Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis 41 (2006) 476–484
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Analysis of lecithin–cholesterol mixtures using Raman spectroscopy

R. Tantipolphan [a], T. Rades [a], C.J. Strachan [a], K.C. Gordon [b], N.J. Medlicott [a,*]

[a] *School of Pharmacy, University of Otago, P.O. Box 913, Dunedin 9015, New Zealand*
[b] *Department of Chemistry, University of Otago, P.O. Box 913, Dunedin 9015, New Zealand*

Received 7 July 2005; received in revised form 2 December 2005; accepted 16 December 2005
Available online 15 February 2006

Abstract

FT-Raman spectroscopy has been used to investigate interactions between lecithin and cholesterol. Raman spectra of lecithin show multiple peaks which can be classified into three regions: hydrophobic chain, interfacial, and headgroup regions. Binary lipid mixtures (1:1, w/w, lecithin:cholesterol) were prepared by physical mixing, granulation, coprecipitation, hydration and heating (65 °C), and heating (120 °C). Regardless of the preparation method, no changes in the spectra were observed in the hydrophobic region. A shift in the wavenumber of the choline methyl asymmetric stretching mode was observed when the samples were prepared by coprecipitation, hydration and heating (65 °C), and heating (120 °C). This may indicate a modification of phospholipids in the headgroup region in these samples. The difference in degrees of frequency shift (physical mixing ≈ granulation < coprecipitation ≈ hydration and heating (65 °C) < heating (120 °C)) suggests that different levels of hydrogen bonding may have occurred in mixtures prepared with these methods. Multivariate analysis utilizing partial least squares regression based on selected wavenumber ranges was applied for the quantitative analysis of the amount of lecithin in lipid mixtures. Calibration models from physical mixing and heating (120 °C) exhibited lower R^2 and root mean square error of cross validation (RMSECV) values compared to the other models suggesting lower sample homogeneity for these preparation methods. Low values of the mean absolute residues and mean Mahalanobis distances imply that the calibration model generated from physical mixing samples may be appropriate for quantitative analysis of lecithin in lipid mixtures prepared by any of the other techniques.

Keywords: Raman spectroscopy; Lecithin; Cholesterol; Interaction; Partial least squares

1. Introduction

Lipid-based systems such as liposomes and solid lipid particles or implants are of interest in drug delivery research as a means of providing targeted or sustained release of incorporated drugs [1–3]. Their performance with respect to retention and release of incorporated drug may be related to the lipid composition [4] making it important to have techniques to quantify lipid compositions. In cholesterol–phospholipid mixtures, cholesterol is reported to: decrease the temperature, enthalpy, and sharpness of the gel to liquid crystalline phase transition of the phospholipid; fluidize or disorder the gel phase; rigidify or order the fluid lamellar phase; reduce membrane permeability above the main transition temperature; and decrease the average molecular surface area of the phospholipid [5–12]. These effects can be investigated using differential scanning calorimetry (DSC) [7–10,13], infrared (IR) [14–16], Raman spectroscopy [15,17–19], fluorescence anisotropy measurements [12,20,21], nuclear magnetic resonance spectroscopy (NMR) [12], and X-ray diffraction [12,13,22]. In general phospholipid–cholesterol interactions have been interpreted using either "condensed complexes" or "superlattice" models.

The condensed complex model proposes that the nonideal behaviour of phospholipid–cholesterol mixtures (known as the cholesterol condensing effect) originates from the complex formation between the compounds [23]. Recently, the presence of nonideal behaviour of lipid mixtures of cholesterol with distearoylphosphatidylcholine (DSPC), stearoyl-oleoylphosphatidylcholine (SOPC), dioleoylphosphatidylcholine (DOPC), and distearoylphosphatidylethanolamine (DSPE) in monolayers at air/water interface was reported in a study by Dynarowicz-Latka and Hac-Wydro [24]. Deviation of the mean molecular area versus composition of phospholipid–cholesterol mixtures at different constant surface pressures from ideal

* Corresponding author. Tel.: +64 3 479 7275; fax: +64 3 479 7034.
E-mail address: natalie.medlicott@stonebow.otago.ac.nz (N.J. Medlicott).

0731-7085/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2005.12.018

systems was clearly illustrated. A positive deviation was observed in DSPE–cholesterol mixtures while negative deviations were seen in the mixtures of phosphatidycholines and cholesterol. The DSPC–cholesterol system presented the strongest shift towards smaller areas on complexation. The condensed complex model often proposes the existence of a single critical cholesterol/phospholipid stiochiometry [16]. Pandit et al. [25] reported, based on molecular dynamic simulations, that bilayers containing a molar ratio of 40:60 cholesterol:dilauroylphosphatidylcholine (DLPC) predominantly formed 1:1 complexes whereas 40:60 cholesterol:dipalmitoylphosphatidylcholine (DPPC) primarily formed 1:2 complexes.

The superlattice model (or regular distribution model) proposes that different lipid species tend to adopt regular, rather than random distribution in the bilayer [16,26]. Unlike the condensed complex model, the superlattice model proposes a series of critical compositions [16]. Utilizing time-resolved fluorescence and Fourier transform infrared spectroscopic measurements, Cannon et al. [16] reported the formation of a superlattice domain in POPC–cholesterol liposomes at cholesterol mole fractions of 0.2, 0.30–0.33, 0.4, and 0.5. The driving force for superlattice formation was suggested to be due to a reduction in the repulsion force between headgroups and an increase in the packing of the acyl chain of phospholipid upon incorporation of cholesterol [16,26]. Bhattacharya and Halda [12] examined the interactions between cholesterol and various lipids which were selected so that the role of the headgroup and ester linkage in the phospholipid upon interaction with cholesterol could be independently investigated. Regardless of the lipid used, inclusion of cholesterol was shown to immobilise lipid chains in the lamellar phase, reduce the permeability of the membrane, and decrease the bilayer thickness. These studies emphasise an important contribution of hydrophobic interactions in phospholipid–cholesterol interactions [22,27,28].

