Cholesteryl N-Monomethoxypoly(ethylene glycol)-succinate-L-phenylalanine: Synthesis and Effect on Liposomes

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Poly(ethylene glycol)-phosphatidylethanolamine conjugate (PEG-PE) has been used in preparing long-circulating liposomes. As a substitute for PEG-PE which can also be used in the long-circulating liposome formualtions, but can be prepared more readily with a lower cost, PEG-Phe-Chol was synthesized from PEG, phenylalanine, and cholesterol. The addition of the PEG derivative to distearoylphosphatidylcholine (DSPC) led to the formation of mixed micelles as well as liposomes when the derivative content was 10 mol% or greater. On the other hand, the addition of just 5 mol% PEG-Phe-Chol to dioleoylphosphatidylethanolamine (DOPE) generated mixed micelles as well as liposomes, but the formation of mixed micelles was completely inhibited by the addition of cholesterol. The leakage of entrapped calcein out of DOPE/cholesterol (7/3) liposomes containing 5 mol% PEG-Phe-Chol was about 45% during the incubation time for 24 h in 50% rabbit plasma, which was similar to that of the same liposomes containing 5 mol% PEG-dipalmitoylphosphatidylethanolamine (DPPE) under the identical conditions.

Keywords : Poly(ethylene glycol), Phenylalanine, Cholesterol, Mixed micelle, Long-circulating liposome.

Introduction

Many types of liposome-based products have been extensively studied for effective drug delivery systems.[1] However, the liposomal drug delivery to cells or tissues other than the reticulo-endothelial system (RES) was difficult since liposomes are rapidly taken up by the RES cells in the liver and spleen.[2,3] To overcome this problem, ganglioside G_{M1} was incorporated into liposomes. Therapeutic applications of G_{M1}-containing liposomes, however, are not practical due to the high cost of G_{M1} and the difficulty in obtaining large quantities either by extraction of natural sources or by synthesis.

Monomethoxypoly(ethylene glycol)-phosphatidylethanolamine congjugate (PEG-PE) was also reported to prolong the circulation lifetime of liposomes.[4] The linkage between PEG and PE can be variable as shown in Figure 1 where dipalmitoylphosphatidylethanolamine (DPPE) was employed as one of the common PEs.[5] The PEG protective action may be due to the flexibility of polymer molecules and the ability to form a dense polymeric cloud over the liposome surface.[6,7] However, even though PEG-PE can be prepared in large quantities compared to G_{M1}, its preparation is still costly since PE is expensive.

The PEG-cholesterol (PEG-Chol) and PEG-dipalmitoylglycerol (PEG-DPG) conjugates were also synthesized by coupling monomethoxypoly (ethylene glycol) with either cholesterol or dipalmitoylglycerol via an ether bond.[8] The concentrations of the circulating liposomes containing PEG-Chol or PEG-DPG in mice blood were relatively high at 2 h post-injection, but much lower at 24 h post-injection compared to those containing G_{M1} or PEG-distearoylphosphatidylethanolamine. The shorter circulating lifetimes were attributed to the lack of effective long-lasting association of those compounds with the liposomes.

We synthesized a PEG derivative of cholesterol, PEG-Phe-Chol. Cholesterol was chosen as a lipid part since it has been frequently used in preparing stable liposomes. Phenylalanine was used as an another spacer in addition to the succinate. The Phe spacer provides an amide bond which may form a hydrogen bond with phospholipid molecules and enhance the retention time of the derivative in liposomes. Furthermore, the phenyl group allows one to monitor conveniently any further reaction and separation by thin layer chromatography since any compounds containing aromatic rings or polyconjugated moiety are easily detected with a UV lamp. The PEG with number-average molecular weight of 2000 was used since it has been most commonly employed in the long-circulating liposome formulations. Of course, all the starting materials are neither relatively toxic nor expensive. This paper describes the synthesis of the PEG derivative and its effects on liposome stability *in vitro*.

