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Synthesis, liposomal formulation and thermal effects on phospholipid bilayers of leuprolide

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Abstract: A novel liposomal formulation was developed for the encapsulation of the oligopeptide leuprolide (GlpHisTrpSerTyr-D-LeuLeuArgProNHEt), a potent analogue of gonadotropin releasing hormone used in the treatment of advanced prostate cancer, endometriosis and precocious puberty. Leuprolide was synthesized using solid phase methodology on a {3-[(ethyl-Fmoc-amino)-methyl]-1-indol-1-yl}-acetyl AM resin and Fmoc/*t*Bu chemistry. The new liposomal formulation, called 'liposomes in liposomes' is composed of egg phosphatidylcholine : dipalmitoylphosphatidylglycerol in a molar ratio of 98.91 : 1.09 (internal liposomes) and egg phosphatidylcholine : dipalmitoylphosphatidylglycerol : cholesterol in a molar ratio of 68.71 : 0.76 : 30.53 (external liposomes). It offers high encapsulation efficiency (73.8% for leuprolide); it can provide new delivery characteristics and it may have possible advantages in future applications regarding the encapsulation and delivery of bioactive peptides to target tissues. Furthermore, the physicochemical characteristics (size distribution and ζ-potential) of the liposomal formulations and the thermal effects on leuprolide in model lipidic bilayers composed of dipalmitoylphosphatidylcholine were studied using differential scanning calorimetry. Finally, the dynamic effects of leuprolide in an egg phosphatidylcholine/cholesterol system were examined using solid state ^{13}C MAS NMR spectroscopy.

Keywords: leuprolide; synthesis; liposomes; differential scanning calorimetry; solid state NMR

INTRODUCTION

Leuprolide is a nonapeptide (GlpHisTrpSerTyr-D-LeuLeuArgProNHEt) synthetic analogue of LHRH that, when given continuously, inhibits pituitary gonadotropin secretion and suppresses testicular and ovarian steroidogenesis. This analogue possesses greater potency than the natural hormone and has proven valuable for the treatment of a wide variety of endocrinological and non-endocrinological disorders such as advanced prostate cancer, endometriosis and precocious puberty [1]. Administration of leuprolide initially increases the circulating levels of luteinizing hormone and follicle-stimulating hormone, resulting in an increase of the levels of gonadal steroids (testosterone, dihydrotestosterone, estrone and estradiol). However, after a period of constant administration, leuprolide decreases the levels of LH and FSH. Finally, chronic administration leads to a suppression of ovarian and testicular steroidogenesis. Its poor pharmacokinetic characteristics when given *per os*, led to research on new technologies for controlled delivery. One pharmaceutical formulation of leuprolide is based on the DepoFoam™ technology [2–5], which consists of multivesicular liposomes characterized by multiple non-concentric aqueous chambers surrounded by a network of lipidic membranes [4]. Liposomes are non-toxic carrier systems, which have been studied for intravenous delivery of, *inter alia*, lipophilic compounds. It has been shown that the physicochemical characteristics of liposomes, including lipid composition, surface charge and size, can modulate their *in vivo* stability and improve the pharmacokinetic properties of the encapsulated drugs [6–13], reducing side effects and enhancing activity: in recent years, liposomes have been successfully applied for drug delivery in numerous cases [14–25]. The liposomal formulation LILs was achieved using the thin film hydration method, following reversed phase evaporation methodology. It is obvious that preservation of the functionality of peptides is required in order to maintain their activity. Most enzymes are fragile and their stabilization during the process of incorporation into the carrier system is required [26,27].

Abbreviations: DPPC, dipalmitoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; DSC, differential scanning calorimetry; EE, encapsulation efficiency; EGPC, egg phosphatidylcholine; LILs, liposomes in liposomes; LUV, large unilamellar vesicles; MAS, magic angle spinning; RCF, relative centrifugal force; REV, reversed evaporation method; SUV, small unilamellar vesicles.

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The overall goal of this study was threefold: first, to develop an improved synthesis of leuprolide; second, to design and to develop a new liposomal formulation for it leading to better pharmacokinetic properties; and third to study its thermal and dynamic effects on phospholipid bilayers.