Raman spectroscopy has been used to characterise phospholipid–cholesterol interactions by monitoring the changes in the shape and frequency of the bands corresponding to molecular vibrations. The samples used to perform the studies are often prepared using the thin-film hydration method. Univariate analysis using the relative peak intensity ratios of suitable bands such as I_{2935}/I_{2880}, I_{1460}/I_{1436}, I_{1130}/I_{1090}, and I_{1720}/I_{1738} has been used to demonstrate alterations in bilayer membranes and to evaluate the spectral regions where these interactions occur [17,20,21,29]. A disadvantage when considering each peak separately is that information contained in multiple peaks may be lost. Interactions between phospholipids and cholesterol have usually been found in spectral ranges, where the peaks associated with atom group vibrations directly involved in the interaction are highly overlapping. For complicated spectra such as these, the use of a multivariate rather than univariate analysis may be favourable since it simultaneously considers multiple spectral features over a wide wavenumber range [30,31].

The objectives of this study were to use Raman spectroscopy to investigate molecular interactions between soybean lecithin and cholesterol in mixtures prepared using different formulation techniques and to evaluate the application of partial least squares analysis (PLS) for the quantitative analysis of lecithin content in lecithin:cholesterol mixtures.

2. Materials and methods

2.1. Materials

Soybean lecithin (Lipoid S100-3, lot 88546-4/10) was purchased from Lipoid GmbH (Germany) and was sieved to obtain particles of less than 63 μm in size. Cholesterol was purchased from Sigma–Aldrich (USA) and was used as received.

2.2. Sample preparation

Binary mixtures of lecithin (0, 20, 40, 50, 60, 80, and 100%, w/w) and cholesterol powders were prepared by one of the following methods: physical mixing, granulation, coprecipitation, hydration and heating (65 °C), and heating (120 °C).

Physical mix samples were prepared by mixing appropriate amounts of lecithin and cholesterol powder by geometric dilution in a beaker using a spatula. Granulated samples were prepared by lightly grinding lipid mixtures in a mortar then mixing with small amount of water (1:0.08, w/w, lipid:water). The granules were dried in an incubator at 37 °C for 48 h and then sieved to obtain particles of less than 710 μm. The granules were then further dried at 37 °C for another 24 h prior to use. Coprecipitation samples were prepared by dissolving various amounts of lipid mixtures in chloroform. The solution was evaporated to dryness at 45 °C for 1 h (Rotavapor R110, Büchi, Switzerland) and the remaining solvent residue was further removed with a stream of dry nitrogen gas for 3 min at room temperature. The film was hydrated with 4 ml water at room temperature. The suspensions were subsequently freeze–dried for 48 h (Freezone 6, Model 79340, Labconco, Missouri, USA) at a condenser temperature of −82 °C and pressure of less than 10^{-1} mbar to obtain a dry powder of the lipid mixtures. Hydration and heating (65 °C) samples were prepared by hydrating lipid mixtures (0.3 g) with 1 ml water to create lipid suspensions. These were subjected to three sequential heat–cool cycles which comprised heating to 65 °C (which is about 10 °C above the transition temperature of fully hydrated lecithin) for at least 30 min then cooling to room temperature over 30 min. The heated suspensions were sonicated and vortexed to ensure thorough mixing at the elevated temperature. The resulting suspensions were freeze–dried using the same conditions as for the coprecipitation samples. Heating (120 °C) samples were prepared by incubating the physically mixed lipids in a 120 °C oven (Contherm Thermotec 2000, Contherm Scientific Ltd., New Zealand) for 10 min then leaving to cool to room temperature in a dessicator overnight prior to use. All samples were individually prepared in triplicate and stored in a dessicator at room temperature until required.

2.3. Raman spectroscopy

Raman spectra were recorded using a FT-Raman instrument consisting of a Bruker FRA 106/S FT-Raman accessory (Bruker Optik, Ettlingen, Germany) with a Coherent Compass 1064-

500N laser (Coherent Inc., Santa Clara, USA) attached to a Bruker IFS 55 FT-IR interferometer, and a D 425 Ge diode detector. Spectral acquisition was carried out at room temperature utilizing a Nd:YAG laser at 1064 nm and a laser power of 150 mW. Samples were packed into an aluminum sample holder and a total of 925 scans were averaged for each sample at a resolution of 4 cm^{-1}. The wavenumber accuracy was monitored using sulfur as reference standard. Spectral analysis and partial least squares (PLS) models were performed using OPUS^{TM} 5.0 (Bruker Optik, Ettlingen, Germany). The observed spectral changes are modest. Thus care was taken to ensure that these observed differences were not caused by calibration drift within the experiment. Data were obtained with a resolution of 4 cm^{-1} and digitization of 1 cm^{-1} per data point. Higher resolution spectra, with increases data point density per wavenumber, did not improve the analysis but did increase acquisition time. The spectral drift was monitored with sulfur during data set acquisition, the peak-to-peak reproducibility was better than 1 cm^{-1}, i.e. 1 data point.

2.4. Quantitative analysis of lecithin/cholesterol mixtures

To construct a calibration model, a total of 21 independent samples were prepared by one of the selected methods to obtain binary mixtures consisting of 0–100%, w/w, lecithin and cholesterol. Calibration models were constructed for each method of preparation by cross validation (leave-one-out). Raman spectra from each set of preparations were first adjusted for scattering differences associated with sample packing and laser intensity fluctuations utilizing multiplicative scattering correction (MSC). The wavenumber ranges were selected to take into account interactions that could occur in the hydrophobic, interfacial, and headgroup regions of the phospholipid. For comparison, all of the models were constructed using the same wavelength ranges and number of PLS factors. In addition to correlation coefficients (R^2), the root mean square error of cross validation (RMSECV) was considered as a criterion to assess linearity and predictive ability of each calibration model:

$$\text{RMSECV} = \sqrt{\frac{1}{M} \cdot \sum_{i=1}^{M} (C_{i,\text{pred}} - C_{i,\text{true}})^2}$$

where $C_{i,\text{pred}}$ is the predicted concentration of sample i, $C_{i,\text{true}}$ the true concentration of sample i, and M is the number of samples. Mahalanobis distance is a measure of similarity between examined spectra and calibration spectra. The algorithm to obtain this parameter is well described in the literature [32,33]. For this study, the Mahalanobis distance values were calculated by the OPUS software. The average of this parameter, when the calibration model constructed from physical mixing samples was used to predict the amount of lecithin present in the lipid mixtures prepared by the remaining methods, and the mean absolute residue was applied to determine the quality of the predicted values. The mean absolute residue is defined as:

$$\text{Mean absolute residue} = \frac{1}{M} \cdot \sum_{i=1}^{M} |C_{i,\text{pred}} - C_{i,\text{true}}|$$