Experimental Section

Synthesis of PEG-Phe-Chol. A solution of N-(*tert*-butoxycarbonyl)-L-phenylalanine (0.200 g, 0.754 mmole), cholesterol (0.350 g, 0.905 mmole), diisopropylcarbodiimide (DIPC, 0.155 g, 0.980 mmole) and N,N-dimethylaminopyridinium *p*-toluenesulfonate (DPTS, 0.235 g, 0.754

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mmole) in dichloromethane (5 mL) was stirred for 3 h at room temperature. The reaction mixture was diluted with hexane (10 mL) and the resulting solid was filtered off. The filtrate was concentrated and the residue was chromatographed (silica gel, hexane/ethyl acetate = 10 : 1) to give 0.445 g of a white solid. The solid was dissolved in dichloromethane/trifluoroacetic acid (2/1, v/v) and stirred for 30 min at room temperature. The reaction mixture was basified with aqueous sodium carbonate solution, extracted with dichloromethane, dried over sodium sulfate, and concentrated to get 0.367 g of cholesteryl ester of L-phenylalanine which was used in the next step without further purification (91%).

A solution of cholesteryl ester of L-phenylalanine (0.149 g, 0.28 mmole) and N-hydroxysuccinimidyl monomethoxypoly(ethylene glycol) succinate (0.531 g, 0.24 mmole) in dichloromethane (5 mL) was stirred for 12 h at room temperature. The reaction mixture was washed with aqueous sodium carbonate solution, concentrated, and chromatographed (ethyl acetate/methanol = 10 : 1 and then dichloromethane/methanol = 10 : 1). The crude product was finally purified with recrystallization from hexane/dichloromethane to obtain pure PEG-Phe-Chol (0.589 g, 94%).

Preparation of Liposomes. 1,2-Distearoylphosphatidylcholine (DSPC) or a mixture of DSPC and either PEG-DPPE or PEG-Phe-Chol in chloroform was dried with a rotary evaporator under a reduced pressure. The dried lipid films were vacuum desiccated for 2 h and suspended with an aqueous calcein solution (25 mM, 300 mOms/L, pH 7.5). The samples were frozen, melt, and vortexed at 50-60 °C. The freeze-thawing cycle was repeated several times. The suspensions were then sonicated at 50-60 °C with a tip type sonicator (Misonic Inc.) for 5 min, followed by a 10 min resting period. The samples were then sonicated for an additional 5 min and rested for 10 min. The samples were finally sonicated for 5 min. The liposome suspensions were chromatographed on a Bio-Gel A column equilibrated with PBS (phosphate buffered saline, 300 mOms/L, pH 7.5) to remove unentrapped calcein. Dioleoylphosphatidylethanolamine (DOPE) liposomes containing PEG-DPPE or PEG-Phe-Chol were prepared as above, but the DOPE suspensions were vortexed and sonicated at room temperature. When necessary, appropriate amounts of cholesterol were added to the solutions of phospholipid and PEG-Phe-Chol in chloroform.

Leakage Test from Liposomes in Buffer and in Serum-Containing Medium. The small unilamellar DOPE liposomes (1.9×10^{-3} M) were incubated in PBS at pH 7.5 and 37 °C. A fluorometer (Simoaminoco Luminescence Spectrometer, Series 2, excitation at 490 nm, and emission at 520 nm) was used to measure the fluorescence intensity of calcein, and the percent leakage was calculated as described earlier.[9] For a study of plasma stability, a suspension of DOPE liposomes containing calcein (3.8×10^{-3} M) and the same volume of rabbit plasma were preincubated at 37 °C and mixed together. The fluorescence of the mixture was measured at different incubation time-points.

Phase Transitions of Lipid Bilayers. DSPC or a mixture of DSPC with 5 or 10 mol% PEG-Phe-Chol was hydrated in PBS with the freeze-thawing procedure (about 50 wt%). The hydrated sample was transferred into an aluminum DSC pan and sealed. The thermal phase transitions of the lipid bilayers were observed with a DSC 2910 differential scanning calorimeter (TA Instrument Co.).

