Liposomes with detachable polymer coating: destabilization and fusion of dioleoylphosphatidylethanolamine vesicles triggered by cleavage of surface-grafted poly(ethylene glycol)

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Abstract Plasma-stable liposomes (100 nm) were prepared from dioleoylphosphatidylethanolamine (DOPE) and 3–6 mol% of a new disulfide-linked poly(ethylene glycol)-phospholipid conjugate (mPEG-DTP-DSPE). In contrast to similar preparations containing non-cleavable PEG-phospholipid conjugate, thiolytic cleavage of the grafted polymer chains facilitated rapid and complete release of the liposome contents. Furthermore, the detachment of PEG from DOPE liposomes resulted in liposomal fusion. Finally, while formulation of pH-sensitive DOPE/cholesterol hemisuccinate liposomes with mPEG-DTP-DSPE abolished the pH sensitivity, cleavage of the PEG chains completely restored this property. These are the first examples of new useful properties of liposomes grafted with cleavable polymer.

Key words: Liposomes; Drug targeting; Phosphatidylethanolamine; Polyethylene glycol derivatives; Membrane fusion; Disulfide bond

1. Introduction

Incorporation of mPEG-lipid conjugates in liposomal bilayer results in formation of so-called sterically stabilized liposomes (SSL), which are distinguished by their low RES uptake, and prolonged circulation lifetimes [1,2]. SSLs also tend to extravasate into pathological foci, which leads to improved delivery of liposomal drugload [3,4]. If a cell-internalizable targeting device is linked to the PEG coating of a SSL, specific uptake by target cells is observed [5–7]. However, grafting of PEG on the liposome surface interferes with the ability of the liposome to undergo membrane fusion and destabilization [8] which are important mechanisms for making SSL-carried pharmaceuticals available to their biological targets. One possible solution is to create SSL capable of losing their protective polymer coating after they reach their destination site. This can be achieved by introducing a cleavable linkage between the polymer chain and the hydrophobic moiety of the liposome bilayer [9].

Here we report the synthesis and properties of SSLs containing mPEG-DTP-DSPE, a novel, thiolytically cleavable conjugate. To ensure fusogenicity, the balance of the liposome lipid was DOPE, a non-bilayer forming, fusion facilitating lipid [10]. We demonstrate that while DOPE/mPEG-DTP-DSPE liposomes are quite stable, thiolytic cleavage of the grafted polymer off the liposomal surface leads to vesicle destabilization and fusion, accompanied by complete release of the entrapped contents. In a different formulation, removal of the polymeric barrier resulted in a lipid vesicle stable at neutral pH, but which released entrapped solute in response to a lower pH. This is the first demonstration of some of the useful properties manifested by SSLs with cleavable PEG-lipid conjugates.

2. Materials and methods

2.1. General

Lipids were purchased from Avanti Polar-Lipids; HPTS and DPX were from Molecular Probes; most other chemicals were from Sigma. Human plasma (Blood Transfusion Center, Moffit Hospital, San Francisco) was thawed and filtered through a 0.45 μm sterile filter. Horse serum (0.1 μm refiltered, tissue culture grade) was from Hyclone. Carbamate-linked mPEG-DSPE [11] and mPEG-NH_2 [12] were synthesized from mPEG (MW 2000) as reported. TLC ($CHCl_3$-CH_3OH-H_2O 90:18:2) on silica gel G (Analtech) was visualized with I_2 vapor, Dragendorff and ninhydrin spray reagents [13].

