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Research Article

Synthesis of Monomethoxypolyethyleneglycol—Cholesteryl Ester and Effect of its Incorporation in Liposomes

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Abstract. The objective of the present study was to synthesize monomethoxypolyethyleneglycol-5000 cholesteryl ester [PEG–CH] as a cost-effective substitute for polyethyleneglycol–phosphatidylethanolamine and to evaluate the influence of its incorporation in liposomal bilayers for surface modification. PEG–CH was synthesized and characterized by infrared (IR), proton nuclear magnetic resonance spectroscopy (^{1}H NMR), and differential scanning calorimetry (DSC) studies. Influence of incorporation of PEG–CH in liposomes was evaluated on various parameters such as zeta potential, DSC, and encapsulation efficiency of a hydrophilic drug pentoxyfylline. Conventional and PEG–CH containing pentoxyfylline liposomes were formulated and their stability was evaluated at 4°C for 3 months. PEG–CH could be successfully synthesized with good yields and the structure was confirmed by IR, DSC, and ^{1}H NMR. The incorporation of PEG–CH in liposomes resulted in reduction of the zeta potential and broadening of the DSC endotherm. Furthermore, incorporation of PEG–CH in liposomes decreased the encapsulation efficiency of pentoxifylline in liposomes when compared to conventional liposomes. Conventional and PEG–CH containing pentoxyfylline liposomes did not show any signs of pentoxyfylline degradation when stored at 4°C for 3 months.

KEY WORDS: liposomes; PEG–cholesteryl ester; steric stabilization.

INTRODUCTION

Liposomes are vesicles consisting of one or more self-assembled lipid bilayers enclosing aqueous compartment(s). They have potential applications as drug carriers due to properties such as sustained release (1), altered pharmacokinetics (2,3), increased drug stability (4), ability to overcome drug resistance (4,5), and target specific tissues (5,6). However, their use as a drug delivery system is limited due to their rapid clearance from systemic circulation by mononuclear phagocytic system. In order to overcome this disadvantage, long circulating liposomes or sterically stabilized liposomes containing poly (ethylene glycol) (PEG) coating were developed. These liposomes are also known as stealth liposomes (7,8). In order to impart stealth properties to the liposomes, use of amphiphilic PEG–lipid derivatives has been recommended (7,8). The most commonly used lipid anchors for PEG are phophotidylethanolamine derivatives such as distearoylphosphatidylethanolamine and dioleoylphosphatidylethamolamine (7,8). However, the expense of phosphatidylethanolamine derivatives and the difficulties in obtaining large quantities of these lipids severely increase cost of the liposomes (9). Hence, low cost and easily available lipid anchors are being explored for the development of stealth liposomes since the last decade. Among the various lipid anchors for PEG, cholesterol (CH) has shown a great promise because of its relatively low cost, as compared to other anchors such as ceramides, and ease of availability. CH is an important constituent of liposomes which also increases rigidity and half-life of liposomes *in vivo* (10). Furthermore, PEGylated CH molecules can be easily synthesized in large amounts on an industrial scale (9). In view of this, various PEGylated CH derivatives have been synthesized by researchers linked by ether, carbamate, and carbonate bonds (9,11–15). However, to date, there are no reports on the synthesis of PEGylated cholesterol derivative based on ester bond.

In the present investigation, a simple method for the synthesis of cholesteryl ester derivative of PEG viz. monomethoxypolyethyleneglycol-5000–cholesteryl ester [PEG–CH] from monomethoxypolyethyleneglycol-5000 monocarboxylic acid and cholesterol is reported. The synthesized PEGylated cholesterol derivative has been incorporated in the pentoxifylline liposomes and the effect of its incorporation on various parameters such as zeta potential, differential scanning calorimetry (DSC), and encapsulation efficiency of the pentoxifylline has been studied.

MATERIALS AND METHODS

Materials

PEG-5000 was purchased from Aldrich, USA. Thionyl chloride and triethylamine were obtained from S. D. Fine-Chem Ltd., Mumbai, India. Phospholipon 90 [PL90] and

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phospholipon 90H [PL90H] were generous gifts from Phospholipid GmBH, Germany. CH and stearyl amine [SA] were purchased from Loba Chemie Pvt. Ltd., Mumbai, India and Sigma, USA, respectively. Pentoxifylline (PTX) was obtained as a gift sample from Sun Pharmaceuticals Ltd., Baroda. All other chemicals and solvents were of analytical reagent grade and were used without further purification..

