Figure 3b). An increase in cholesterol content from 0 to 33% reduced propofol bilayer loading by ~12% (from 24 to 12%; Figure 3b), whereas with tetradecanol drug loading only reduced by ~4% (from 22 to 18%) over a similar concentration range (Figure 3b). In terms of vesicle size and surface charge no significant difference in vesicle size was observed with the incorporation of propofol or increasing tetradecanol content with all formulations, being 5–6 μm in size with zeta potential values near neutral (results not shown).

The effect of the phospholipid alkyl chain length used in combination with a fatty alcohol (tetradecanol) on bilayer drug loading (propofol) was also investigated. Liposomes prepared from phosphatidyl choline (average C_{12} alkyl chain length) and its derivatives, DMPC (C_{14} alkyl chain length), DPPC (C_{16} alkyl chain length) and DSPC (C_{18} alkyl chain length) were prepared in combination with 11 total molar % tetradecanol or cholesterol (Figure 3c). These studies showed that propofol incorporation increased slightly with increasing lipid alkyl chain length in the order of DSPC > DMPC = DPPC > phosphatidyl choline, with incorporation values increasing from ~20 to 25% mol/mol. A similar trend was generally seen with the equivalent cholesterol-based formulations (Figure 3c).

Fatty alcohols as alternatives to cholesterol: drug release studies

Devaraj *et al.*[14] showed that whilst niosomes containing fatty alcohol exhibited a release pattern similar to that of the cholesterol formulation, the release rates from the fatty alcohol formulations were slower than that of cholesterol formulations. To investigate this with drugs loaded within the bilayer of liposomes, the effect of the particular fatty alcohol on the release profile of propofol from liposome-based systems was examined by preparing liposomes using the alcohol series and comparing them with formulations containing cholesterol.

Cumulative percentage propofol release vs time plots of liposomes are shown in Figure 4a. Initially, the cumulative release rate of propofol from the various formulations was

Table 3 Summary of key features of fatty alcohols used

Fatty alcohol	Synonym	Alkyl chain length	Molecular weight	Molecular formula
Tetradecanol	Myristyl alcohol	14	214	$CH_3(CH_2)_{13}OH$
Hexadecanol	Palmitoyl alcohol, cetyl alcohol	16	242	$CH_3(CH_2)_{15}OH$
Octadecanol	Octadecyl alcohol, stearyl alcohol	18	270	$CH_3(CH_2)_{17}OH$