MATERIALS AND METHODS

Materials

Leuprolide was synthesized using standard Fmoc chemistry. Fmoc-protected amino acids and peptide reagents were purchased from Bachem AG (Bubendorf, Switzerland) and Novabiochem (Läufelfingen, Switzerland). All solvents and reagents used for solid-phase synthesis were of analytical quality. EGPC was obtained from Lipoid (Ludwigshafen, Germany), DPPG, DPPC were obtained from Avanti Polar Lipid Inc. (Alabaster, AL, USA); phosphate buffer solution (pH 7.4) was obtained from Life Technologies Ltd (Gibco BRL, Paisley Scotland); all chemicals, i.e. methanol, chloroform, ether, were analytical grade. HPLC grade water was obtained using a PRO™PS Labconco system.

Synthesis of Leuprolide

The synthesis was performed by solid phase methodology on a {3-[(ethyl-Fmoc-amino)-methyl]-1-indol-1-yl}-acetyl AM resin [19] using Fmoc/*t*Bu chemistry [20]. The Fmoc group was removed with 25% piperidine in DMF. Activation was performed *in situ* using DIC/HOAt in DMF [21,22]. Couplings were performed with 3/3.3/4.5 molar excesses of Fmoc-amino acid/DIC/HOAt, respectively. Completeness of the reaction was monitored by the Kaiser test [23]. Treatment of the peptide–resin conjugate with TFA/DCM/1,2-ethanedithiol/anisole/water (90 : 5 : 1 : 2 : 2, v/v) (15 ml/g peptide resin) for 4 h afforded the desired product. The peptide was precipitated upon evaporation *in vacuo* and the addition of ether. The crude peptide was purified by gel filtration chromatography on Sephadex G-15 using 15% AcOH. Final purification was achieved by RP-HPLC. ES-MS was in agreement with the expected result. The desired product was obtained in 55% overall yield.

Preparation and Physicochemical Characterization of Liposomes

Liposome preparation. Liposomes consisting of EGPC, DPPG were prepared by the reverse phase evaporation method [28,29] and were characterized by measuring the z-average mean diameter and ζ-potential. Briefly, following the reverse phase evaporation procedure, EGPC and DPPG at a molar ratio of 98.91 : 1.09 were dissolved in a mixture of ether and chloroform (1 : 1 by volume). Phosphate buffer solution (pH 7.4) was then added (organic phase: aqueous phase 3 : 1 by volume), and the mixture was vortexed and briefly sonicated in a bath-type sonicator until a relatively stable emulsion was formed. The organic solvents were removed under reduced pressure and the reversed emulsion was transformed into an aqueous vesicle dispersion consisting of LUVs [27,29,30]. SUVs were prepared from the resultant liposomal suspension, which was subjected to sonication for two 5 min periods interrupted by a 5 min resting period, in an ice bath using a probe sonicator (amplitude 100, cycle 0.7–UP 200S, dr. hielsher GmbH, Berlin, Germany). The resultant vesicles were allowed to anneal any structural defects for 3 min. The liposomal suspension was then centrifuged (fixed angle rotor, Sorvall T-880, RCF value = 28 684, 20 min, 4 °C) in order to separate SUVs from titanium and from LUVs.

Liposome size and ζ-potential measurements. Size and ζ-potential of liposomes are the parameters that indicate the physical stability of the liposomal preparations. The liposome dispersion was stored in glass vials at 4 °C. The first measurements of size and ζ-potential were performed immediately after the preparation of the liposomes and thereafter at fixed time intervals for a 2 month period. A 100 µl liposome dispersion was diluted 10-fold in a phosphate buffer solution (pH 7.4), and the z-average mean and ζ-potential of the empty and loaded SUVs were measured immediately in order to determine the effect of leuprolide loading on the liposomal physicochemical properties. Samples were scattered (633 nm) at an angle of 90° and measurements were made at 25 °C in a photon correlation spectrometer (Zetasizer 3000, Malvern K) and analysed by the CONTIN method (MALVERN software) (Table 1).

