Notes

Synthesis of Dihexadecyl *N*-[Monomethoxypoly(ethylene glycol)] -L-glutamate and Its Effect on Liposomes

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Many types of liposome-based procucts have been extensively studied for effective drug delivery systems.[1] However, 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] Two factors that govern the affinity of liposomes to the RES may be the nonspecific hydrophobic interactions of liposomes with RES cells and a specific opsonization reaction involving blood components.[4-6] To overcome this problem, ganglioside GM1 was incorporated into liposomes. The liposomes containing ganglioside GM1, which was called "stealth liposomes", showed considerably prolonged circulation lifetimes *in vivo*.[7-9] Therapeutic applications of GM1-containing liposomes, however, are not practical due to the high expense of GM1 and the difficulty in obtaining large quantities either by extraction of natural sources or by synthesis.

Diacyl phosphoethanolamine-*N*-[monomethoxypoly (ethylene glycol) succinate] (PEG-PE) was also reported to prolong the circulation time of liposomes.[10] The long circulation time may allow concentration of PEG-coated liposomal drugs in solid tumors.[11] 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.[12,13]

A new type of immunoliposomes carrying monoclonal antibodies at the distal ends of the PEG chains enhanced the lung binding.[14] Allen *et al.* synthesized various types of PEG derivatives and studied their effect on the RES uptake.[15] Parr *et al.* reported the influence of different chemical links between PEG and lipid moieties on circulation lifetime.[16] Huang *et al.* studied interaction of PEG-PE with cholesterol-phospholipid mixtures,[17] and effects of PEG chain length and lipid acyl chain composition on the interaction of PEG-PE with phospholipid.[18] The pH-sensitive liposomes containing PEG-PE decreased the pH-dependent release of entrapped calcein in buffer or cell culture medium, but sterically stabilized the liposomes *in vivo*.[19] The effect of PEG-PE on liposome structure and leakage at submicellar concentrations was also reported.[20]

Even though PEG-PE can be prepared in relatively large quantities, the preparation of PEG-PEs is still costly due to the high price of PE. Thus we attempted to investigate an alternative to PEG-PE which can be readily prepared in large quantities. Dihexadecyl *N*-[monomethoxypoly(ethylene glycol) 2000 succinate]-L-glutamate (PEG-DHG) was synthesized, and its interaction with phospholipid bilayers was compared with that of PEG-DPPE (1,2-dipalmitoylphosphatidylethanolamine) (Figure 1).

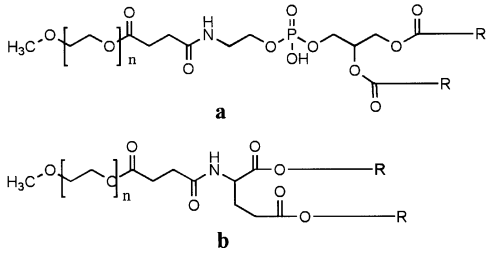


Figure 1. Chemical structures of (a) PEG-DPPE and (b) PEG-DHG.

Experimental Section

Synthesis of PEG-DHG. Compound **1** was prepared as previously described procedure.[21] A solution of compound **1** (0.60 g, 0.84 mmol) and Pd/C (10%, 0.10 g) in hexane/ethyl acetate (1/2, 10 mL) was stirred at room temperature under 1 atmosphere of hydrogen until TLC showed that the reaction was completed. After filtration, the filtrate was concentrated and the resulting compound **2** was used in the next step without further purification.

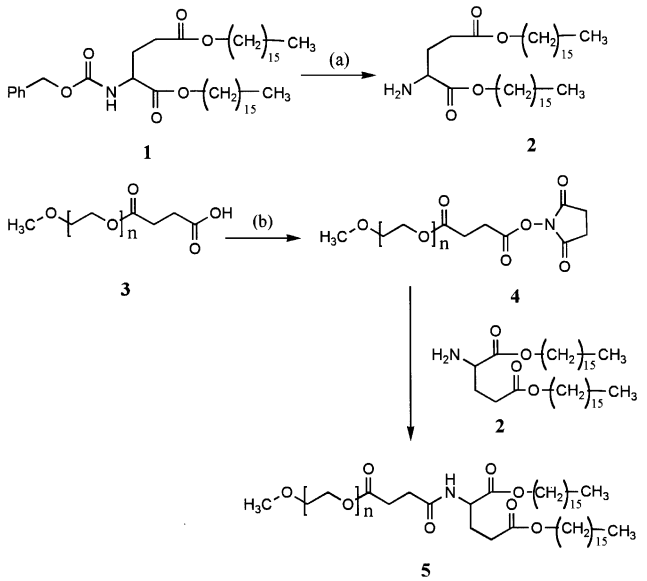
Compound **3** was prepared according to the previously reported procedure.[15] A solution of compound **3** (0.80 g, 0.38 mmol), DCC (0.10 g, 0.49 mmol), and *N*-hydroxysuccinimide (0.048 g, 0.42 mmol) in dichloromethane (20 mL) was stirred for 1 h at room temperature in order to obtain compound **4**. The crude compound **2** (0.27 g, 0.46 mmol) was added to the above solution and stirred for an additional 3 h. The precipitate was filtered off and the filtrate was concentrated. The residue was subjected to silica gel column in ethyl acetate/methanol (10/1) and then in dichloromethane/methanol (15/1) to obtain compound **5**, PEG-

DHG (0.86 g).