2.2. Preparation of mPEG-DTP-DSPE

Solution of DTSP [14] (873 mg, 2 mmol) in dimethylformamide (10 ml) was treated with mPEG-NH_2 (2 g, 1 mmol) and triethylamine (140 ml). After 15 min TLC showed that the reaction was complete. The polymer was recrystallized twice from isopropanol and dried in vacuo over P_2O_5. Yield 1.7 g (73%). ^{1}H-NMR (CD_3OD): δ 2.6 (m, SCH_2CH_2CON), 2.85 (s, Su, 4H), 3.0 (overlapping m, $SCH_2CH_2CO_2$-Su and SCH_2CH_2CON), 3.38 (s, CH_3, 3H), 3.64 (s, PEG, ≈180H). The composition of the product mixture, mPEG-DTP-OSu and (mPEG-)$_2$DTP (mol% ratio ≈75:25), was deduced from the integration of the peaks at 2.6, 2.85, and 3.0 ppm. Solid DSPE (100 mg) was added to the $CHCl_3$ solution of the recovered

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Abbreviations: CHEMS, cholesteryl hemisuccinate; Chol, cholesterol; DOPC, 1,2-dioleoyl-*sn*-glycerophosphorylcholine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphorylethanolamine; DPX, *p*-xylene-bis-pyridinium bromide; DSPE, 1,2-distearoyl-*sn*-glycero-3-phosphorylethanolamine; DTSP, dithiobis(succinimidyl propionate); DTT, dithiothreitol; HEPES-NS, 20 mM *N*-hydroxyethyl-piperazinoethanesulfonic acid, 144 mM NaCl, pH 7.2; HPTS, trisodium 8-hydroxypyrenetrisulfonate; MES, *N*-morpholinoethanesulfonic acid; NBD-PE, *N*-(7-nitro-2-1,3-benzoxadiazol-4-yl)-phosphatidylethanolamine (egg transphosphatidylated); PEG, poly(ethylene glycol); mPEG, methoxypoly(ethylene glycol); mPEG-DSPE, *N*-(ω-methoxypoly(oxyethylene)-α-oxycarbonyl)-DSPE; mPEG-DTP-DSPE, *N*-(2-(ω-methoxypoly(oxyethylene)-α-aminocarbonyl)ethyl-dithiopropionyl)-DSPE; mPEG-DTP-OSu, *N*-succinimidyl-(2-(ω-methoxypoly(oxyethylene)-α-aminocarbonyl)ethyl-dithiopropionate; PL, phospholipid; Rho-PE, *N*-lissamine-rhodamine B sulfonyl-phosphatidylethanolamine (egg transphosphatidylated); RES, reticuloendothelial system; SSL, sterically stabilized liposomes

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polymer (600 mg) and triethylamine (240 μl). The suspension was incubated at 45°C until it clarified. Complete consumption of DSPE was confirmed by TLC. The polymer lipid conjugate (mPEG-DTP-DSPE) was purified by dialysis as described [11]. Yield 269 mg (70%). ^{1}H-NMR (CD_3OD): δ 0.88 (t, CH_3, 6H), 1.26 (s, CH_2, 56H), 1.58 (br m, $CH_2CH_2C{=}O$, 4H), 2.31 (2×t, $CH_2C{=}O$, 4H), 2.62 (2×t, SCH_2CH_2CON 4H), 2.98 (t, CH_2-CONHDSPE, 4H), 3.36 (s, CH_3O, 3H), 3.4 (2×t, CH_2N, 4H), 3.64 (s, PEG, ≈180H), 3.9 (q, CH_2CHCH_2OP, 2H), 4.0 (t, NCH_2CH_2OP, 2H), 4.17 and 4.39 (2× dd, OCH_2CHCH_2OP, 2H), 5.2 (m, OCH_2CHCH_2O, 1H).