Preparation of LILs

Preparation of the 'inner liposomes' and encapsulation of leuprolide. The internal liposomes of LILs formulation were prepared by the reversed evaporation method. Briefly, the lipid mixture EGPC/DPPG (98.91 : 1.09 molar ratio) was dissolved in chloroform/ether and mixed with the solution of leuprolide (8 mg/ml) in phosphate buffer solution. The organic solvent was removed until the reverse emulsion transformed into aqueous vehicle dispersion. The sample was then sonicated in a probe sonicator and centrifuged. Additional phosphate buffer solution at pH = 7.4 was slowly added to the prepared liposomal suspension and the solution was subjected to magnetic stirring for 30 min in order to ensure that the outer phase of the suspension was aqueous and the liposomal formulation was a water/oil/water emulsion [2,31–33].

Preparation of the final LILs suspension. The final LILs were prepared as follows: using the thin film hydration method. EGPC, DPPG and cholesterol at a molar ratio of 68.71 : 0.76 : 30.53 were dissolved in chloroform and the solvent was completely removed leading to the formation of a dry lipid film, which was left in a desiccator for 24 h.

Subsequently, the aqueous liposome suspension consisting of the 'inner liposomes' was then added to the round flask containing the dry lipid film and then the lipid film was slowly hydrated, following the thin film hydration method [29,30]. The liposomes were frozen with dry ice and then thawed at a temperature above the transition temperature. The freeze-thaw cycles were repeated 10 times [26,34,35].

Liposome images. Liposomes were observed under an epifluorescence Zeiss Axioplan2 microscope equipped with a rhodamine long pass filter (excitation LP 510 + KP 560, beamsplitter FT 580 and emission LP 560). Observations were made with an ×40 Plan Neofluar dry lens (NA 0.75), or an

Table 1 Physical Stability of Liposomes during 2 months Storage at 4 °C

Time (weeks)		EGPC : DPPG 98.91 : 1.09 (mol%)	EGPC : DPPG : leuprolide 86.54 : 0.96 : 12.5 (mol%)	'liposomes in liposomes'
0	Size[a]	109.2 ± 5.0	74.2 ± 4.5	>1500
	PI[b]	0.44	0.28	1.00
	ζ-Potential[c]	−7.2 ± 1.4	−9.9 ± 3.4	−13.9 ± 1.8
2	Size	111.5 ± 3.2	78.2 ± 3.7	>1500
	PI	0.47	0.33	1.00
	ζ-Potential	−6.8 ± 1.9	−9.7 ± 3.2	−11.6
4	Size	110.2 ± 2.6	77.6 ± 3.0	>1500
	PI	0.46	0.31	1.00
	ζ-Potential	−6.6 ± 2.5	−10.3 ± 2.8	−12.8
8	Size	114.4 ± 3.3	81.4 ± 4.2	>1500
	PI	0.44	0.32	1.00
	ζ-Potential	−7.7 ± 10.9	−10.9 ± 6.3	−36.4
14	Size	118.0 ± 5.5	83.2 ± 6.5	—
	PI	0.46	0.33	
	ζ-Potential	−8.4 ± 3.5	−12.4 ± 4.6	—

[a] z-average mean (nm).
[b] Polydispersity index.
[c] ζ-Potential (mV).

×100 Plan Neofluar oil immersion lens (NA 1.30). Images were captured with a CCD b/w camera in front of which two lenses were used alternately, providing a final magnification of ×1000 or ×1500. The camera was under the control of Image Pro Plus v3.1. Following capture, images were corrected for background by subtracting an out of focus image, and then filtered using a HiGauss filter (7 × 7 pixel size, one pass, strength 3).