Synthesis of monomethoxypolyethyleneglycol-5000 monocarboxylic Acid [PEG-COOH]

PEG–COOH was synthesized according to the method reported by Lele *et al.* (16). Briefly, PEG 5000 (5 g) was dissolved in 40 mL acetone with the aid of intermittent heating. To this solution, 0.85 mL Jone's reagent (containing 0.01 M CrO3) was added in a single portion and the reaction mixture was stirred with a magnetic needle overnight at room temperature. Isopropyl alcohol was added to this mixture to quench the reaction. Finely powdered activated charcoal (0.5 g) was added to the reaction mixture to remove chromium salts formed during reaction. The reaction mixture was stirred for 2 h and was filtered to obtain a colorless acetone solution. The solution was concentrated in vacuo and a white powdered product was isolated. The yield of the reaction was 88%.

Synthesis of Monomethoxypolyethyleneglycol-5000 Cholesteryl Ester

PEG–COOH (20 g, 3.35 mM based on its acid value) was dissolved in 200 mL of tetrahydrofuran (THF) by warming, and two to three drops of dimethyl formamide were added to the solution followed by addition of 0.25 mL (3.35 mM) of thionyl chloride. The solution was stirred at room temperature for 24 h to yield monomethoxypolyethyleneglycol-5000 acid chloride [PEG–COCl] *in situ*. The acid chloride thus formed was used immediately for further reaction. To the solution of PEG–COCl, cholesterol (1.29 g, 3.35 mM) and triethylamine (0.47 ml, 3.35 mM) was added at room temperature. The reaction mixture was stirred for 24 h at room temperature and then filtered to remove the precipitate of triethylamine hydrochloride. The clear solution was added dropwise to petroleum ether with continuous stirring to obtain solid product. The product was redissolved in THF and precipitated in petroleum ether. This step was repeated twice for purification and the final product vacuum dried to yield a yellowish white powder.

Characterization of PEG–CH

The synthesized PEG–CH was characterized by various methods such as DSC, Fourier transformed-infrared spectroscopy (IR) and ^{1}H nuclear magnetic resonance spectroscopy (NMR) in order to confirm the structure.

Preparation of Conventional and PEG–CH Containing Pentoxifylline Liposomes

Empty and PTX-loaded liposomes were prepared by a minor modification of thin film hydration method as described by Taylor *et al.* (17). Briefly, PL90, PL90H, CH, and SA were taken in molar ratio of PL90:PL90H:CH:SA:: 5.5:5.5:11:0.22 or PL90:PL90H:CH:SA:: 3:8:4:2.22, respectively, in a 250-ml round bottom flask (RBF) and dissolved in chloroform. Chloroform was evaporated under reduced pressure on a rotary evaporator (Superfit, India) at 40°C to form a thin film of phospholipids on the inner surface of the flask. The lipid film was hydrated by phosphate-buffered saline, pH 7.4 [PBS] or PTX solution (20 mg/mL) in PBS above the gel to liquid crystalline phase transition temperature of phospholipids. The RBF was hand shaken vigorously for 5 min followed by reheating for a predetermined time period to anneal liposomes. The RBF was further shaken on horizontal shaker bath (Expo, India) for 6 h with intermittent sonication in a bath sonicatior (Expo, India) for 30 s after each hour, to reduce the vesicle size. For preparation of PEG–CH containing liposomes, 5 or 10 mol% of PEG–CH was added to the phospholipid mixture before lipid film formation.

Study of Influence of Incorporation of PEG–CH in Liposomes

Particle Size

The particle size of pentoxifylline-loaded conventional and PEG–CH containing liposomes was evaluated by laser light scattering on a Malvern Mastersizer MS 3 (Malvern Instruments, USA).

Zeta Potential Measurements

Conventional and PEG–CH containing empty liposomes (hydrated with double-distilled water) of two different compositions were prepared as described above. The first composition was PL90:PL90H:CH:SA::3:8:4:2.2 containing high concentration (20 mol%) of SA, a compound that imparts positive charge to the liposomal bilayer. To liposomes of this combination, PEG–CH (10 mol%) was added in the lipid film for surface modification. The second composition viz. PL90:PL90H:CH:SA::5.5:5.5:11:0.22 contained a much lower concentration (2 mol%) of SA. To this formulation, 5 mol% and 10 mol% of PEG–CH was added for surface modification. For zeta potential measurements, an appropriately diluted liposome sample was taken and zeta potential of each sample was measured in triplicate by using Malvern Zeta-sizer. Zeta potential values of conventional and PEG–CH containing liposomes were compared by Student's *t* test.