3. Results and discussion

3.1. Raman spectra of cholesterol/lecithin mixtures prepared by physical mixing

Raman spectra of lecithin and cholesterol are shown in Fig. 1. The assignment of the Raman bands is summarized in Table 1. According to the molecular structure of phospholipid, vibrational spectroscopy presents various peaks which can be classified into three regions: the hydrophobic chain consisting of C–H stretching modes, CH_2 deformation, CH_2 twisting, and C–C stretching modes, the interfacial region containing C=O stretching, and the polar headgroup region comprising of a band of PO_2^- asymmetric stretching and C–N stretching [17,20,29,34].

In the lecithin spectrum, the most intense bands at 2881 and 2846 cm^{-1} in the C–H stretching region are assigned to asymmetric and symmetric methylene stretching, respectively [17]. Vibration of terminal methyl groups attributes to a symmetric C–H stretching band at 2932 cm^{-1} [35,36]. Bunow and Levin described the 2959 cm^{-1} line as characteristic of both the methylene asymmetric stretching and the terminal methyl symmetric stretching [36]. The choline methyl asymmetric stretching mode is located at 3035 cm^{-1} [17,20,37]. CH_2 deformation peaks appear at 1456 and 1438 cm^{-1}. The 1295 cm^{-1} line was assigned to the CH_2 twisting mode [38]. The C–C stretching

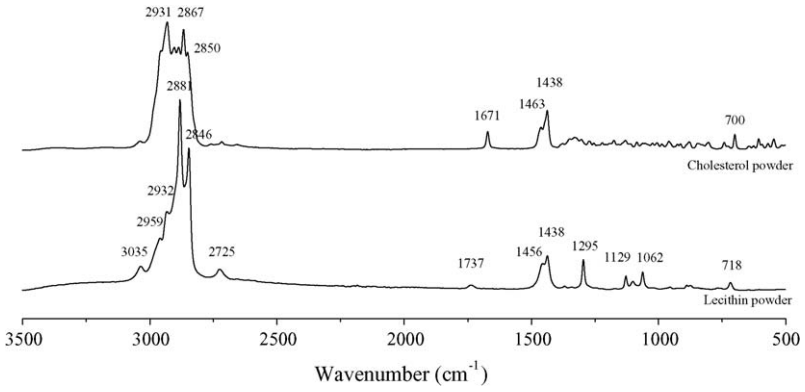

Fig. 1. Raman spectra of lecithin and cholesterol powder.

Table 1
Assignment of Raman bands observed in lecithin and cholesterol powder

Raman bands (cm^{-1})		Assignment
Measurement	Reference	
Lecithin powder		
3035	3039[b]	ν_{asym} $N(CH_3)$ choline
2959	2962[k,l]	ν_{asym} $(-CH_3)$ + ν_{asym} $N(CH_3)$ choline
2933	2932[a,c]	ν_{sym} $(-CH_3)$
2881	2880[c,d,f,I]	ν_{asym} $(-CH_2)$
2846	2845[f]	ν_{sym} $(-CH_2)$
2725	2725[d]	δ_s $(-CH_2)$ + ρ_w $(-CH_2)$
1737	1735[e]	ν (C═O)
1456	1460[f]	δ_d $(-CH_2)$
1438	1436[f]	δ_d $(-CH_2)$
1295	1296[e]	ρ_t $(-CH_2)$
1129	1128[f,j]	ν $(C-C)_{trans}$
1100	1100[j]	ν $(C-C)_{trans}$
1062	1062[f,j]	ν $(C-C)_{trans}$
874	874[a,g]	ν_{sym} $(C-N)_{gauche}$ and δ_d $(-CH_2)$
718	717[a,g,i]	ν_{sym} $(C-N)_{gauche}$
Cholesterol powder		
2931	2936[h]	ν_{sym} $(-CH_2)$
2903	2906[h]	ν_{asym} $(-CH_2)$
2867	2868[h]	ν_{sym} $(-CH_3)$
2850	2850[h]	ν_{sym} $(-CH_2)$
1671	1671[f]	ν (C═C)
1463	1468[h]	δ_d $(-CH_2)$
1438	1444[h]	δ_d $(-CH_2)$
700	700[h]	δ_r $(-CH_2)$

References: (a) Sailer et al. [34], (b) Gaber and Peticolas [40], (c) Schrader [35], (d) Capelle et al. [42], (e) Kint et al. [38], (f) Bush et al. [17], (g) Lhert et al. [43], (h) Faiman [39], (i) O'Leary [29], (j) Levin [20], (k) Bunow and Levin [36], (l) Mendelsohn [18]. *Abbreviations:* ν, stretching vibration; sym, symmetric; asym, asymmetric; δ_d, deformation; δ_s, scissoring; ρ_w, wagging; ρ_r, rocking; and ρ_t, twisting.

modes present three bands at 1129, 1100, and 1062 cm^{-1} corresponding to all-*trans* conformation of the acyl chain [20]. Vibration of the carbonyl group in the interfacial region gives a weak band at 1737 cm^{-1} [38]. In accordance with other studies, the only Raman band found in the headgroup region originates from C–N stretching and appeared at 718 cm^{-1} [18,29].

The spectrum of cholesterol shows two regions of strong bands; the first are C–H stretching modes at 2700–3100 cm^{-1} and the second CH_2 deformation modes which have bands at 1400–1500 cm^{-1} [39]. More specifically, methylene asymmetric stretching is located at 2903 cm^{-1} and symmetric stretching bands appear at 2931 and 2850 cm^{-1} [39]. In addition there is a band at 1671 cm^{-1} which is assigned to the C=C stretch [15,17].