2.3. Preparation and measurements of liposomal samples

Liposomes were prepared from the mixture of DOPE or DOPC with either mPEG-DSPE or mPEG-DTP-DSPE at the molar ratios indicated in the text. pH-sensitive liposomes were prepared from DOPE and CHEMS (4:1 molar ratio) [15,16]. Lipid films were hydrated in HEPES-NS or HPTS-DPX solution (30 mM HPTS, 30 mM DPX, pH 7.2, adjusted to 290 mOs with NaCl) and extruded through two 0.1 μm polycarbonate membranes [17,18]. Untrapped dye was removed by gel filtration. Liposome size was determined by dynamic laser light scattering, and lipid concentration by phosphate assay [19]. Entrapped volume of the liposomes was calculated from the amount of incorporated HPTS determined by fluorometry as below. The release of entrapped solute was studied by fluorescence-dequenching assay using liposomes with entrapped HPTS-DPX. Percent of released HPTS was determined as the increase in sample fluorescence (λ_{em} = 512 nm, λ_{ex} = 413 nm – pH-independent isosbestic point [20]) over that of the preincubation sample (zero release) normalized to the increase in fluorescence obtained after lysis of preincubation sample with 0.2% Triton X-100 (100% release). Lipid mixing was studied by energy transfer assay based on the label dilution [21,22]. Liposomes containing 1 mol% each of NBD-PE and Rho-PE were incubated with 'unlabeled' liposomes (without fluorescent lipids) in the ratio 1:3 (total PL ≈ 10 mM). To prepare the reference sample with 100% lipid mixing, an aliquot of the reaction mixture was dried, solubilized in chloroform and evaporated in vacuum. The lipids were rehydrated in HEPES-NS and dispersed by sonication. The fluorescence intensities of NBD at 525 nm (F_{525}) and Rho at 590 nm (F_{590}) (λ_{ex}=468 nm) were used to calculate the degree of lipid mixing from the formula:

$$\text{Percent lipid mixing} = 100\times(R-R_0)/(R_{100}-R_0),$$

where R is F_{525}/F_{590} of the sample, R_0 is F_{525}/F_{590} of the 'labeled' liposomes (no mixing), and R_{100} is F_{525}/F_{590} corresponding to 100% lipid mixing. To assess stability in blood serum or plasma, liposomes with entrapped HPTS-DPX were incubated in 75% human blood plasma or 75% horse blood serum, at 37°C and 2–2.5 mM PL. At designated intervals, aliquots were taken and the released dye was determined as described above. The fluorescence intensity of HPTS was not affected by serum, plasma or added detergent.

3. Results and discussion

In order to study the properties of lipid vesicles surface-modified with cleavable PEG chains, we prepared a new disulfide-linked PEG-phospholipid conjugate. Our choice was governed by the well documented stability/lability properties of disulfides in both chemical and biological systems. The synthesis of the disulfide conjugate is summarized in Fig. 1. Substitution of only one of the succinimidyl esters of DTSP with mPEG-NH_2 was facilitated by using an excess of the bifunctional reagent. Under these conditions the desired product, mPEG-DTP-OSu, constituted the bulk of the polymer recovered from the reaction mixture (70–80% by NMR). The minor product, symmetrical disulfide of *N*-(mPEG)-mercaptopropionamide, did not interfere with the DSPE-coupling reaction. Hence the recovered polymer was reacted with DSPE until the latter was completely consumed. The conjugate, mPEG-DTP-DSPE, was purified by removing the excess of PEG-reagents by dialysis. The structure of the purified

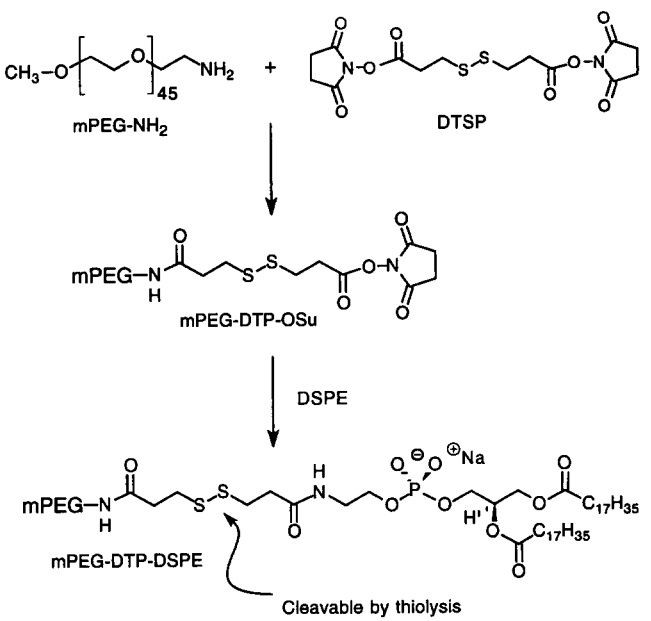

Fig. 1. Schematic depiction of synthesis of thiolytically cleavable mPEG-DTP-DSPE.