Determination of the Encapsulation Efficiency of Leuprolide into Liposomes

Encapsulation efficiency or loading efficiency is usually defined as the percent fraction of the total input drug encapsulated (in lipid bilayers and/or aqueous compartments) in the liposomes at a particular phospholipid concentration and expressed as a weight percentage (w/w) [10,30]. The determination of the EE of leuprolide into liposomes was based on its UV absorbance at λ_{max} 287 nm. SUVs were prepared following the reverse phase evaporation method using EGPC and DPPG at a molar ratio of 98.91 : 1.09 and leuprolide, which was added at a concentration 8.0 mg/ml. In order to determine the exact percentage of the drug incorporated in the SUVs (inner liposomes), the leuprolide encapsulated in liposomes was purified from the drug, which was not encapsulated, using Sephadex (G 75) column chromatography.

The LILs formulation encapsulating leuprolide was prepared following the method described above. Purification of the leuprolide encapsulated in LILs from the free drug was performed by centrifugation (fixed angle rotor, Sorvall T-880, relative centrifugal force value = 28 684, 20 min, 4 °C). The UV absorption of leuprolide was measured and the EE of liposomes was determined.

Release Characteristics

Briefly, release experiments were performed as follows: the LILs suspensions were diluted 5-fold into a phosphate buffer solution (pH 7.4). Samples were kept at room temperature 25 °C. The solutions were analysed spectrophotometrically at 287 nm. The release ratio was calculated by subtracting the remaining encapsulated drug from the initial amount leuprolide (100%).

Methods

Differential scanning calorimetry. A conventional DSC technique was applied for the study of the samples, using a Perkin Elmer DSC-7 calorimeter. DPPC with or without cholesterol was dissolved in equal volumes of chloroform and ether. Leuprolide was added in the form of a solution (in phosphate buffer solution, pH 7.4). The solvent was then evaporated *in vacuo* (0.1 mmHg) at a temperature above the transition temperature of the phospholipid. An aliquot of the sample (ca 5 mg) was sealed into stainless steel capsules obtained from a Perkin Elmer DSC 7 calorimeter. Prior to scanning, the samples were held above their phase transition temperature for 1–2 min to ensure equilibration. All samples were scanned at least twice until identical thermograms were obtained using a scanning rate of 2.5 °C/min. The temperature scale of the calorimeter was calibrated using indium (T_m = 156.6 °C) as the standard reference sample [36]. The samples tested were either binary mixtures of DPPC/cholesterol and DPPC/leuprolide or ternary mixtures of DPPC/cholesterol/leuprolide. For the ternary mixtures an equimolar concentration of cholesterol and leuprolide was used. The molar concentration of cholesterol, leuprolide or equimolar mixture of cholesterol/leuprolide was expressed relative to DPPC and was in ascending order of 4.8, 9.1 and 16.7 mol%.

Solid state NMR spectroscopy. High-resolution NMR spectra were obtained at 100.63 MHz on a Bruker MSL 400 NMR spectrometer capable of high-power ^{1}H-decoupling and

equipped with a MAS unit. The spinning rate used for MAS NMR experiments was 1 KHz and was kept constant at the temperature range run in the experiments. Each spectrum was an accumulation of 2000 scans. The lipid content for the two samples was ca 50 mg. The delay time was 6 s, the 90° pulse width was 5 μs and the acquisition time was 40 ms. Chemical shift values of DPPC bilayers were obtained from the literature [37].

RESULTS AND DISCUSSION

The role of the carrier on the pharmacological activity of drugs is of great importance since it drastically affects its pharmacokinetic properties. Liposomal formulations are often proposed for the improvement of the therapeutic action of drugs, especially for those used against cancer. Liposomes can modulate the bioavailability and improve the pharmacokinetic properties of the encapsulated drug [10]. In this study, SUV liposomes composed of EGPC and DPPG at a molar ratio of 98.91 : 1.09 were prepared. The physical stability ($t = 4\,^{\circ}C$, phosphate buffer solution pH = 7.4) of the SUVs was monitored for 2 months. The results showed that the SUVs were found to retain their stability with particle sizes ranging from 109.2 ± 5.0 nm to 118.0 ± 5.5 nm; ζ-potential: -7.2 ± 1.4 to -8.4 ± 3.5 mV. Leuprolide was encapsulated into SUVs and its EE was calculated at 53.3% (w/w), after separation of the unentrapped drug using column chromatography (Sephadex G-75). The remaining leuprolide was assayed by UV spectrometry at 287 nm. The stability studies showed that leuprolide encapsulating liposomes were stable during a 2 month period with a vesicle size ranging from 74.2 ± 4.5 nm (PI: 0.28) to 83.2 ± 8 nm (PI: 0.31); ζ-potential: -9.9 ± 3.4 to -12.4 ± 4.6 mV. The results of the experiments are shown in Table 1.