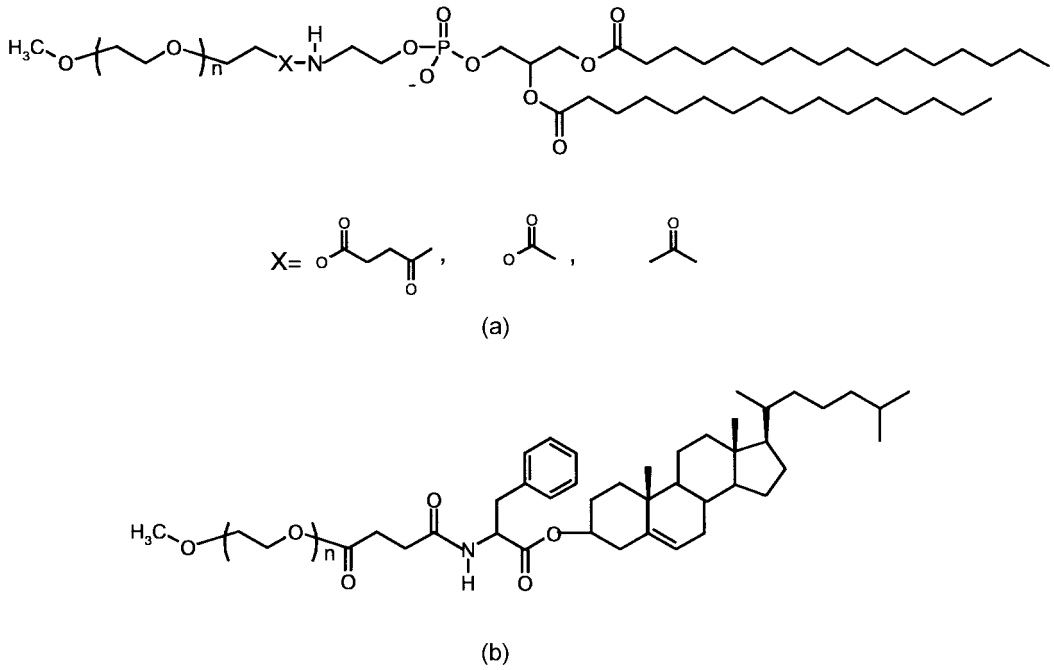
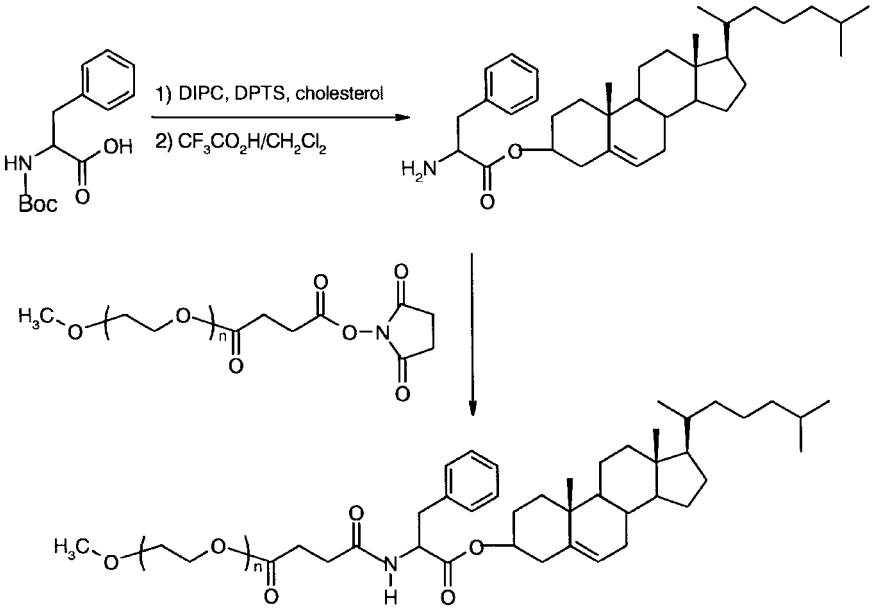


Figure 1. Chemical structures of (a) PEG-DPPE and (b) PEG-Phe-Chol.



Scheme 1. A chemical synthetic route of PEG-Phe-Chol.

Results and Discussion

N-(*tert*-Butoxycarbonyl)-L-phenylalanine was coupled to cholesterol via an ester bond in the presence of DIPC and DPTS as shown in Scheme 1, and the intermediate was treated with trifluoroacetic acid in order to obtain cholesteryl ester of L-phenylalanine. N-Hydroxysuccinimidyl monomethoxypoly(ethylene glycol) succinate was synthesized from monomethoxy poly(ethylene gylcol), succinic anhydride, and N-hydroxysuccinimide according to the previously reported procedure.[10] The final coupling reaction of cholesteryl ester of L-phenylalanine with N-hydroxysuccinimidyl monomethoxypoly(ethylene glycol) produced PEG-Phe-Chol as a white powder. The overall yield of the two steps from *N*-(*tert*-butoxycarbonyl)-L-phenylalanine was about 85%. Any side product was not detected in the final coupling reaction. The ^{1}H-NMR spectrum of PEG-Phe-Chol showed all of the expected signals without any appreciable impurity peaks, as shown in Figure 2a. The ^{13}C-NMR spectrum also shows the three characteristic carbonyl peaks at 170.6-172.5 ppm (Figure 2b), which are corresponding to the carbon atoms of the two different ester bonds and an amide bond. Another six characteristic peaks at 122.7-139.1 ppm are corresponding to the phenyl carbon atoms and the two olefinic carbon atoms in the cholesterol moiety. Thin layer chromatography experiment revealed that there was a single component in the final product, indicating that the isolated PEG-Phe-Chol was quite pure.