conjugate (single spot by TLC) was corroborated by ^{1}H-NMR. Thiolytic lability of the conjugate was confirmed by its incubation with an excess of DTT resulting in the disappearance of the original spot (Rf = 0.55) on TLC, and the appearance of two new spots (Rf = 0.68 and 0.23 for PEG and lipid components respectively).

At physiological pH and ionic strength DOPE does not form bilayers, but rather exists in an inverted hexagonal (H_{II}) phase. To produce liposomes from a DOPE-rich lipid, it must be stabilized in a bilayer phase by an amphiphile with bulky and/or repulsing hydrophilic moieties [23], the requirement perfectly satisfied by PEG-phospholipid conjugates. DOPE readily formed liposomes with either mPEG-DSPE or mPEG-DTP-DSPE (2.9 or 5.7 mol% of PL). These liposomes were similar in size (94–105 nm) and entrapped volume (1.27–1.58 l/mol PL) to their DOPC counterparts. These values agreed well with the reported parameters of membrane-extruded unilamellar vesicles [18]. Both phospholipid-PEG conjugates were not only potent stabilizers of DOPE bilayers, but also provided excellent solute retention in the resulting vesicles. Even prolonged (36 h) incubation with plasma or serum at 37°C did not result in significant loss of entrapped membrane-impermeable solute, HPTS (Fig. 2). In fact the solute retention of DOPE liposomes in the presence of plasma or serum exceeded that of their DOPC counterparts. This finding may be explained by tighter packing of PE molecules in the bilayer as compared with the PC of the same fatty acid composition [24]. Liposomes prepared from phospholipids of low transition temperature usually require Chol to achieve plasma stability [25]. It was recently shown that equimolar mixtures of DOPE and Chol containing various amounts of PEG-lipids form liposomes under physiological conditions [26,27]. Our results indicate that the cholesterol requirement is circumvented by the presence of PEG-phospholipid. The high stability of DOPE liposomes with PEG-phospholipids is also in contrast to DOPE liposomes stabilized with low-molecular weight amphiphiles, which leak in the presence of plasma or serum [23].

When DOPE/mPEG-DTP-DSPE (100:3) liposomes were treated with DTT, a potent thiolytic agent, massive and complete release of entrapped dye occurred after a short lag phase, while the same treatment had no effect on the dye release from DOPE liposomes stabilized with the same amount of mPEG-DSPE (Fig. 3A). DTT-induced release of the dye from mPEG-DTP-DSPE-stabilized liposomes was accompanied by visible aggregation and precipitation of the lipid. These observations can only be explained by the thiolytic cleavage of grafted PEG off the liposome surface and the subsequent loss of bilayer stability leading to the transition of DOPE into hexagonal and/or isomorphic phase. Such phase transition is usually accompanied by fusion and disintegration of the vesicles and by the release of entrapped solute [28]. Inherent instability of the bilayer phase of the bulk liposomal lipid was necessary for the release of liposome contents since the treatment of DOPC/mPEG-DTP-DSPE liposomes with DTT under the same conditions produced no leakage (Fig. 3A). It is noteworthy that DTT itself, although used in relatively high concentration, did not have any effect on the solute release from DOPE/mPEG-DSPE liposomes.