The preparation procedure for LILs (day of preparation: z-average mean > 1500 nm; ζ-potential -13.9 ± 1.8 mV; week 7: z-average mean > 1500 nm; ζ-potential -36 mV) was started from SUVs as described above. The LILs formulation offered a very good encapsulation efficiency of leuprolide up to 73.8%. The presence of different kinds of vesicles in the same formulation leads to the assumption that in the biological environment, the formulation can provide a sustained release of the drug. The free (not encapsulated) drug that is present in the aqueous solution is expected to have a quick action while that encapsulated in LILs is expected to present different release rates, depending on the vesicle (SUVs or LUVs).

The results from the release study of the drug from LILs at room temperature (Table 2) showed a constant release rate from 15 to 45 h and 50% of the drug remained encapsulated at 53.2 h (R^2 0.955) (Figure 1). It is speculated that better drug release could be achieved by liposomes composed of more rigid lipid bilayers. This could be possible using lipid mixtures with high transition temperatures (>50 °C) [12], resulting in effective stability after administration.

Table 2 Release of Leuprolide from the LILs Formulation

Time (h)	Drug release (%)	Time (h)	Drug release (%)
1	3.72	32	32.53
12	6.56	37	38.90
16	14.13	41	43.20
20	18.24	60	51.40

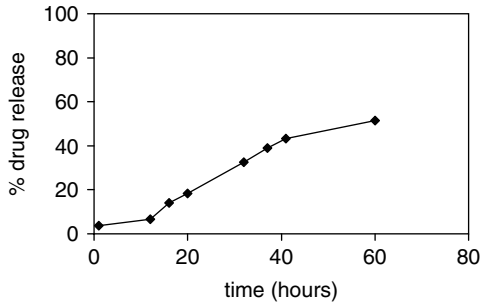

Figure 1 % Release rate of leuprolide from LILs formulation in phosphate buffer solution.

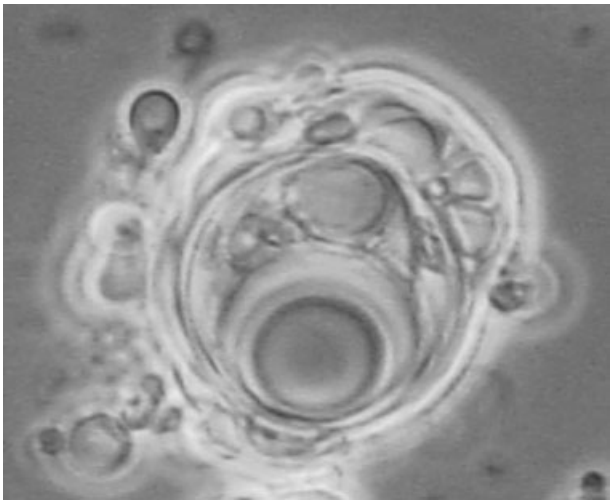

Figure 2 LILs suspension labeled with rhodamine-PE.

Images of the new liposomal formulation taken using a fluorescence microscope, show that the encapsulation of SUVs into LUVs was performed effectively. The image (Figure 2) shows the presence of the liposomal formations, SUVs and LUVs in the same formulation.