It is known that DSPC undergoes a pretransition and main transition at 51 °C and 55 °C, respectively.[11] DSPC bilayers prepared in this experiment show the pretransition at about 51 °C as a very small shoulder, but clearly show the main transition at 56 °C as shown in Figure 3. DSPC bilayers containing 5 mol% PEG-Phe-Chol show the main transition at 57 °C with a shoulder at 52 °C. On the other hand, the

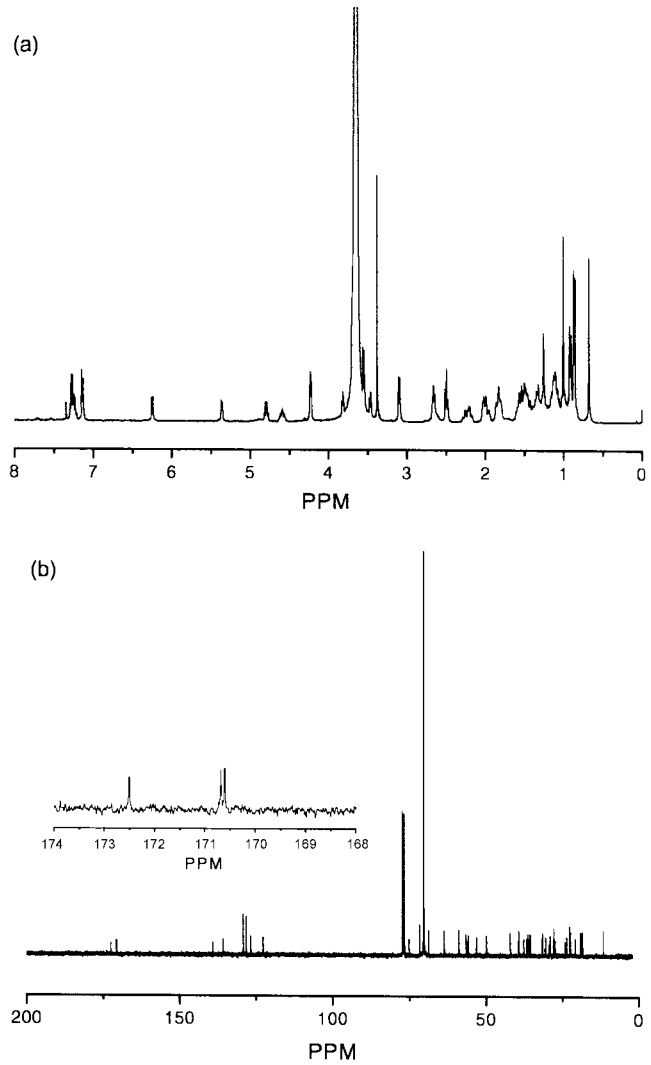


Figure 2. (a) ^{1}H and (b) ^{13}C NMR spectra of PEG-Phe-Chol in $CDCl_3$ (400 MHz).