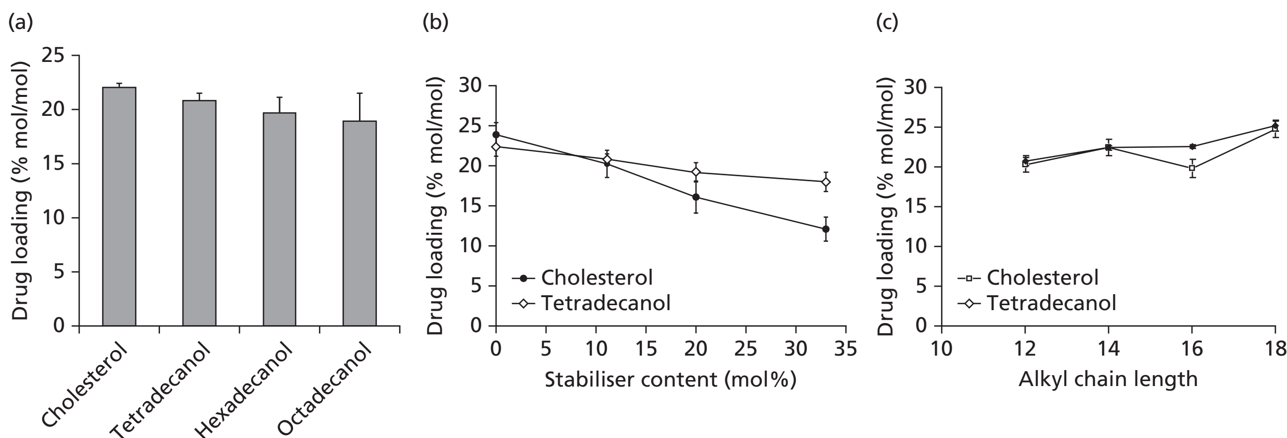

Figure 3 The influence of fatty alcohols on propofol loading in liposomes. (a) The effect of alkyl chain length of fatty alcohol on propofol entrapment with comparison against an equivalent cholesterol-based preparation. Liposomes encapsulating propofol were prepared from 16 μmol phosphatidyl choline and 11 total molar % fatty alcohol or cholesterol. (b) The influence of cholesterol or tetradecanol content (0–33 mol%) on propofol loading and (c) the influence of phospholipid alkyl chain length on propofol entrapment in liposomes containing 11 total molar % fatty alcohol or cholesterol. All formulations were prepared in the presence of 1.0 mg drug. Results are expressed as the means of four experiments ± SD.

not significantly different, with 20–25% propofol released after 12 h. Similar patterns were noted up to 48 h, however by 72 h differences in the formulations became apparent with cumulative release rate decreasing in the order of tetradecanol > hexadecanol > octadecanol > cholesterol. The release data from the fatty alcohol formulation (Figure 4a) was again treated according to zero-order (cumulative amount of drug released vs time; Figure 4b) and first-order (log cumulative percentage of drug released vs time; Figure 4c) models. Unlike the release kinetics of liposomes composed of phosphatidyl choline : cholesterol, no strong correlation was found for the zero-order or first-order release with any of the three liposome formulations prepared with the fatty alcohol replacing cholesterol, nor was there any strong correlation when the data was plotted using the Higuchi model (Table 4).

The longer term stability of these fatty alcohol liposome formulations was tested over a 28-day period and compared with cholesterol formulations. Formulations were stored at either 4 or 25°C and drug retention and particle size monitored at weekly intervals (Figure 5). Propofol retention profiles were similar for the fatty alcohol and cholesterol formulations at 4 or 25°C over the period of the study (Figure 5a and b, respectively). After seven days storage at 4 or 25°C, drug retention dropped to approximately 70–80% for all formulations. At time points thereafter, retention values for all preparations remained constant with no further significant loss of propofol being detected up to day 28 when stored at 4°C, whilst those stored at 25°C showed a slight reduction in drug loading to 65–70%. The mean volume of distribution was monitored with no significant change in either property