The absence of interactions in phospholipid–cholesterol mixtures prepared by the physical mixing method was demonstrated by Khan and Tucker [9]. They reported the transition peak at 46 °C obtained by DSC analysis of hydrated egg lecithin was not affected by physically mixing lecithin with cholesterol. Therefore, alterations in the spectrum of binary mixtures from the physical mixing samples are likely to originate solely from the different ratios of characteristic peaks from each component in the lipid mixtures at particular concentrations. Raman spectra of binary mixtures of lecithin and cholesterol in the hydrophobic chain and headgroup regions are shown in Fig. 2. Increasing the amount of cholesterol progressively shifts a number of bands to higher wavenumbers; the symmetric CH_2 stretching mode shifts from 2846 to 2850 cm^{-1}, the asymmetric CH_2 stretching mode shifts from 2881 to 2883 cm^{-1}, and the CH_3 choline headgroup mode shifts from 3035 to 3039 cm^{-1}. Shifts to lower wavenumber with increasing cholesterol concentration are also observed; the symmetric C–H stretching of the chain terminal CH_3 at 2933 cm^{-1} shifts slightly to 2931 cm^{-1}. The Raman spectra of phospholipid and cholesterol contain a similar band at 1438 cm^{-1} (CH_2 deformation) which is unshifted with addition of cholesterol.

3.2. Effect of preparation methods of 1:1, w/w, lecithin/cholesterol mixtures on Raman spectra

The preparation methods for lecithin/cholesterol mixtures were selected so that the lipid mixtures were exposed to different levels of mixing, water content, and temperature as these parameters were expected to affect the degree of molecular interaction. Table 2 summarizes the expected relative levels of sample homogeneity, water content and temperature exposure for each of the mixture preparation methods. Granulation was expected to reduce mixing errors compared to physical mixing and result in improved sample homogeneity. The use of water as binder in wet granulation enhances the water content in the mixtures and in turn this could increase interactions in these mixtures. Granulation samples were not heated above the chain melting transition temperature for lecithin, so effects of temperature on the potential for interaction are minimal. Samples prepared by coprecipitation should result in improved sample homogeneity compared with physical mixing and granulation if lecithin and cholesterol recrystallize together from the organic solvent. Lower sample homogeneity would occur if lecithin and cholesterol fractions recrystallized at different rates from the organic solvent. This phenomenon was reported by Huang et al. [22] when thin film hydration or lyophilization methods were used. Moreover, if an excess amount of water is used to hydrate the samples an opportunity for molecular interaction exists. Hydration of lipid films was performed at room temperature so the effects of temperature on the interaction were considered to be similar to the first two methods. Among the selected methods, the hydration and heating (65 °C) method was expected to be

Table 2
Summary of the variables in each preparation method

Preparation method	Homogeneity	Water content	Temperature exposure
Physical mixing	+	+	+
Granulation	++	++	+
Coprecipitation	++ or +++	+++	+
Hydration and heating (65 °C)	+++	+++	+++
Heat (120°)	+	− or +	+++

Abbreviations: homogeneity: +++ = excellent, ++ = very good, and + = good; water content: +++ = added excess water, ++ = added small amount of water, + = no added water, − = loss of water; temperature exposure: + = room temperature or temperature under the phase transition temperature of lecithin and +++ = temperature above the phase transition temperature of lecithin.