Differential Scanning Calorimetry

The fully hydrated DPPC bilayers (Figure 3) showed a characteristic thermogram consisting of a broad low enthalpy transition at 35.3 °C and a sharp enthalpy main transition at 41.2 °C. The DPPC bilayer

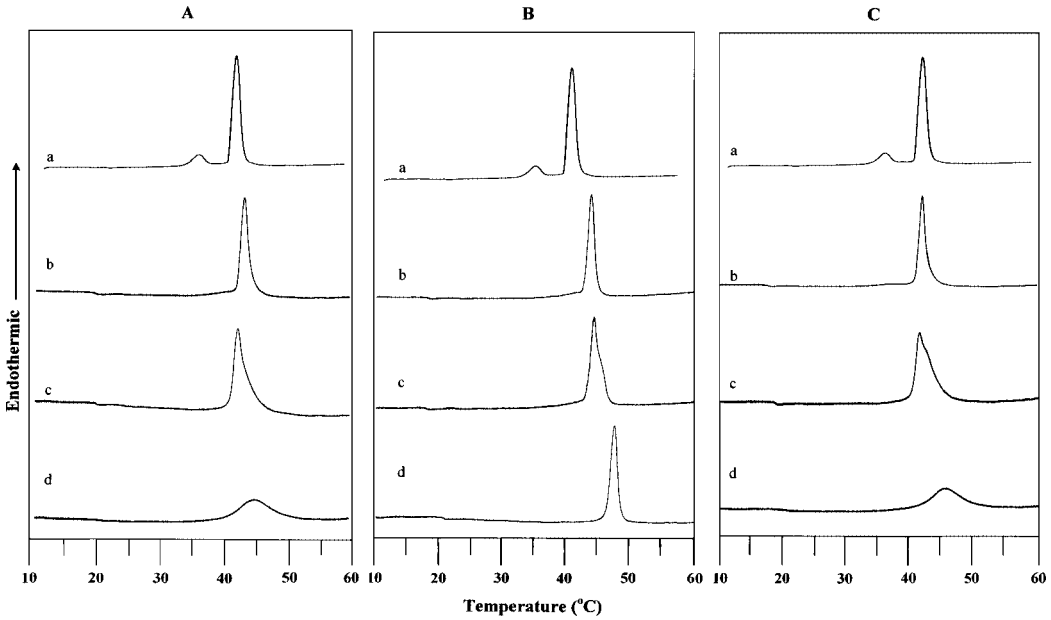

Figure 3 Normalized DSC thermograms of fully hydrated bilayers of DPPC with varying amounts (in mol%) of: cholesterol (left A), leuprolide (middle B) or equimolar cholesterol and leuprolide (right C): 0 (pure DPPC) (curve a), 4.8 mol% (curve b), 9.1 mol% (curve c), 16.7 mo% (curve d).

Table 3 Transition Temperatures and Enthalpy Changes of the Studied Multilamellar Vesicles in the Absence or Presence of Leuprolide or Equimolar Mixture of Leuprolide/Cholesterol

Sample	T_{onset} (°C)	SD	T_m (°C)	SD	$T_{m1/2}$	SD	ΔH (J/g)	SD
DPPC	40.32	0.17	41.20	0.51	0.5	0.12	41.01	0.26
DPPC/Chol (4.8% mol)	42.80	0.01	43.61	0.01	1.1	0.00	28.61	0.34
DPPC/Chol (9.1% mol)	41.80	0.00	42.88	0.01	1.4	0.00	26.19	0.16
DPPC/Chol (16.7% mol)	41.00	0.00	42.11	0.01	2.2	0.00	24.14	0.30
DPPC/Leupr (4.8% mol)	42.87	0.01	44.55	0.01	1.6	0.00	29.47	0.48
DPPC/Leupr (9.1% mol)	43.88	0.11	44.68	0.43	1.8	0.11	28.08	0.51
DPPC/Leupr (16.7% mol)	44.22	0.08	45.19	0.07	1.0	0.00	21.52	0.01
DPPC/Chol/Leupr (4.8% mol)	43.30	0.01	44.81	0.01	1.6	0.00	18.11	0.38
DPPC/Chol/Leupr (9.1% mol)	41.34	0.36	42.57	0.01	1.2	0.00	26.88	0.18
DPPC/Chol/Leupr (16.7% mol)	40.83	0.01	41.91	0.00	2.9	0.00	23.25	0.05

exists in the gel phase (L_β') for temperatures lower than 33 °C, and in the liquid crystalline phase for temperatures higher than 42 °C (L_α'). Between 33°–42 °C the phospholipid bilayer exists in the $P_{\beta'}$ or ripple phase.