DSC Studies of Liposomes

Conventional and PEG–CH containing empty liposomes were centrifuged at 21,000×g for 30 min in a high-speed centrifuge (Remi, India) to obtain a solid pellet of liposomes. An accurately weighed amount (4–8 mg) of pellet or individual components was transferred to aluminum pans and sealed hermetically. The samples were scanned from 30°C to 180°C at the heating rate of 10°C/min using empty hermetically sealed aluminum pans as reference.

Determination of Encapsulation Efficiency of Conventional and PEG–CH Containing Liposomes

Liposomes were diluted with PBS and centrifuged at 21,000×*g* for 30 min. The supernatant was analyzed for PTX at 274 nm by using a validated UV spectrophotometric method after suitable dilution (18).

The entrapment efficiency was calculated by the following equation:

$$\%EE = \left[\frac{M\text{initial drug} - M\text{free drug}}{M\text{initial drug}}\right] \times 100 \qquad (1)$$

where, "$M_{\text{initial drug}}$" is the mass of initial drug used for the fabrication of liposomes and the "$M_{\text{free drug}}$" is the mass of free drug detected in the supernatant after centrifugation of the liposomal dispersion.

Stability of PTX-Loaded Conventional and PEG–CH Containing Liposomes

PTX-loaded conventional and 10 mol% PEG–CH containing liposomes were stored at 4°C for a period of 3 months. Samples (n=3) were removed at 0, 15, 30, 60, and 90 days and were assessed for PTX content by a validated stability indicating high-performance liquid chromatography (HPLC) method developed in-house. The particle size of the liposomes and their zeta potential was also measured at all time points. The statistical significance of differences in the data was analyzed utilizing analysis of variance followed by Bonferroni's test (GraphPad InStat Demo Version). Differences were considered statistically significant at $P<0.05$.

HPLC Analysis of PTX

The PTX content of the various liposomal formulations was determined by a stability-indicating, validated reverse-phase HPLC method developed in-house. The HPLC apparatus consisted of Jasco PU-2080 Plus Intelligent HPLC pump (Jasco, Japan) equipped with a Jasco UV-2075 Intelligent UV/VIS detector (Jasco, Japan), a Rheodyne 7725 injector (Rheodyne, USA), a Jasco Borwin Chromatography Software (version 1.50) integrator software and a Bondapak RP-18 (4.6×300 mm and 5 μ particle size) column. The mobile phase consisted of a mixture of methanol/water (55:45 *v*/*v*) at a flow rate of 1 mL/min that led to retention time of 5.2 min when detection was carried out at 275 nm. The assay was linear (r^2=0.999) in the concentration range 1–16 μg/mL with the lowest detection limit of 0.5 μg/mL of PTX. The method was validated with respect to accuracy and inter- and intra-day precision as per International Conference on Harmonisation guidelines, and the relative standard deviation was less than 2% in both the cases.