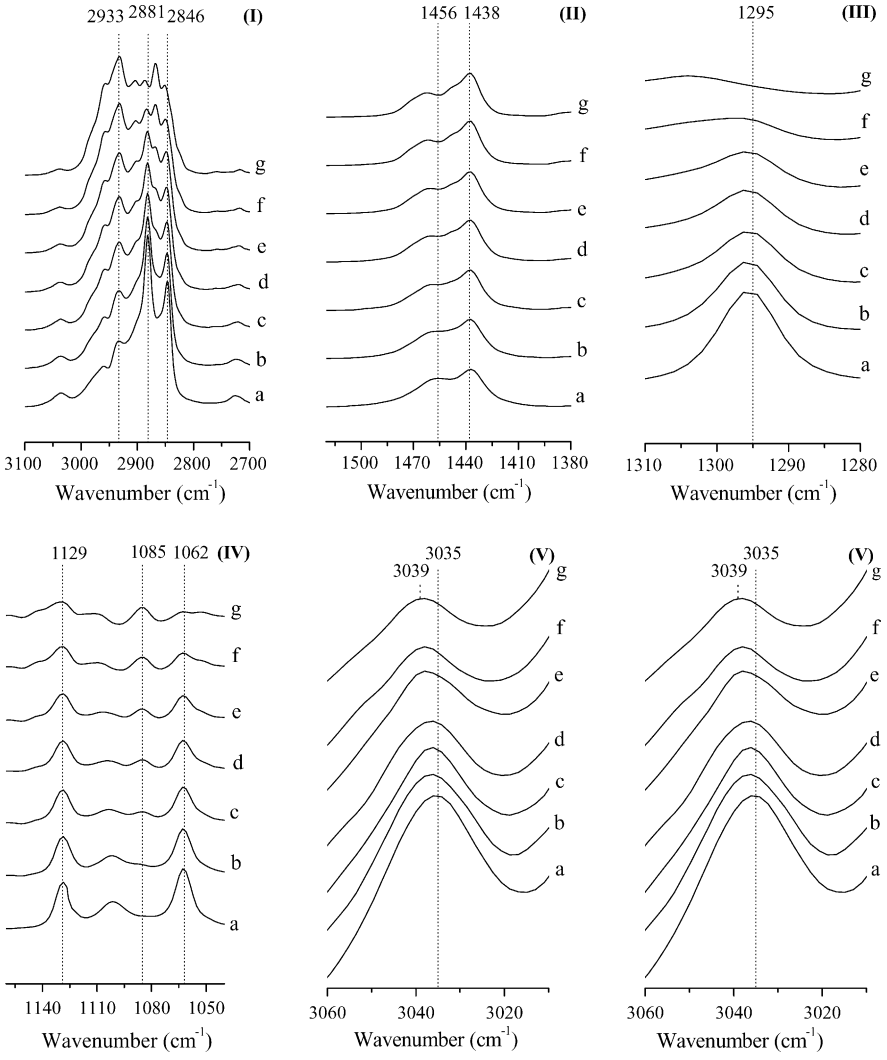

Fig. 2. Raman spectra of lecithin, cholesterol, and lipid mixtures at (I) C—H stretching, (II) CH_2 deformation, (III) CH_2 twisting, (IV) C—C stretching, (V) C—H stretching of the methyl groups at the ammonium terminal of choline headgroup, and (VI) C—N stretching modes; where (a) 100%, w/w, lecithin; (b) 80%, w/w, lecithin; (c) 60%, w/w, lecithin; (d) 50%, w/w, lecithin; (e) 40%, w/w, lecithin; (f) 20%, w/w, lecithin; (g) 0%, w/w, lecithin.

the most likely to create mixtures with high degree of interactions. This is because water was present and the suspended lipid mixture was heated above the chain melting transition temperature of hydrated lecithin. Heating (120 °C) method was also expected to create opportunities for molecular interaction because although water was not added the samples were heated to above the chain melting transition temperature for dry lecithin.

Fig. 3 shows the Raman spectra of the hydrophobic regions for 1:1, w/w, mixtures of lecithin:cholesterol for each preparation method (a–e). In addition, a computed spectrum created by mathematically adding a pure lecithin powder spectrum with a pure cholesterol spectrum was also included in Fig. 3(f). No significant modifications from the computed spectra were seen with any of the preparation methods suggesting cholesterol did not interact with the hydrophobic region of lecithin. C–H stretching modes are highly sensitive to modifications of the acyl chain of the phospholipid. Disruption of regular ordering of phospholipid leads to a decrease in the intensity but an increase in the frequency of the asymmetric methylene stretching band at 2881 cm^{-1} [20,40]. Simultaneously, a rise in the intensity and wavenumber of the 2933 cm^{-1} band is also observed. In addition, a decrease in the intensity of the main CH_2 deformation peak at 1460 cm^{-1} and an increase in the width of this peak into a shoulder of the 1436 cm^{-1} line is often detected upon lattice disordering [17]. Furthermore, the CH_2 twisting band often generates a shift from 1296 to 1300 cm^{-1} together with band broadening upon increasing chain mobility with decreasing conformational order [38]. Contrary to our results, Bush et al. [17] reported a slight increase in the intensity of the 2938 cm^{-1} line associated with C–H stretching and a decrease in the intensity of 1460 cm^{-1} band associated with CH_2 deformation mode on increasing cholesterol concentrations in anhydrous DPPC up to 30 mol% at ambient temperature. However, consistent with our results they reported C–C stretching modes at 1130 and 1062 cm^{-1} were unchanged for anhydrous DPPC–cholesterol mixtures of cholesterol [17]. The authors concluded that the addition of up to 30 mol% cholesterol to anhydrous bilayers

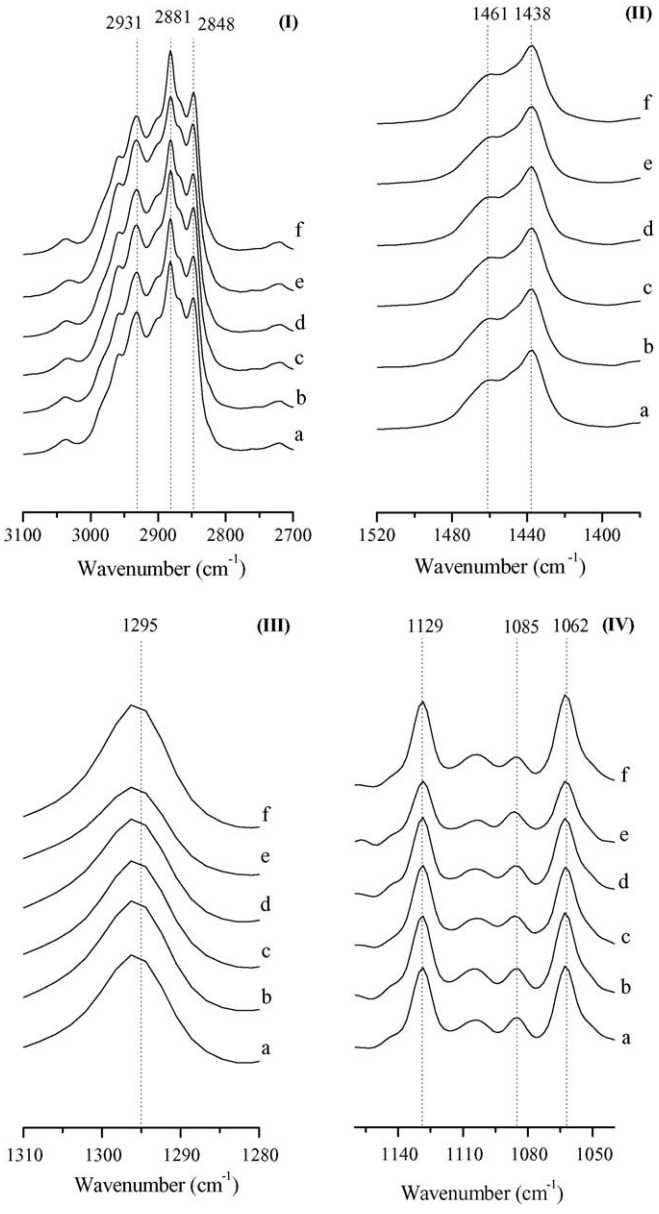

Fig. 3. Raman spectra of lecithin and cholesterol mixtures (1:1, w/w) at (I) C—H stretching, (II) CH_2 deformation, (III) CH_2 twisting, (IV) and C—C stretching modes. (a) Physical mixing; (b) granulation; (c) coprecipitation; (d) hydration and heating (65 °C); (e) heating (120 °C); (f) computed spectrum.