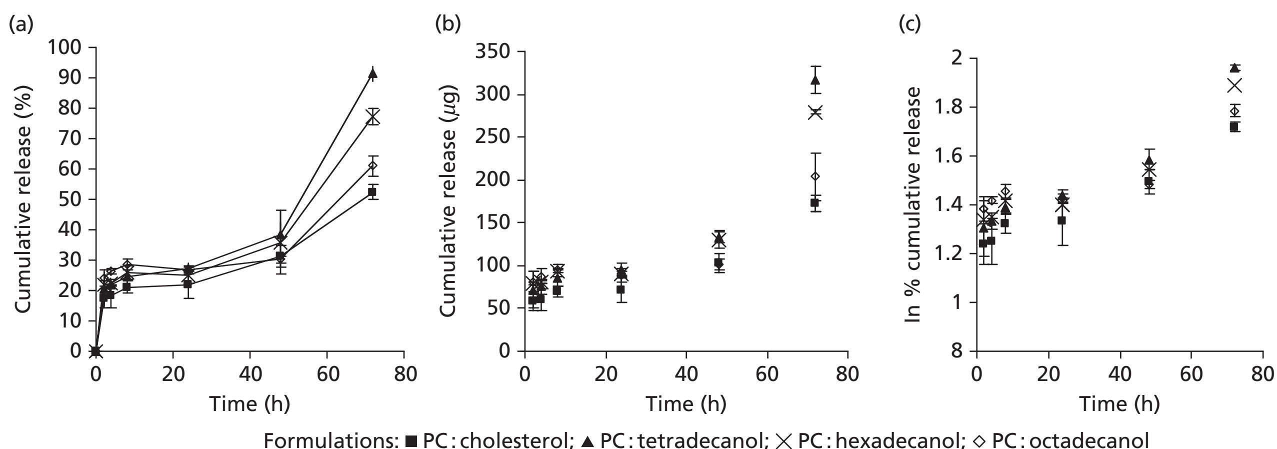

Figure 4 The effect of fatty alcohol alkyl chain length on drug release. Propofol release under physiological conditions from various formulations in aqueous buffer, pH = 7.4, at 37°C. Propofol was encapsulated and drug release was measured on the basis of un-entrapped propofol recovered in the suspension following centrifugation. PC, phosphatidyl choline. Results represent percentage cumulative release of initially incorporated propofol and are expressed as the means of four experiments ± SD.

Table 4 Kinetic values of release of propofol from liposomal formulations containing different stabilisers using the correlation coefficient parameter

Stabiliser	R^2		
	Zero-order plot	First-order plot	Higuchi model
Tetradecanol	0.8301	0.9337	0.8077
Hexadecanol	0.8202	0.8847	0.7940
Octadecanol	0.7274	0.7701	0.7107

Correlation coefficient parameter, R^2.

measured across the range of the study, with vesicles remaining 5–6 μm in size (results not shown).

Langmuir monolayer studies

Langmuir monolayers are a good model system for the lipid – water interface of cell membranes to probe processes at surfaces of membranes yielding phase diagrams of phospholipids in the form of surface pressure/mean molecular area (Π-A) isotherm curves.[22] The technique can be used to thermodynamically analyse interactions between components in mixed monolayers, enabling conclusions to be drawn with respect to both the level of affinity between two components and their molecular arrangement at the air/water interface.[23] For example, studies by Hac-Wydro and Wydro[24] found that a saturated fatty acid (i.e. stearic acid) rendered phospholipid membranes more rigid, while the inclusion of an unsaturated fatty acid (i.e. oleic acid) increased its fluidity. Furthermore, an increasing quantity of the saturated fatty acid gradually destabilised the model membrane whereas in comparison, a small proportion of its unsaturated analogue made the membrane thermodynamically more stable.

Studies were undertaken to compare the geometrical interactions between lipid and fatty alcohol molecules vs those with a cholesterol monolayer. In these studies DSPC was used in place of phosphatidyl choline, as a pure sample of DSPC can be obtained whilst phosphatidyl choline is a mixture of compounds. The compression modulus-area and mean molecular area parameters were used to investigate the interactions at the air/water interface between DSPC and tetradecanol and compared with an equivalent cholesterol-based formulation. The surface pressure–mean molecular area (Π-A) isotherms of one-component films and mixtures of DSPC and tetradecanol or cholesterol at a molar ratio of 89 : 11% are shown in Figure 6, with calculations presented in Table 5. The Π-A isotherm for pure DSPC was in agreement with results collated by Cardenas *et al*.[25] with a molecular area calculated as 51.0 Å^2 per molecule. For cholesterol, the swift, practically linear increase of surface pressure up to the point of collapse beyond 39.5 Å^2 per molecule indicated a closely-packed monolayer structure that exists as a solid-phase during the compression with mean molecular area avoiding radical alterations during the process, similar to studies reported recently.[26]

In the case of surfactant mixtures, all isotherms were found to lie between the ranges of those of the pure components (Figure 6). The measured mean molecular areas for