The DSC scans of DPPC/cholesterol have been previously extensively analysed [37–41]. In summary, cholesterol caused the abolition of the pretransition temperature even at very low concentrations, a gradual broadening of the phase transition and a decrease of ΔH as its incorporated concentration increased and it did not affect significantly the T_m of DPPC bilayers alone.

The presence of leuprolide up to 16.7 mol% caused a significant increase of T_m of $4° \pm 0.2$ °C at the different concentrations run in the experiments. This is an important finding since it shows that strong specific interactions between the leuprolide and membrane bilayers must exist. Interestingly, the $T_{m1/2}$ of the bilayer in the presence of leuprolide was not decreased but slightly increased. This means that cooperativity of the phospholipids was not increased by the strong interactions between the additive and phospholipid bilayers. The ΔH of DPPC bilayers was lowered meaning that *trans : gauche* isomerization increased with the presence of drug molecule (see Table 3 for the quantitative results).

The experimental data can be interpreted as follows. The strong association of the leuprolide most probably with polar groups results in an increase of T_m. In addition, this association affects the hydrophobic region by causing an increase of *trans : gauche* isomerization. The resulting subsequent increase of the fluidity explains the decrease of the observed ΔH and cooperativity (increase of $T_{m1/2}$).

The DPPC/cholesterol/leuprolide ternary mixture showed the predominant effect of cholesterol at all samples used in the experiments. Thus, the samples of ternary mixture resembled mostly the DPP/cholesterol rather than DPPC/leuprolide. This was expected since the molar ratio of cholesterol/leuprolide was in the range 5–100. However, the effect of the leuprolide in the ternary mixtures should not be ignored. For example, in the ternary mixture where the molar ratio between cholesterol and leuprolide was 100, the T_m of the preparation was 44.81 °C, that is 0.26 °C higher than that observed for DPPC/leuprolide binary mixture (45.16 °C) and 1.20 °C higher than that observed in DPPC/cholesterol binary mixture (43.61 °C).

This finding may be of biological significance. The presence of leuprolide exerted thermal changes not only in model DPPC bilayers alone, but also in DPPC bilayers with high cholesterol content, which resemble more the biological ones. The presence of leuprolide in DPPC or DPPC/cholesterol bilayers resulted in a shifting of the T_m at higher values. Such a property may be desirable for certain diseases.

Solid State ^{13}C NMR Spectroscopy

The promising results obtained by DSC, especially in DPPC/cholesterol bilayers, triggered our interest in applying solid state ^{13}C NMR spectroscopy, using instead of DPPC, EGPC for the formation of the lipid bilayers. The reason that the same system was not used in DSC experiments is that the thermal scans in such a system are too broad to be analysed.

When a drug molecule is incorporated into a membrane bilayer, one can observe: (a) changes in peak intensity and line-width due to modified membrane fluidity; (b) changes in chemical shift values of individual carbon nuclei for the membrane lipid due to modified phase transition profiles; and (c) the appearance of a specific subset of peaks from the carbon nuclei of the incorporated drug molecule.

The ^{13}C MAS spectra of EGPC/cholesterol ($x =$ 16.7) and EGPC/cholesterol ($x = 16.7$) for the region 0–80 ppm are shown in Figure 4. The broad featureless peak at 30 ppm was attributed to $(CH_2)_n$ of the DPPC alkyl chains and was the most diagnostic of the mesomorphic changes which occurred in DPPC bilayers as the temperature increased. Its upfield shift and narrowing depict the phase transition of the phospholipid bilayer from the gel phase or solid state to the liquid crystalline phase.