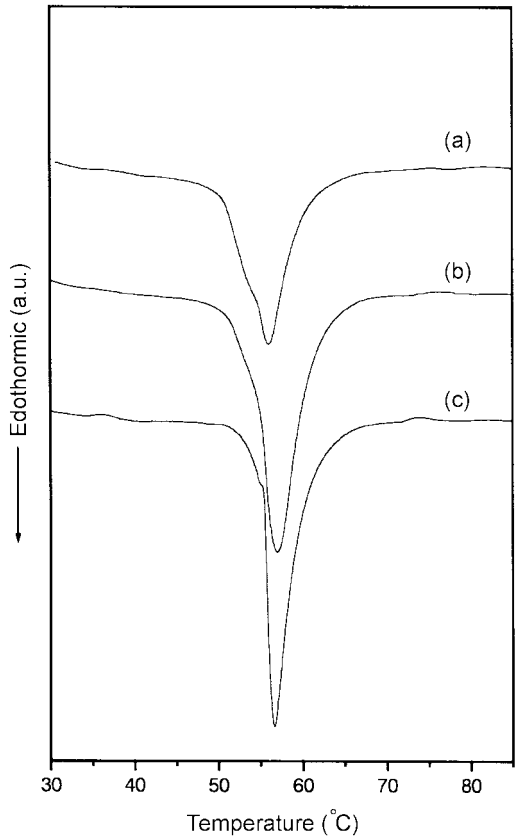


Figure 3. DSC thermograms of hydrated bilayers in PBS (about 50 wt%) of (a) DSPC only, (b) DSPC/PEG-Phe-Chol (95/5), and (c) DSPC/PEG-Phe-Chol (90/10). All of the thermograms were obtained from the second heating scans and the scan rate was 5 °C/min.

main transition of DSPC bilayers containing 10 mol% PEG-Phe-Chol appeared at 55 °C. The main transition width at the half height increased from 2.5 to 5.6 °C as the PEG-Phe-Chol content increased from 0 to 10 mol%. The enthalpy changes related to the transition peak size was not compared in this experiment since the concentrations of DSPC in the DSC samples could be different from the initial concentrations due to the procedure of sample transfer from the stocks to the DSC pans.

The pretransition is very sensitive to the presence of molecules which can interact with lipid head groups.[12] The pretransition of DSPC bilayers was shifted progressively toward a lower temperature region as the PEG-Phe-Chol content increased, indicating that PEG-Phe-Chol molecules interact with DSPC head groups. Any changes of the main transition in temperature, enthalpy, and the peak width result from a change of organization of the lipid molecules in bilayers. Thus this result indicates that PEG-Phe-Chol disturbs DSPC bilayers, and the extent of the disruption increases with the PEG-Phe-Chol content.

In order to determine the amount of PEG-Phe-Chol which can be added to DSPC or DOPE to prepare the corresponding liposomes, light-scattering measurements were performed for DSPC and DOPE dispersions prepared by the sonication method. In the case of DSPC, the size distribution

Table 1. Size distribution of lipid assemblies[a]

host lipid	PEG-Phe-Chol (mol%)	cholesterol (mol%)	size distribution (nm)
DSPC	0		89±8 (88), 300±60 (12)
	5		106±9 (95), 212 (4), 388 (1)
	10		8±1 (47), 13±2 (22), 100±25 (31)
	20		8±1 (59), 80±25 (41)
	10	30	27±3 (49), 99±21 (51)
DOPE	5		35±3 (25), 130±25 (75)
	10		47±10 (48), 116±20 (52)
	20		29±5 (50), 120±25 (50)
	5	30	110±23 (100)
	10	30	38±6 (46), 162±35 (54)

[a]In case of more than one population, the number in parenthesis indicates percentage of total population falling within theindicated size range.

was the bimodal type of small (89 ± 8 nm) and large (300 ± 60 nm) unilamellar liposomes as shown in Table 1. When the PEG-Phe-Chol content was 5 mol%, most DSPC liposomes were in the range of 106 ± 9 nm. However, when the PEG-Phe-Chol content was increased to 10 mol%, a significant amount (about 70%) of mixed micelles appeared. Cholesterol is known to largely affect the physical properties of lipid membranes. For example, the addition of cholesterol inhibited the formation of mixed micelles from liposomes of dipalmitoylphosphatidylcholine (DPPC) containing PEG-PE.[13] Thus 30 mol% cholesterol was added to the mixture of DSPC and 10 mol% PEG-Phe-Chol, but the mixed micelles were still formed.

On the other hand, the effect of PEG-Phe-Chol on DOPE liposomes was somewhat different from that of DSPC liposomes since a mixture of DOPE and PEG-Phe-Chol formed mixed micelles (25%) as well as liposomes (75%) when the PEG-Phe-Chol content was only 5 mol%. However, the incorporation of 30 mol% cholesterol into the mixture resulted in the formation of liposomes only. In other words, the formation of mixed micelles was completely inhibited by cholesterol. Even though the exact stabilization mechanism of the DOPE bilayers by cholesterol was not studied here, the inhibition of the liposome induction to mixed micelles may be due to the decreased curvature at the lipid-water interface since the total volume of the hydrocarbon region should be increased by cholesterol.