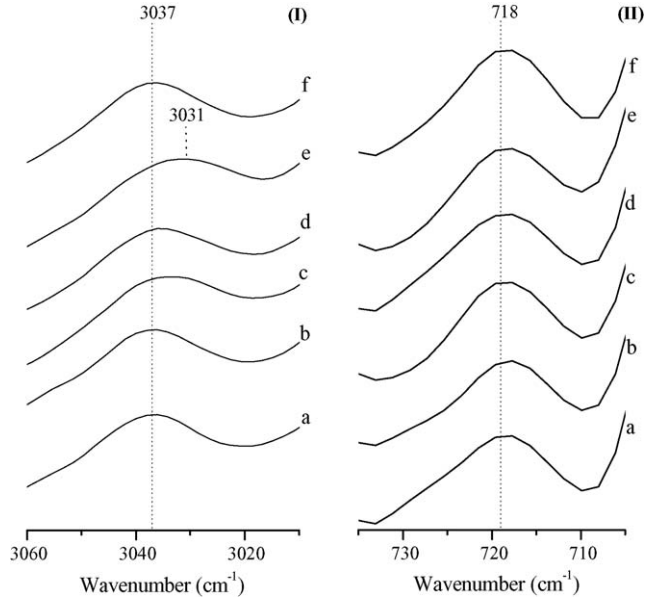

Fig. 4. Raman spectra of lecithin and cholesterol mixtures (1:1, w/w) at headgroup regions: (I) C—H stretching of the methyl groups at the ammonium terminal of choline headgroup and (II) C—N stretching modes. (a) physical mixing; (b) granulation; (c) coprecipitation; (d) hydration and heating (65 °C); (e) heating (120 °C); (f) computed spectrum.

alters lateral chain interaction without developing *trans*/*gauche* isomerisation.

Fig. 4 shows the regions of phospholipid headgroup vibrations in the Raman spectra. The most obvious spectral shift as a function of lipid composition involves the 3037 cm^{-1} choline headgroup CH_3 stretch (Fig. 4(I)). The wavenumber of this band is found at 3037 cm^{-1} in the spectra of physical mixing (a), granulation (b), and computed (f) spectra. However in co-precipitation (c) hydration and heating (65 °C) (d), and heating (120 °C) (e) samples this band shifts to 3034, 3035, and 3031 cm^{-1}, respectively. These shifts were observed in measurements made on three independently prepared mixtures and may suggest a conformational modification of phospholipid headgroup. However, the absence of change in the C–N stretching band at 718 cm^{-1} (Fig. 4(II)) indicates that such an alteration is not associated with this atom group.

Although the main driving force of phospholipid and cholesterol interactions is thought to be hydrophobic interactions, the possibility of an interaction via direct and indirect hydrogen bonding between hydroxyl group of cholesterol and polar groups of phospholipid has been demonstrated [14,25]. The insertion of cholesterol by placing its hydroxyl group close to the ester carbonyl group of phospholipid and its sterol tetracyclic ring extending towards the hydrophobic region of phospholipid theoretically permits hydrogen bonding [5,41]. Considering the species of molecules presented in the lipid mixtures (phospholipid, cholesterol, and water), the potential modes of hydrogen bonding are: (1) a direct hydrogen bond between cholesterol hydroxyl group and any oxygen in a lecithin molecule (carbonyl or phosphate oxygen) [6,25], (2) an indirect hydrogen bond between two molecules (two phospholipids, two cholesterols or phospholipid and cholesterol) via a water bridge [6,25], and (3) a hydrogen bond [25] or charged pair interaction [6] between the hydroxyl group of cholesterol and the methyl group of the choline moiety (Fig. 5). In the preparations to which no water was added, the importance of hydrogen bonding via a water bridge cannot be eliminated at this stage owing to the fact that the crystalline structure of phospholipid often contains a few molecules of water strongly bound to the headgroup region. Since the carbonyl and phosphate vibrations do not give adequate Raman signals, no interpretation regarding the hydrogen bonding to these modes could be obtained in this study. Recently, computer modeling studies have suggested hydrogen bonding between the methyl group of the choline moiety of the phospholipid and the hydroxyl oxygen of cholesterol play an important role in phospholipid–cholesterol interactions [25]. The red shift

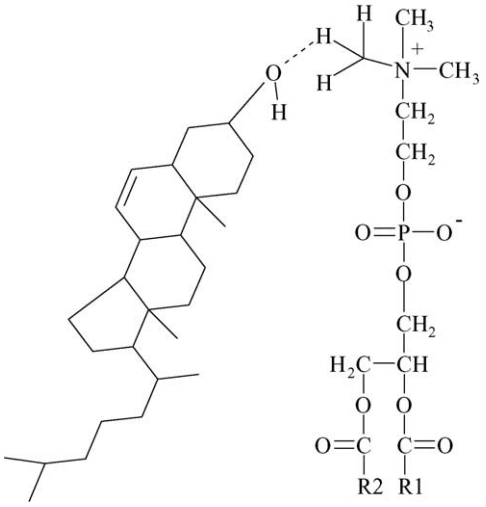

Fig. 5. Schematic drawing illustrating a possible hydrogen bonding leading to an elongation of the C—H bond and, consequently, resulting in a lowered C—H stretching frequency. R_1 and R_2 are referring to CH_2— hydrocarbon chains at positions 1 and 2 of the glycerol backbone of lecithin.