RESULTS AND DISCUSSION

Synthesis of PEG–COOH

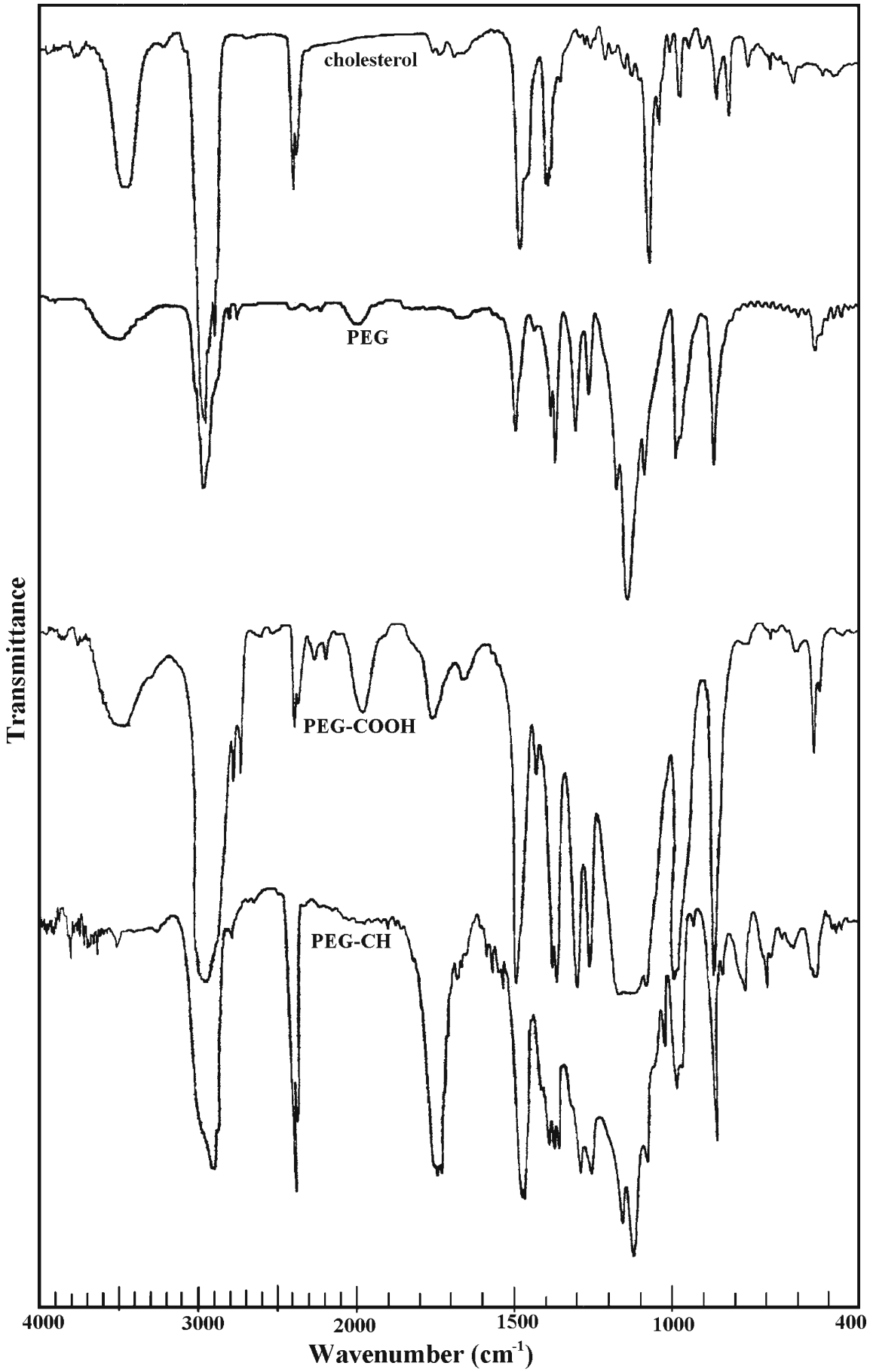
PEG–COOH was obtained as a white powder in 84.76% yield. Its acid value of 0.167 mM carboxyl groups/g was in agreement with the theoretical value of 0.19 mM carboxyl groups/g and that previously reported by Lele *et al.* [0.16 mM carboxyl groups/g] (16). The IR spectra of PEG and PEG–COOH are shown in Fig. 1. IR spectrum of PEG did not show any peak in the range of 1,700–1,750 cm^{-1} indicating absence of carbonyl functional moiety, while appearance of a peak at 1,747 cm^{-1} in the spectra of PEG–COOH indicates oxidation of terminal $–CH_2OH$ group to –COOH group. Both spectra showed a broad peak at about 3,450 cm^{-1} corresponding to –OH stretching vibrations. ^{1}H NMR spectrum of PEG–COOH in $CDCl_3$ showed a singlet at 3.4δ corresponding to $–O–CH_3$ group, a singlet at 3.5δ for $–CH_2$ group vicinal to $–O–CH_2–COOH$, a broad singlet at 3.63δ corresponding to $(–CH_2–CH_2–O)_n$ of PEG chain, and a singlet at 3.8δ corresponding to $–OCH_2$ vicinal to –COOH group that was in agreement with the previously reported values (3). The DSC scans of PEG and PEG–COOH are shown in Fig. 2. The thermogram of PEG indicated an onset of melting endotherm at 58.4°C, maximum peak temperature at 65.6°C, and peak recovery temperature at 78.4°C. The thermogram of PEG–COOH showed a melting endotherm with the onset temperature at 52.8°C, maximum peak temperature at 61.4°C, and peak recovery temperature at 73.5°C.