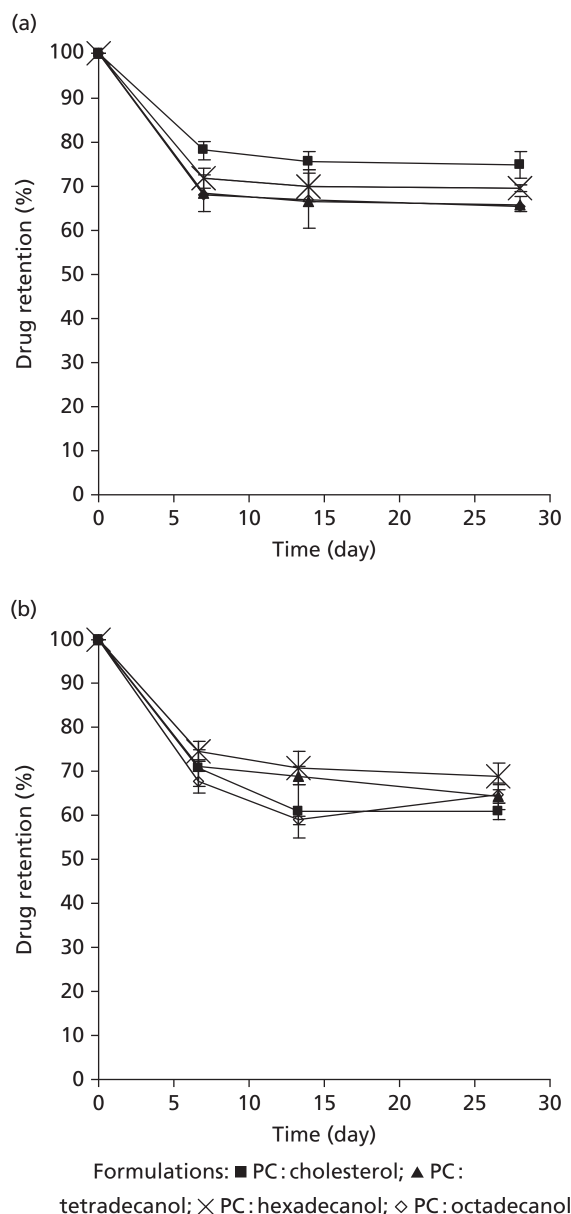

Figure 5 Propofol retention of various vesicle formulations. The various formulations were in in aqueous buffer, pH = 7.4, stored at 4°C (a) and 25°C (b) for 28 days. Propofol was encapsulated and drug retention was measured on the basis of un-entrapped propofol recovered in the suspension following centrifugation at 27 200*g* for 30 min. PC, phosphatidyl choline. Results represent percentage retention of initially incorporated propofol and are expressed as the means of four experiments ± SD.

mixed surfactant monolayers (Table 5) were smaller than the theoretical predicted ones for the ideal mixtures, deviating negatively from the additive rule.[24]

Discussion

From the initial drug loading studies, where the effect of cholesterol was investigated, a trend of increasing molecular

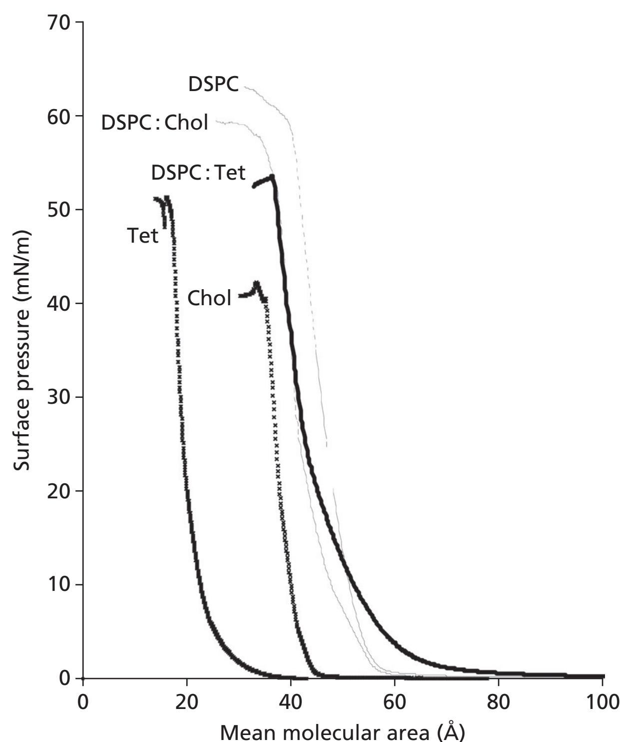

Figure 6 The surface pressure–area isotherms of mixed and pure monolayers at the air/water interface. The air/water interface was at 20°C. The mixed and pure monolayers were of 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), tetradecanol (Tet) or cholesterol (Chol). Results are expressed as the means of three experiments ± SD.

weight with reducing drug loading was noted with the lowest molecular weight drug, propofol (178 Da; Table 1), showing the highest incorporation. However, with the exception of midazolam, a similar pattern could be linked to the lipophilicity of the drug. Therefore, given the small number of drugs tested, it was difficult to draw firm conclusions but there was an indication that molecular weight and lipophilicity will play contributing factors on drug loading within bilayers, as would be expected.