As can be observed in the EGPC/cholesterol spectra this peak (due to the $(CH_2)_n$) was increased in magnitude as the temperature rose and became narrower. In addition, the peaks due to the head group became more pronounced. This was not observed with bilayers containing leuprolide meaning that the lipid bilayers containing leuprolide were almost

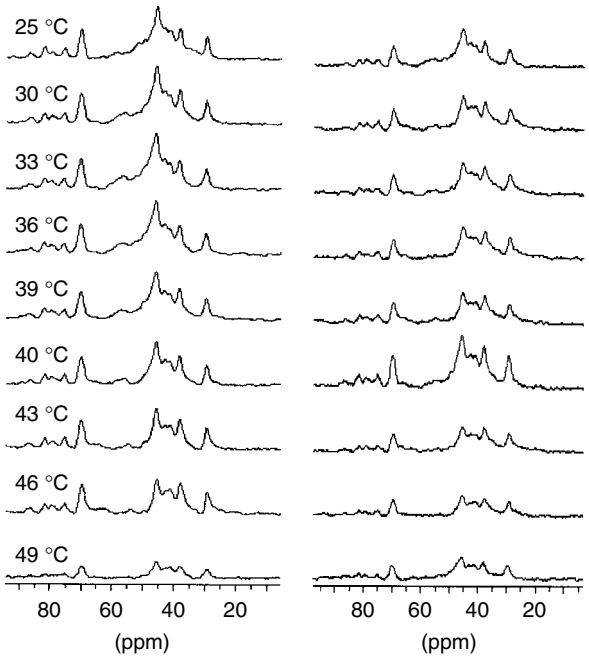

Figure 4 ^{13}C MAS spectra of EGPC/cholesterol (16.7 mol%) (left), EGPC/cholesterol/leuprolide (16.7 mol) (right) for the region 0–80 ppm at a temperature range of 25°–49 °C.

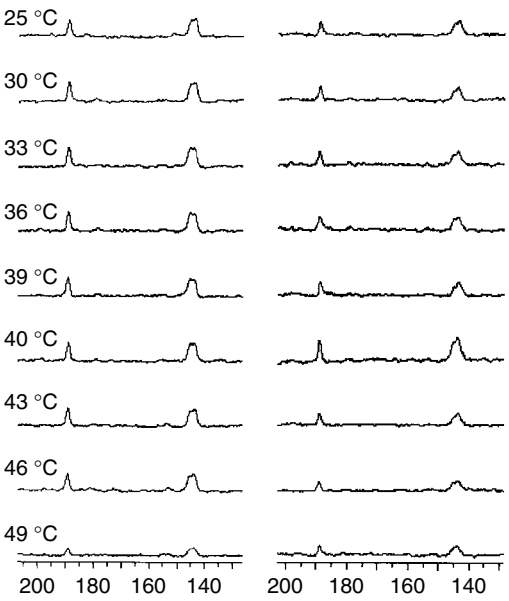

Figure 5 ^{13}C MAS spectra of EGPC/cholesterol (16.7 mol%) (left), EGPC/cholesterol/leuprolide (16.7 mol%) (right) for the region 100–180 ppm at a temperature range of 25°–49 °C.

constant with the increase of temperature. Thus, this ternary system may serve as a better buffer of bilayers than the binary one. This observation was not limited to the hydrophobic region but also to the head-group as can be observed in the same figure.

The ^{13}C MAS spectra of EGPC/cholesterol (16.7% mol) and EGPC/cholesterol/leuprolide (16.7% mol) for

the region 100–180 ppm are shown in Figure 5. During the phase transition, the line shape of the peak assigned to the ester carbonyl groups changed. The broad, low intense peak in the gel phase narrowed and increased in intensity in the liquid crystalline phase. Phospholipid bilayers containing leuprolide appeared to have similar spectra in this region at all temperature ranges. This was not observed with the EGPC/cholesterol sample where gradual changes in the shape of the observed peaks occurred as the temperature rose.

CONCLUSIONS

In conclusion, this research led to the development of an improved synthesis of leuprolide in high yield and a new liposomal formulation, which we have called 'liposomes in liposomes', (LILs). This formulation offers very good encapsulation efficiency and opens an avenue for further research in this area. Future experiments will be focused on the study of the improvement of the release of leuprolide from the LILs formulation. Leuprolide modified both the thermal and dynamic properties of model lipid bilayers used in this study. The results are promising for the development of controlled release liposomal systems for the encapsulation of other bioactive peptides.

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