Preparation of liposomes. 1,2-Distearoylphosphatidylcholine (DSPC) or a mixture of DSPC with PEG-DPPE or PEG-DHG in chloroform was dried with a rotary evaporator. The dried lipid films were vacuum desiccated for 1 h and suspended with an aqueous calcein solution (50 mM, 300 mOsm/L, pH 8.0). The samples were frozen, melt, and vortexed at 70-80 °C. The freeze-thawing cycle was repeated several times. The mixture was then sonicated at 70 °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. The transparent liposome suspension was chromatographed on Bio-Gel A column equilibrated with PBS (phosphate buffered saline, 300 mOms/L, pH 7.5) to remove unentrapped calcein. The phospholipid concentration was determined by employing the Bartlett method.[22] DOPE liposomes containing PEG-DPPE or PEG-DHG were prepared as above, but the DOPE suspensions were vortexed and sonicated at room temperature.

Measurements of % leakage from liposomes. Small liposomes (4.4×10^{-4} M) were incubated in PBS at pH 7.5 and 37 °C. A fluorometer (Simoaminoco Luminescence Spectrometer, Series 2) was used to measure the fluorescence intensity of calcein, and the percent leakage was calculated as described earlier.[23] For a study of plasma stability, calcein-containing liposome suspensions (1.7×10^{-4} M) and the same volume of human plasma were preincubated at 37 °C and mixed together. The fluorescence of the mixture was measured at different incubation time-points.

Measurements of phase transition temperatures. DSPC or a mixture of DSPC with 6 mol% PEG-DPPE, or PEG-DHG was hydrated in PBS with the freeze-thawing procedure (15 wt%). The hydrated sample was transferred into an aluminum DSC pan and sealed. Thermal phase transitions of the bilayers were observed with a DSC 2910 differential scanning calorimeter (TA Instrument Co.).



Scheme 1. A chemical synthetic route of PEG-DHG: (a) H_2 (1 atm), Pd/C (10%), hexane/ethyl acetate (1/2); (b) DCC, N-hydroxysuccinamide, CH_2Cl_2.

Results and Discussion

Deprotection of the *N*-carbobenzyloxy group of compound **1** was performed with Pd/C in the hydrogen atmosphere to obtain compound **2**. Compound **3** was prepared from monomethoxy PEG (MW 2000) and succinic anhydride, and converted to compound **4**,[16] which reacts readily with compound **2** to yield the final target compound. The synthesis of PEG-DHG was efficient, and the overall yield was 85% based on compound **3**.

A proton NMR spectrum of PEG-DHG showed all of the expected signals without any significantly appreciable impurity peaks. A carbon-13 NMR spectrum of the compound indicated four peaks in the range of 171-173 ppm, corresponding to four carbon atoms of the carbonyl groups in PEG-DHG: three ester groups and one amide group.

Small DSPC liposomes containing 6 mol% PEG-DHG, which is greater than the minimum required for full protection,[15] were prepared. The released calcein from the DSPC liposomes with or without PEG-DHG was less than a few

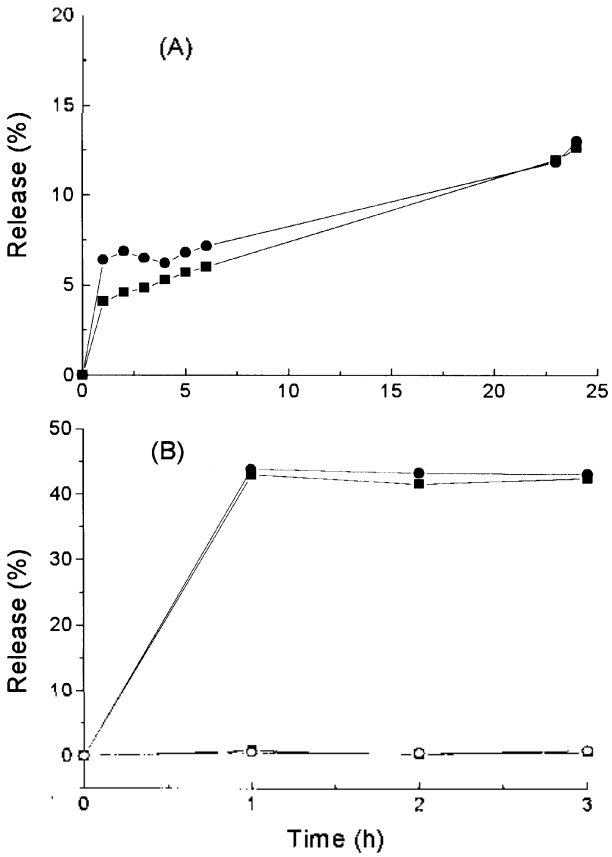


Figure 2. (A) Release of entrapped calcein from DSPC liposomes containing (●) 6 mol% PEG-DPPE and (■) 6 mol% PEG-DHG in 50% human plasma at 37 °C; (B) Release of entrapped calcein from DOPE liposomes containing (○) 10% PEG-DPPE, or (□) 10% PEG-DHG in PBS, and (●) 10% PEG-DPPE, or (■) 10% PEG-DHG in 50% human plasma at 37 °C.