Synthesis of PEG–CH and its Characterization

The intermediate for the esterification reaction, i.e., PEG–COCl was very hygroscopic and unstable. Therefore, it was not isolated from the reaction mixture but prepared *in situ*, and esterification with cholesterol was continued immediately in the same reaction mixture. PEG–CH was obtained as a yellowish white fine powder in 86.8% (18.63 g) yield. IR spectrum of PEG–CH showed absence of –OH stretching vibrations in the range of 3,400–3,450 cm^{-1} although both starting materials viz. CH and PEG showed –OH stretching vibrations in this range as shown in Fig. 1. Disappearance of this peak suggested complete reaction of PEG–COOH and CH to form PEG–CH. IR spectrum of PEG–CH also exhibited a peak at 1,734 cm^{-1} corresponding to carbonyl of ester functional group. DSC scans of cholesterol and PEG–CH are shown in Fig. 2. CH had a melting endotherm showing onset temperature at 145.8°C, maximum peak temperature at 151°C, and peak recovery temperature at 160.6°C. In DSC thermogram of PEG–CH, the endotherm corresponding to CH was absent indicating absence of unreacted cholesterol in the product. It showed a melting endotherm with onset temperature at 43.9°C, peak temperature at 58.3°C, and recovery temperature at 68.9°C. In addition, the thermogram was dissimilar to that of PEG.

^{1}H NMR of PEG–CH in $CDCl_3$ showed multiplets in the region of 0.7–2.1δ indicating the presence of cholesteryl moiety, a broad singlet at 3.6δ corresponding to $(–CH_2–CH_2–O)_n$ of PEG chain and a singlet at 4.2δ corresponding to $–OCH_2$ moiety vicinal to –COOH group (Fig. 3).

The advantage of this synthetic methodology is that the reaction can be carried out economically, at room temperature, with inexpensive reagents and does not require any special laboratory equipment. Also, the method gives good yields and can be used for synthesis of cholesteryl esters of PEG of any chain length. It is reported that the *in vivo* behavior of sterically stabilized liposomes depends upon PEG


cholesterol
PEG
PEG-COOH
PEG-CH
Transmittance
4000
3000
2000
1500
1000
400
Wavenumber (cm-1)

◀ **Fig. 1.** IR spectra of cholesterol, PEG, PEG–COOH, and PEG–CH

molecular weight and amount of PEG derivatives added to liposomes (19). The methodology presented here affords precise control of the molecular weight of PEG–cholesteryl ester derivative by simply selecting PEG of appropriate molecular weight.

Study of Influence of Incorporation of PEG–CH in Liposomes

Presence of polymers like PEG or amphiphilic PEG derivatives on surface of liposomes influences physical properties like zeta potential, internal volume, thermal transitions, and encapsulation efficiency of the drug. Therefore, it can reasonably be expected that changes in physical properties of liposomes by incorporation of polymers like PEG–CH will indicate whether the polymer is imparting steric stabilization to liposomes.

Particle Size

Laser light scattering measurements showed unimodal distributions for both conventional and PEG–CH containing pentoxifylline liposomes. The mean particle size of conventional PTX liposomes was 2.33 μm and that of PEG–CH containing PTX liposomes was 1.63 μm ($n=3$).

Incorporation of PEG–CH in liposomes led to a decrease in the mean volume diameter. PEG–CH may have surface-stabilizing properties which may be responsible for the reduction in the particle size.

Zeta Potential Measurements

The zeta potential of conventional liposomes of composition PL90:PL90H:CH:SA::3:8:4:2.2 was found to be +11.7± 1.3 mV while that of PEG–CH (10 mol%) containing liposomes was found to be +7±1.5 mV ($n=3$). Thus, significant ($P<0.001$) reduction of the zeta potential was observed by incorporation of PEG–CH in liposomes. Zeta potential of liposomes of composition PL90:PL90H:CH: SA::5.5:5.5:11:0.22 containing lower concentration of SA was found to be +5.1±1.3 mV. It is evident that the reduction in the concentration of SA resulted in significant decrease in the zeta potential of liposomes. The incorporation of the PEG–CH in these liposomes resulted in decrease of zeta potential value in concentration-dependant manner. Liposomes containing 5mol% of PEG–CH yielded zeta potential value of +4.1±2.3 mV ($n=3$, $P>0.05$) whereas liposomes containing 10 mol% PEG–CH showed zeta potential value of +2.9± 0.9 mV ($P<0.05$). Zeta potential of liposomes is due to the presence of charged moieties like SA on the surface. Concentration-dependent decrease in zeta potential by addition of PEG–CH is probably observed due to strong shielding effect on the positive charge of stearyl amine by the hydro-