Surface-grafted PEG chains even at low concentration are an efficient steric barrier that prevents fusion of liposome membranes [8]. Since the fusion potential of a liposome stripped of its PEG coating was an important prerequisite in the design of the liposomes described in the present work, we used a lipid mixing assay to ascertain whether fusion may occur after the detachment of PEG from the surface of DOPE/mPEG-DTP-DSPE liposomes (Fig. 3B). Indeed, incubation of DOPE/mPEG-DTP-DSPE liposomes with DTT resulted in lipid mixing consistent with fusion. After a short lag phase the degree of lipid mixing gradually increased and reached the value of 70% in 2 h (Fig. 3B); then the fusion process slowed down but continued until 100% mixing was achieved in 24 h (not shown). Note that in the absence of DTT neither DOPE/mPEG-DSPE nor DOPE/mPEG-DTP-DSPE liposomes exhibited any appreciable lipid mixing, confirming that surface-attached PEG prevents fusion. These results indicate that removal of PEG chains from the surface of DOPE-liposomes causes not only release of the liposome-entrapped solute, but also mixing of lipid bilayers consistent with fusion. Evidently the removal of PEG is complete enough to allow close contact of lipid bilayers required for fusion. Interestingly, the lag phase for lipid mixing was shorter ($\approx$10 min) than that for contents release ($\approx$30 min). This suggests that leakage follows fusion and may result from the same bilayer perturbations that lead to fusion.

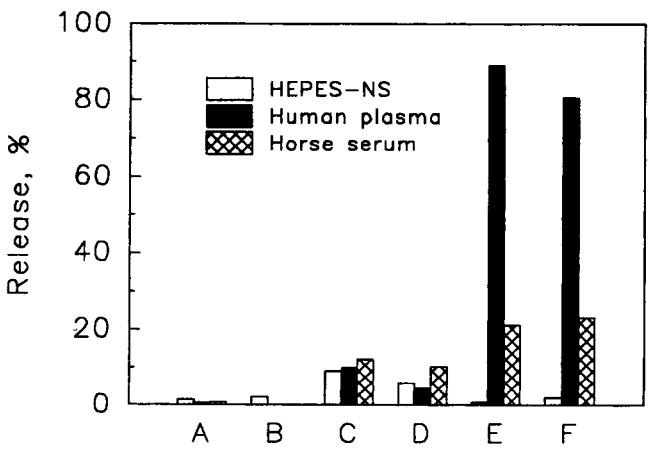

Fig. 2. Release of entrapped fluorescent marker from the liposomes at 37°C after 36 h incubation in various media. Lipid compositions: DOPE/mPEG-DSPE 100:3 (A) or 100:6 (B); DOPE/mPEG-DTP-DSPE 100:3 (C) or 100:6 (D); DOPC/mPEG-DSPE 100:3 (E) or 100:6 (F).