However, what was demonstrated with all of the drugs tested was the competitive action of cholesterol in bilayer loading, as was previously reported with studies on ibuprofen-loaded liposomes.[3] The ability of cholesterol to stabilise liposomes has been well documented: early studies by Gregoriadis and Davis[16] showed that equimolar concentrations of phospholipid and phosphatidyl choline increased the stability and reduced the bilayer permeability of liposomal bilayers. This has been attributed to the increased packing densities of phospholipid molecules and subsequent space-filling action of cholesterol, which is thought to result from cholesterol accommodating within the molecular cavities formed by surfactant monomers assembled into vesicles.[14,27,28] Therefore, it was possible that there was competition for packaging space between cholesterol and the incorporated drug within the bilayer with the bulky nature of cholesterol, when entrapped in the lipid bilayer, reducing bilayer drug loading.

Within Figure 2, the release profile of propofol from liposomes was shown to be influenced by the presence of cholesterol, with its addition to the liposome bilayers stabilising and reducing propofol leakage. Similar conclusions were reported by Hathout *et al.*,[19] who demonstrated that the increase in the molar ratio of cholesterol in the prepared liposomal formulations progressively decreased the release of acetazolamide from the vesicles. However, whilst in a separate study Gopinath *et al.*[7] did not show any consistent relation between release rate of zidovudine and cholesterol, they did reveal a significant reduction in permeability when the content of cholesterol was enhanced from 18 to 45%, therefore indicating a more stable formulation in the presence of the higher cholesterol concentrations.

The higher membrane permeability of cholesterol free liposomes compared with cholesterol-containing formulations could be due to the presence of defects in the former, more fluid, membrane resulting from the absence of cholesterol.[29] The presence of cholesterol and saturated phospholipids is understood to be key factors for reducing membrane permeability of amphipathic drugs.[30] This ability of cholesterol to induce membrane stability is thought to occur via an interaction between the rigid hydrophobic ring structure of the molecule and the alkyl side-chains of phospholipids, which ultimately decreases membrane fluidity.[31] Cholesterol, by increasing the orientation order of the relatively mobile hydrocarbon chains of liquid-crystalline phospholipid bilayers, decreasing bilayer permeability and reducing the efflux of the entrapped drug, resulted in

Table 5 The experimental and ideal extrapolated area and area compressibility of mixed and pure monolayers at the air/water interface

	Extrapolated area at zero pressure (Å^2 per molecule)	Ideal extrapolated area at zero pressure (Å^2 per molecule)	Deviation from ideality (%)	Collapse pressure (mN/m)
DSPC	51.0	–	–	63.2
Cholesterol	39.5	–	–	44.2
Tetradecanol	21.0	–	–	51.8
DSPC : cholesterol (89 : 11%)	45.3	49.7	–8.9	59.3
DSPC : tetradecanol (89 : 11%)	45.2	47.7	–5.2	54.0

Air/water interface was at 20°C. Mixed and pure monolayers were of DSPC, cholesterol or tetradecanol. Results are expressed as the means of three experiments.

prolonged drug retention and, when present in sufficient quantity, it is understood to abolish the gel-to-liquid phase transition endotherm of bilayers e.g. Ohtake *et al*.[6] and Vilcheze *et al*.[32] The presence of cholesterol also appeared to transform the kinetics of drug release from the liposomes from a zero-order for formulations with no cholesterol content stabiliser to a first-order release when cholesterol was present. The zero-order release profile (constant rate of drug release from delivery system) of phosphatidyl choline only formulations possibly reflects its relatively 'porous', more fluidised membrane structure compared with its more condensed and theoretically less permeable cholesterol-containing counterparts.