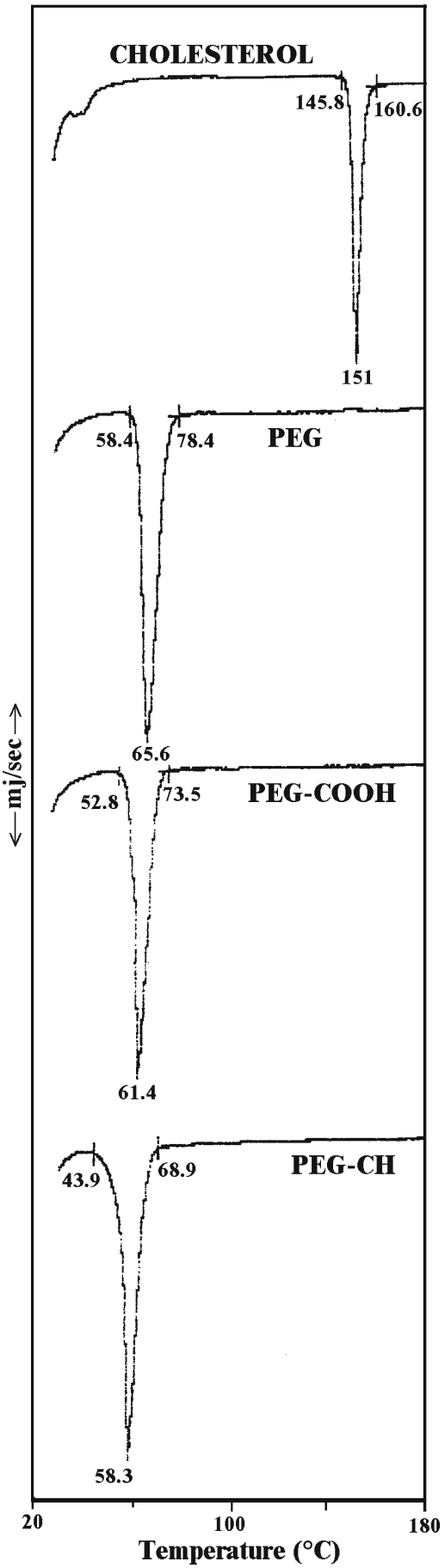


Fig. 2. DSC thermograms of cholesterol, PEG, PEG–COOH, and ▶ PEG–CH

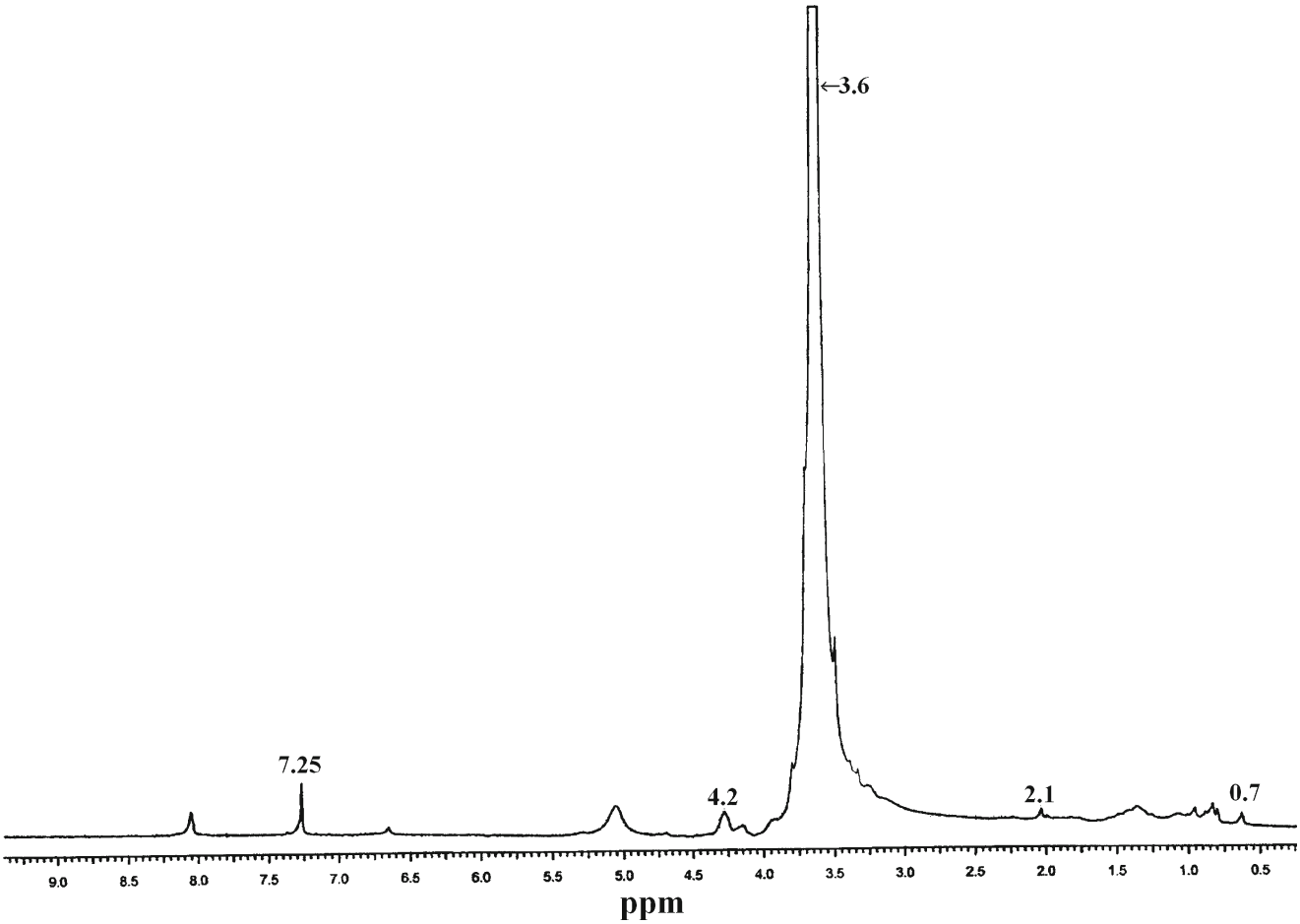


Fig. 3. ^{1}H NMR spectra of PEG–CH

philic headgroups of PEG–CH. This suggests successful association of PEG–CH with liposomal bilayers. Schneider *et al.* (11) have reported similar concentration-dependent decrease in the zeta potential of liposomes by incorporation of cholesteryl hemisuccinate-PEG and PEG–distearoyl phosphatidylethanolamine.

DSC Studies of Liposomes

The DSC scans of PL90, PL90H, CH, SA, PEG–CH, conventional liposomes, and PEG–CH (10 mol%) containing liposomes are shown in Fig. 4. All the individual components in the liposomes showed sharp endothermic transitions in their DSC thermograms. Conventional empty liposomes showed a broad endothermic peak with onset temperature at 88.9°C, peak transition temperature at 113.6°C, and recovery temperature at 140.8°C. Upon addition of PEG–CH, this endothermic transition was further broadened by downward shift in onset temperature at 48.1°C, peak transition temperature at 108.8°C, and recovery temperature at 152.6°C. The broadening of the endothermic transition in PEG–CH containing liposomes indicates its interaction with liposomal bilayer and provides further evidence of its incorporation in liposomes. These results are in agreement with earlier reports that have shown that incorporation of halofantrine, an amphiphilic drug, in dipalmitoyl phosphatidylcholine [DPPC]-cholesterol liposomes results in broadening of gel to liquid crystalline phase transition endotherm of phospholipids because of the perturbations in phospholipids (20). In another report, Bedu-Addo *et al.* (21) have shown that incorporation of increasing concentrations of monomethoxypolyethyleneglycol–phosphatidylethanolamine [PEG–PE] in DPPC/PEG–PE liposomes leads to decrease in the phase transition cooperativity. Further, Bedu-Addo *et al.* (22) have also shown that interaction of PEG–PE with phosphatidylcholine–cholesterol liposomes results in the broadening of the phase transition and the inhibition of phase separation.