DOPE does not form liposomes by itself under the physiological conditions, and needs a second component such as fatty acid or other types of lipid.[14] It has been frequently used along with a protonatable component in preparing pH-sensitive liposomes which were designed to more efficiently deliver large or highly charged molecules into cells. In order to prolong circulation lifetime of the liposomes *in vivo*, sterically stabilized pH-sensitive DOPE liposomes were also developed by incorporation of PEG-PE.[13]

The stability of DOPE/cholesterol (7/3) liposomes containing 5 mol% of PEG-DPPE or PEG-Phe-Chol was studied by monitoring the leakage of entrapped calcein from the liposomes. The PEG derivative of 5 mol% is known to be more than enough for protection of the small liposomes surface.[16,17]

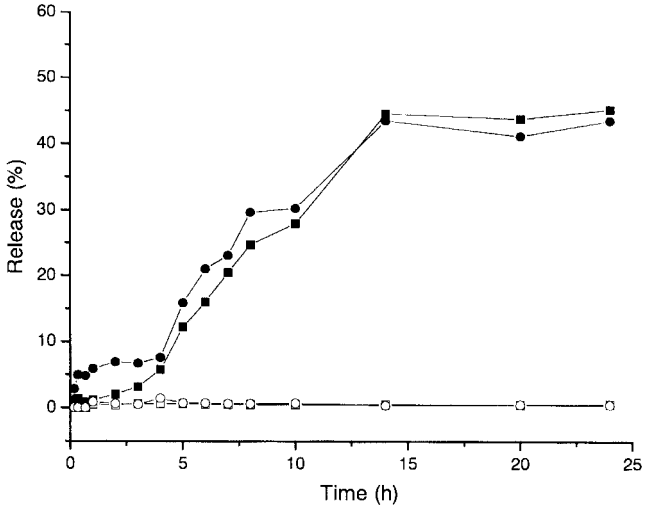


Figure 4. Percent release of entrapped calcein from DOPE/cholesterol (7/3) liposomes containing (●) 5 mol% PEG-DPPE or (■) PEG-Phe-Chol in 50% rabbit plasma at 37 °C. The opened symbols represent percent release of entrapped calcein from the same liposomes, but incubated in PBS at 37 °C.

The liposomes were incubated in PBS at 37 °C for 24 h, but the leakages from the two different liposomes were almost negligible since the amounts of the released calcein were less than 1% in both cases. This result indicates that DOPE molecules were tightly packed in the liposomes containing 30 mol% cholesterol and 5 mol% PEG-Phe-Chol.

During the incubation in 50% rabbit plasma at 37 °C, however, the leakage of the entrapped calcein from the liposomes increased almost linearly during the incubation for 14 h and reached a plateau of about 45% leakage as shown in Figure 4. If most of the PEG derivative molecules in outer monolayers of the DOPE liposomes are removed by the plasma proteins, the DOPE liposomes will be collapsed completely to release the entrapped calcein into the aqueous media. However, after the leakage for 14 h, the DOPE liposomes in the plasma did not release any more during the next several hours.

The leakage behavior of DOPE/cholesterol liposomes containing PEG-Phe-Chol was very similar to that of the liposomes containing PEG-DPPE. This result suggests that PEG-Phe-Chol in the DOPE liposomes may not be easily extracted out by the plasma proteins, and the degree of retention of PEG-Phe-Chol in DOPE liposomes may be similar to that of PEG-DPPE even though the retention test should be performed *in vivo* for further direct comparison. The amide bond in PEG-Phe-Chol may play an important role in retaining the new PEG derivative in the liposomes *via* hydrogen bonding with lipid molecules.

Conclusion

PEG-Phe-Chol was successfully synthesized from the activated PEG intermediate and cholesterol ester of L-phenylalanine. A mixture of DSPC and PEG-Phe-Chol formed liposomes only when the PEG derivative content was 5 mol%, but formed mixed micelles as well as liposomes when the derivative content was 10 mol%. DOPE formed mixed micelles as well as liposomes when the PEG-Phe-Chol content was just 5 mol%. However, the incorporation of 30 mol% cholesterol to the mixture completely inhibits the fromation of the mixed micelles. According to the DSC result, PEG-Phe-Chol destabilzes the DSPC bilayers, resulting from the formation of the mixed micelles. The relative amount of mixed micelles depended on the content of the PEG derivative in the lipid mixtures. The leakages of entrapped calcein from DOPE/cholesterol (7/3) liposomes containing 5 mol% PEG-Phe-Chol were similar to those of the DOPE/cholesterol (7/3) liposomes containing 5 mol% PEG-DPPE during the incubation in PBS or 50% rabbit plasma for 24 h at 37 .

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