Given the previously reported potential of fatty alcohols to replace cholesterol in niosomes, their potential in bilayer drug-loaded liposomes was investigated.[14] The more favourable conditions for propofol-encapsulation at higher portions of tetradecanol compared with cholesterol (Figure 3b) could have been due to less competition in packaging in the bilayer between the fatty alcohol and the drug compared with those previously noted with cholesterol.[3] However the choice of phospholipid used in the preparation of liposomes influenced the bilayer loading of a drug independent of the choice of bilayer stabiliser (cholesterol or fatty alcohol; Figure 3c). This increased drug loading could be related to the increased hydrophobic volume and/or hydrophobic bonding offered by the longer alkyl chain lipids, as was shown by work investigating cholesterol and the role of alkyl chain length on the entrapment of water soluble and low solubility drugs.[3,33,34]

In terms of the size and charge of the vesicles, neither the hydrocarbon chain length of fatty alcohol nor the inclusion of cholesterol yielded any significant change in the size or zeta potential of the liposomes, similar to the investigation on niosomes.[14]

Fatty alcohols as alternatives to cholesterol: drug release studies

Whilst the choice of fatty alcohol used in the liposome composition was not shown to significantly influence drug loading, it was shown to influence drug release (Figures 3a and 4, respectively). The data suggested that the longer length of the fatty alcohol alkyl chain length played a key role in bilayer permeability and influenced bilayer incorporated drug release. Formulations containing octadecanol within their composition released propofol at a significantly lower rate when compared with formulations containing tetradecanol or hexadecanol ($P < 0.05$). Given that bilayer drug release is recognised to be influenced by the hydrophobicity of the lipid excipients within the vesicles this could explain the slower drug-release rate for the octadecanol formulation when compared with the shorter chain tetradecanol- and hexadecanol-containing formulations.[3,35] However all fatty alcohol formulations displayed higher drug release than the formulation containing cholesterol, therefore indicating fatty alcohol to be a less effective bilayer excipient than cholesterol in drug retention of propofol. The reduced permeability of cholesterol-containing bilayers compared with fatty alcohol membranes could be due to an enhanced space-filling action offered by cholesterol compared with the fatty alcohols tested.

From the Langmuir monolayers (Figure 6) of lipid interactions with cholesterol and tetradecanol, there was evidence of a strong interaction between DSPC and cholesterol or tetradecanol and a 'condensing effect' by both tetradecanol and cholesterol. The 'condensing effect' of cholesterol has been presented by Devaraj *et al*.,[14] and was attributed to the accommodation of cholesterol in the molecular cavities generated via the assembling of surfactant monomers into vesicles. However, the results obtained in Figure 6 show that effective mean area per molecule for the DSPC : cholesterol mixture deviated nearly 3% more from ideality than the fatty alcohol formulation (−8.9 vs −5.2%, respectively; Table 5). This evidence combined with cholesterol being a much more bulky structure (39.5 Å^2 per molecule for cholesterol compared with 21.0 Å^2 per molecule for tetradecanol; Table 5) indicated that the condensing influence by tetradecanol was less compared with cholesterol. This enhanced intercalation of the DSPC : cholesterol compared with DSPC : tetradecanol could explain the higher release rates of propofol from the tetradecanol liposomes compared with the cholesterol systems (Figure 4) which offer stronger complexing and reducing bilayer permeability.[3,36]

Conclusions

A key aspect in the pharmaceutical application of liposomes as delivery systems for poorly soluble drugs is their efficient and stable encapsulation of such drugs. We have shown that phosphatidyl choline liposomes could be prepared to solubilise low solubility drugs, with the drug solubilisation capacity of the liposomes being dependent on the lipids used and the characteristics of the drug. Liposomes were also effectively formulated from fatty alcohols as an alternative bilayer stabiliser to cholesterol. The general similarities in the characterisation of liposomes containing fatty alcohol and cholesterol suggested a common behavioural influence for both compounds within the bilayer. However, evidence obtained from Langmuir studies suggested that tetradecanol may have a reduced influence within the bilayer compared with cholesterol, manifesting as relatively decreased packing densities of phospholipid molecules.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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