percents within 4 h at 37 °C (data not shown), suggesting that PEG-DHG molecules do not destabilize the DSPC liposomes. The stability of DSPC liposomes containing 6 mol% PEG-DPPE or PEG-DHG was also tested in 50% normal human plasma. The leakage rate of calcein from the liposomes containing PEG-DHG was similar to that for liposomes containing PEG-DPPE (Figure 2A). This result indicates that PEG-DHG does not increase the leakage rate of DSPC liposomes incubated in PBS or in 50% plasma compared to PEG-DPPE.

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.[24] DOPE/PEG-DPPE (10/1, mol/mol) and DOPE/PEG-DHG (10/1, mol/mol) liposomes released only a few percents of entrapped calcein within 3 h in PBS at 37 °C (Figure 2B). If most of PEG derivative molecules in outer monolayers of the DOPE liposomes are removed by plasma proteins, the DOPE liposomes will be collapsed completely to release the entrapped calcein into the aqueous media. However, the DOPE liposomes in 50% human plasma released only about 45% of the entrapped calcein within 1 h, and did not release any more during the next 2 h. Regardless of the exact mechanism about the loss of the PEG derivatives from the liposomes, the present result suggests that the PEG derivatives in the outer monolayers of DOPE liposomes may not be easily extracted out by plasma proteins, and the degree of retention of PEG-DHG 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 with PEG-DPPE.

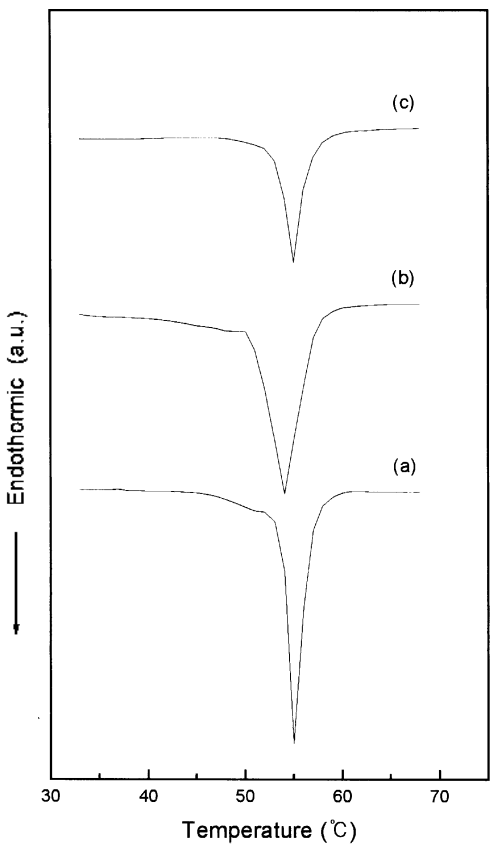


Figure 3. DSC thermograms of hydrated bilayers of (a) DSPC only, (b) DSPC with 6 mol% PEG-DPPE, (c) DSPC with 6 mol% PEG-DHG. All of the thermograms were obtained from the second heating scans and the scan rate was 5 °C/min.

It is known that DSPC undergoes the pretransition and the main transition at 51 and 55 °C, respectively.[25] DSPC bilayers prepared in this experiment show the pretransition as a very small shoulder at about 51 °C, but clearly show the main transition at 55 °C as shown in Figure 3. DSPC bilayers containing PEG-DPPE or PEG-DHG show only the main transition at 54 or 55 °C, respectively. 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 sample transfer procedure from the stocks to DSC pans.

The pretransition is very sensitive to the presence of molecules which can interact with the lipid head groups.[26] The pretansition of DSPC bilayers was not detected when PEG-DPPE or PEG-DHG molecules were present in the DSPC bilayers, indicating that both PEG-DPPE and PEG-DHG molecules interact with DSPC head groups. Any modification of the main transition in temperature, enthalpy, and the peak width results from a change of organization of the lipid molecules in bilayers. With PEG-DHG molecules, the main transition temperature was not shifted. On the other hand, the transition peak width was slightly increased compared to that for pure DSPC bilayers, but narrower than that for the bilayers containing PEG-DPPE. This result indicates that incorporation of PEG-DHG into DSPC bilayers may not disturb significantly DSPC bilayers, and at least does not disturb them as much as PEG-DPPE.

In summary, PEG-DHG was successfully synthesized. Retention of PEG-DHG in DOPE liposomes was comparable to that of PEG-DPPE *in vitro*. Disturbance of DSPC bilayers by PEG-DHG may be comparable to or less than that by PEG-DPPE. The influences of PEG-DHG on the circulation lifetime of liposomes and biodistribution of liposomal contents are under investigation and will be reported in the near future.

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