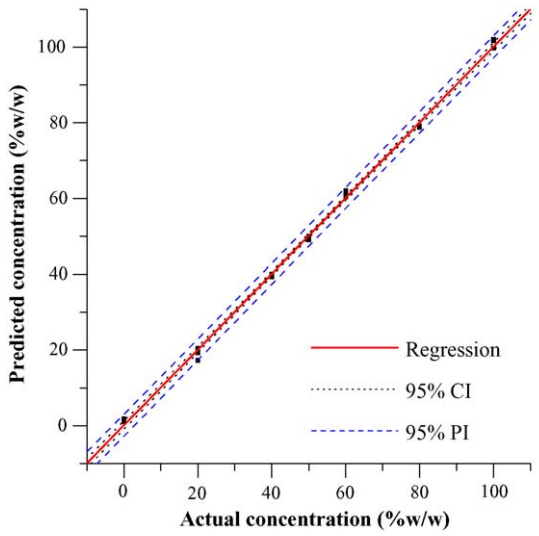

Fig. 6. Actual vs. predicted concentration of lecithin from binary lecithin/cholesterol mixtures prepared by the physical mixing method.

we observed in the choline methyl group vibration in Fig. 4(I) for formulations where lecithin and cholesterol were intimately mixed (spectra c, d, and e) gives experimental evidence to suggest that this group is involved.

In conclusion, no considerable difference in the spectra in the hydrophobic region was detected by visual observation of the spectra obtained from different methods of lecithin/cholesterol preparations. However slight increases in the frequency of methyl groups at choline headgroup vibration were observed. Progressive decrease in the wavenumber may imply increasing degrees of interaction occurred at the headgroup region in the following order: physical mixing ≈ granulation < coprecipitation ≈ hydration and heating (65 °C) < heating (120 °C).

3.3. Quantitative analysis of lecithin–cholesterol mixtures

A representative PLS regression with 95% confidence prediction intervals of actual versus predicted concentration of lecithin from physical mixing samples is given in Fig. 6. The plot illustrates that there is a well defined relationship between the actual and predicted concentrations over the entire concentration range. Similar results were obtained when the regressions were generated using the data from the other preparation methods. A summary of the calibration models is shown in Table 3. Calibration models from physical mixing and heating (120 °C) methods exhibited lower R^2 and higher RMSECV parameters compared to the other methods. This implies the calibration models from granulation, coprecipitation, and hydration and heating (65 °C) samples have less variability compared to those of physical mixing and heating (120 °C) methods. As mentioned previously (Table 2) both physical mixing and heating (120 °C) methods could suffer from poor mixing, and this could partly be responsible for a higher variability in their calibration models.

Using the calibration model built from physically mixed samples, multivariate analysis was used to predict lecithin concentrations from spectra obtained by different preparation methods (granulation, coprecipitation, hydration and heating (65 °C), and heating (120 °C)). Table 4 reports the mean absolute residual and the mean Mahalanobis distances. The low absolute residuals

Table 4
A summary of the assessment of quality of predicted values when the calibration from physical mixing samples was used to predict lipid mixtures prepared by the other methods

Data	Mean absolute residual ± S.D.	Mean Mahalanobis distance ± S.D.
Physical mixing	0.70 ± 0.45	0.10 ± 0.08
Granulation	0.81 ± 0.70	0.13 ± 0.16
Coprecipitation	2.72 ± 1.16	0.32 ± 0.33
Hydration and heating (65 °C)	2.68 ± 1.12	0.23 ± 0.20
Heating (120 °C)	2.78 ± 1.49	0.22 ± 0.23

Table 3
Characteristics of the various calibration models for lecithin–cholesterol mixtures

Data	Wavenumber ranges	Number of PLS factors	R^2	RMSECV
Physical mixing	3061.2–2798.8 and 1780.4–1018.6	2	99.85	1.22
Granulation	3061.2–2798.8 and 1780.4–1018.6	2	99.93	0.81
Coprecipitation	3061.2–2798.8 and 1780.4–1018.6	2	99.95	0.69
Hydration and heating (65 °C)	3061.2–2798.8 and 1780.4–1018.6	2	99.94	0.79
Heating (120 °C)	3061.2–2798.8 and 1780.4–1018.6	2	99.73	1.65

and mean Mahalanobis distances suggest the model generated from the physical mix standards adequately describes mixes prepared by any of the other techniques. Therefore, this model which has considerably simpler sample preparation may be used for the quantitative analysis of lecithin in the lipid mixtures. Nonetheless, higher mean Mahalanobis distances were obtained when the samples were prepared by coprecipitation, hydration and heating (65 °C), and heating (120 °C) methods indicating some dissimilarity between the spectra obtained from these procedures and those of physical mixing samples. A plausible reason for the difference in the spectra between physical mixing/granulation methods and coprecipitation/hydration and heating (65 °C)/heating (120 °C) methods could originate from higher levels of interactions between lecithin and cholesterol in these three mixtures. Considering the granulation method, this technique is very similar to physical mixing apart from an addition of a small amount of water in to the mixtures. The small amount of the added water at room temperature may not be sufficient to induce a phase change in lecithin. Even though the hydration of lipid films in the coprecipitation method was also done at temperatures lower than the transition temperature, the lipid powders while co-dissolving in chloroform might develop a certain degree of interaction. This could lead to a higher degree of interactions in this set of lipid mixtures compared to physical mixing and granulation. The interactions between lecithin and cholesterol in the samples prepared by hydration and heating (65 °C), and heating (120 °C) methods are even higher, since the incorporation of cholesterol into lecithin happened while the hydrocarbon chain of lecithin was in the melted state. This is consistent with the Raman spectral changes that were observed in the phospholipid headgroup regions.

4. Conclusions

- Physical mixing of lecithin and cholesterol powders resulted in a modification of Raman spectra corresponding to the amount of each component in the mixtures.
- No considerable changes in the spectra of (1:1, w/w) lecithin/cholesterol mixtures prepared by different methods were observed in the hydrophobic regions.
- The red shift of the choline methyl stretching, when the mixtures were prepared by coprecipitation, hydration and heating (65 °C), and heating (120 °C), may indicate a modification of phospholipids in the headgroup region. Different degrees of frequency shift (heating (120 °C) > hydration and heating (65 °C) ≈ coprecipitation > granulation ≈ physical mixing) could come from different levels of hydrogen bonding occurring from the use of different preparation methods.
- The calibration models suggest that multivariate analysis employing PLS regression can be used as an alternative to conventional univariate analysis in quantitative analysis the amount of lecithin in lipid mixtures.
- Physically mixed standards could be used to build a model for prediction of the lecithin content of lecithin:cholesterol mixtures. Calculation of the mean absolute residue and mean Mahalanobis distance of predicted concentrations provides a method to monitor interactions in mixtures prepared by different formulation techniques.