Encapsulation Efficiency of Conventional and PEG–CH Containing Liposomes

The effect of incorporation of PEG–CH in liposomes (PL90:PL90H:CH:SA::5.5:5.5:11:0.22) on encapsulation efficiency of PTX, a water-soluble compound, was determined. Encapsulation efficiency of conventional PTX liposomes was found to be 10±0.99% while that of PEG–CH containing liposomes was found to be 4.5±0.35% (n=5). This decrease in encapsulation by incorporation of PEG–CH is probably due to the reduction in internal vesicular volume by bulky PEG chains covering both the inner and outer surfaces of liposomes. The results are in agreement with previous reports

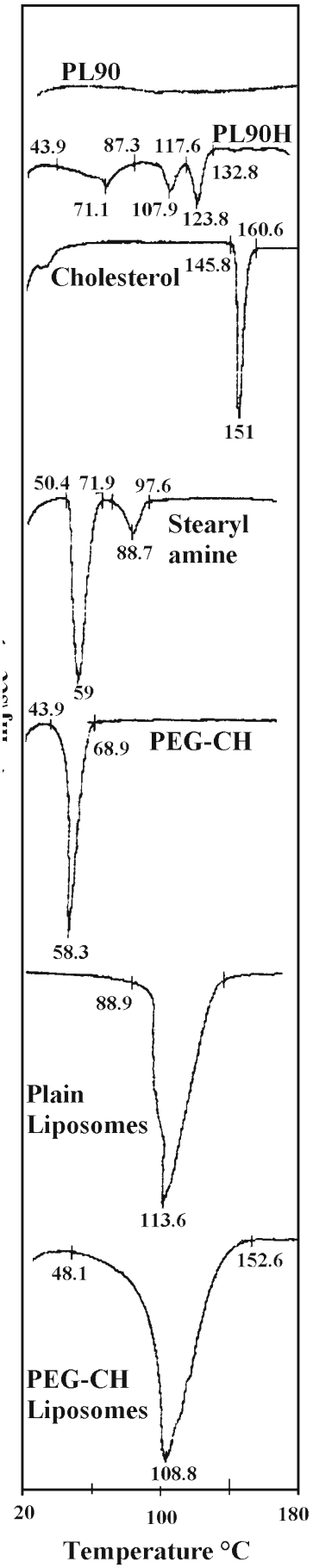


Fig. 4. DSC thermograms of empty conventional and PEG–CH (10 mol%) containing liposomes and their components

by Schneider *et al.* (11) who observed sharp concentration-dependent reduction in encapsulation efficiencies of water-soluble contrast agents in liposomes containing different concentrations of cholesteryl hemisuccinate–PEG and PEG–distearoyl phosphatidylethanolamine.

Stability of PTX-Loaded Conventional and PEG–CH Containing Liposomes

Both, conventional and PEG–CH containing liposomes did not reveal any signs of degradation of PTX after 3-month storage at 4°C (Table I). There were no signs of any color change. Thus, PTX did not undergo any chemical degradation in the liposomes. We also evaluated the encapsulation efficiency of the PTX in the liposomes at all the time intervals and found that the difference in the encapsulation efficiency was not statistically different at all the time points (data not shown). This indicated that there was negligible leakage of pentoxifylline from liposomes during stability studies. The mean particle size and zeta potential of conventional PTX liposomes (2.45±0.3 μm, 11.9±1.5 mV) and PEG–CH containing PTX liposomes (1.7±0.37 μm, 7.2±1.5 mV) did not show any significant change during the stability studies. Thus, it could be assumed that the prepared conventional and PEG–CH containing PTX liposomes have good physical stability.

CONCLUSIONS

Synthesis of PEG–CH was successfully achieved by a simple method. Incorporation of PEG–CH to liposomes altered the properties of liposomal bilayer as indicated by zeta potential measurements, DSC, and encapsulation efficiency of PTX. The conventional and PEG–CH containing PTX liposomes did not show any degradation of PTX at 4°C even at the end of 3 months.

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Table I. Chemical Stability of Pentoxyfylline in Liposomes Stored at 4°C (n=3)

	% Pentoxyfylline content in liposomes (±SD)	
Month	Conventional liposomes	PEG–CH containing liposomes
0.5	95.5 (±0.8)	96.7 (±0.7)
1	94.6 (±0.3)	96.5 (±0.6)
2	92.6 (±0.5)	95.2 (±0.7)
3	92.3 (±0.6)	93.9 (±0.8)

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