Acknowledgements

The authors would like to thank the School of Pharmacy, University of Otago for a Ph.D. stipend for Ruedeeporn Tantipolphan and University of Otago for a University of Otago Research Grant.

References

[1] A. El-Aneed, J. Control. Release 94 (2004) 1–14.
[2] G.F.A. Kersten, D.J.A. Crommelin, Vaccine 21 (2003) 915–920.
[3] M. Singh, D.T. O'Hagan, Pharm. Res. 19 (2002) 715–728.
[4] M. Anderson, A. Omri, Drug Deliv. 11 (2004) 33–39.
[5] A.M. Smondyrev, M.L. Berkowitz, Biophys. J. 77 (1999) 2075–2089.
[6] M. Pasenkiewicz-Gierula, T. Rog, K. Kitamura, A. Kusumi, Biophys. J. 78 (2000) 1376–1389.
[7] C. Huang, S. Li, Biochim. Biophys. Acta 1422 (1999) 273–307.
[8] T.P.W. McMullen, R.N. McElhaney, Biochim. Biophys. Acta 1234 (1995) 90–98.
[9] M.Z. Khan, I.G. Tucker, Chem. Pharm. Bull. 40 (1992) 3056–3061.
[10] T.P.W. McMullen, R.N.A.H. Lewis, R.N. McElhaney, Biophys. J. 79 (2000) 2056–2065.
[11] A.M. Smondyrev, M.L. Berkowitz, Biophys. J. 80 (2001) 1649–1658.
[12] S. Bhattacharya, S. Haldar, Biochim. Biophys. Acta 1467 (2000) 39–53.
[13] J.B. Finean, Chem. Phys. Lipids 54 (1990) 147–156.
[14] P.T.T. Wong, S.E. Capes, H.H. Mantsch, Biochim. Biophys. Acta 980 (1989) 37–41.
[15] S.F. Bush, H. Levin, I.W. Levin, Chem. Phys. Lipids 27 (1980) 101–111.
[16] B. Cannon, G. Heath, J. Huang, P. Somerharju, J.A. Virtanen, K.H. Cheng, Biophys. J. 84 (2003) 3777–3791.
[17] S.F. Bush, R.G. Adams, I.W. Levin, Biochemistry 19 (1980) 4429–4436.
[18] R. Mendelsohn, Biochim. Biophys. Acta 290 (1972) 15–21.
[19] J.L. Lippert, W.L. Peticolas, Proc. Natl. Acad. Sci. U.S.A. 68 (1971) 1572–1576.
[20] I.W. Levin, in: R.J.H. Clark, R.E. Hester (Eds.), Advances in Infrared and Raman Spectroscopy, Wiley, Heyden, New York, 1987, pp. 1–48.
[21] M.M. Batenjany, L.T. Boni, Y. Guo, M.E. Neville, S. Bansal, R.J. Robb, M.C. Popescu, Biochim. Biophys. Acta 1514 (2001) 280–290.
[22] J. Huang, J.T. Buboltz, G.W. Feigenson, Biochim. Biophys. Acta 1417 (1999) 89–100.
[23] H.M. McConnell, A. Radhakrishnan, Biochim. Biophys. Acta 1610 (2003) 159–173.
[24] P. Dynarowicz-Latka, K. Hac-Wydro, Colloid Surf. B 37 (2004) 21–25.
[25] S.A. Pandit, D. Bostick, M.L. Berkowitz, Biophys. J. 86 (2004) 1345–1356.
[26] P. Somerharju, J.A. Virtanen, K.H. Cheng, Biochim. Biophys. Acta 1440 (1999) 32–48.
[27] J. Huang, G.W. Feigenson, Biophys. J. 76 (1999) 2142–2157.
[28] J. Huang, Biophys. J. 83 (2002) 1014–1025.
[29] T.J. O'Leary, in: L. Finegold (Ed.), Cholesterol in Membrane Models, CRC Press, Boca Raton, Fla., 1993, pp. 175–195.
[30] T. Iliescu, M. Baia, V. Miclaus, Eur. J. Pharm. Sci. 22 (2004) 487–495.
[31] M. Prochazka, J. Stepanek, P.Y. Turpin, Chem. Phys. Lipids 132 (2004) 145–156.
[32] H. Mark, in: D.A. Burns, E.W. Ciurczak (Eds.), Practical Spectroscopy, M. Dekker, New York, 2001, pp. 363–400.
[33] R.G. Whitfield, M.E. Gerger, R.L. Sharp, Appl. Specstrosc. 41 (1987) 1204–1213.
[34] K. Sailer, S. Viaggi, M. Nusse, Biochim. Biophys. Acta 1329 (1997) 259–268.
[35] B. Schrader, Infrared and Raman Spectroscopy: Methods and Applications, Weinheim, New York, 1995.
[36] M.R. Bunow, I.W. Levin, Biochim. Biophys. Acta 487 (1977) 388–394.

[37] J. Grdadolnik, D. Hadzi, Spectrochim. Acta A Mol. Biomol. Spectrosc. 54A (1998) 1989–2000.
[38] S. Kint, P.H. Wermer, J.R. Scherer, J. Phys. Chem. 96 (1992) 446–452.
[39] R. Faiman, Chem. Phys. Lipids 18 (1977) 84–104.
[40] B.P. Gaber, W.L. Peticolas, Biochim. Biophys. Acta 465 (1977) 260–274.
[41] H. Ohvo-Rekila, B. Ramstedt, P. Leppimaki, J.P. Slotte, Prog. Lipid Res. 41 (2002) 66–97.
[42] F. Capelle, F. Lhert, D. Blaudez, H. Kellay, J.M. Turlet, Colloid Surf. A 171 (2000) 199–205.
[43] F. Lhert, F. Capelle, D. Blaudez, C. Heywang, J.M. Turlet, J. Phys. Chem. B 104 (2000) 11704–11707.