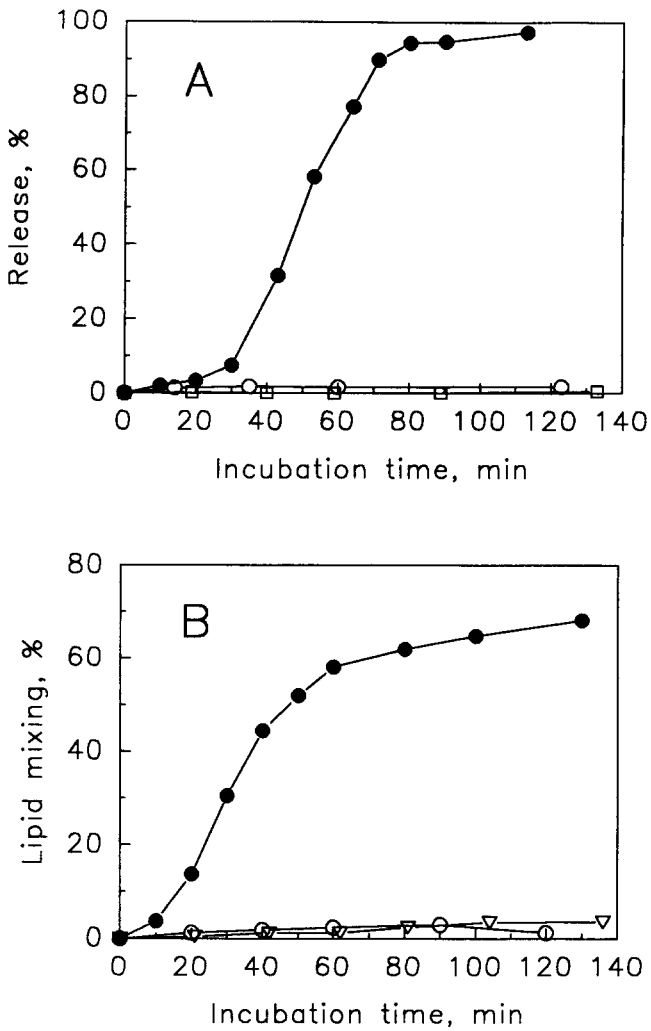

Fig. 3. (A) Release of entrapped fluorescent marker from the liposomes incubated with DTT: DOPE/mPEG-DTP-DSPE (filled circles); DOPE/mPEG-DSPE (open circles); DOPC/mPEG-DTP-DSPE (squares). (B) Effect of DTT on lipid mixing in the liposomes containing thiolytically cleavable or non-cleavable PEG-phospholipid. DOPE/mPEG-DTP-DSPE with DTT (filled circles) or without DTT (open circles); DOPE/mPEG-DSPE with DTT (triangles). PEG-lipid, 2.9 mol%; incubation in HEPES-NS (pH 7.2) at 37°C; DTT (10 mM).

Addition of weakly acidic, carboxyl-bearing amphiphiles, e.g. CHEMS, stabilizes DOPE in the bilayer phase in a pH-dependent manner. When pH drops to levels suppressing ionization of the amphiphile's carboxyl, the lipid undergoes phase transition with concomitant release of liposomal contents. Such pH-sensitive liposomes are potentially promising as carriers specific to the acidic environment of endosomes and many tumors [15,16,22,23,29,30]. Yet their potential use in vivo is hampered by their fast clearance by RES. It is well established that RES clearance of liposomes is dramatically reduced by surface-grafted PEG chains [1,2,4]. It is not clear, however, how steric stabilization with PEG-phospholipids affects pH sensitivity of liposomes composed of a non-bilayer

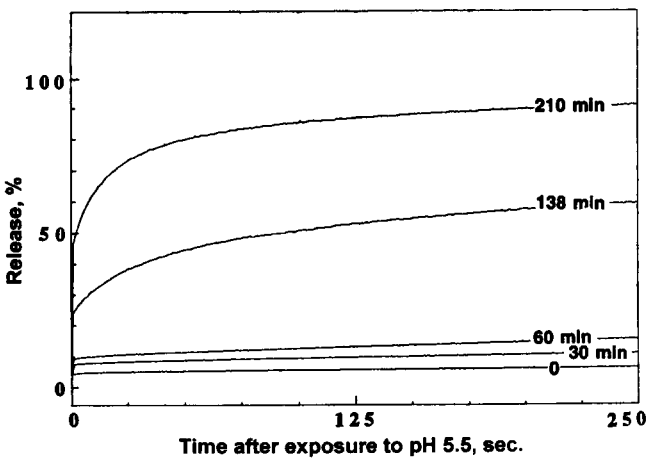

Fig. 4. Effect of incubation with DTT on the release of entrapped dye from the liposomes of DOPE/CHEMS/mPEG-DTP-DSPE (80:20:3). Liposomes were incubated in HEPES-NS with 10 mM DTT at 37°C and 10 mM PL. At times indicated on each curve, aliquots of liposomes were transferred into fluorometer cuvette containing 20 mM MES, 144 mM NaCl, pH 5.5, at room temperature, and the dye release was monitored.

forming lipid and an ionizable amphiphile. To answer this question, we have prepared liposomes containing DOPE, CHEMS, and mPEG-DSPE (80:20:3), and studied the solute release of entrapped DPX-quenched HPTS after acidification to pH 5.5. In contrast to DOPE/CHEMS (80:20) liposomes [15], which are stable at pH 7.4 but at pH 5.5 completely released the entrapped dye in a few minutes, there was only minor dye leakage under the same conditions from DOPE/CHEMS liposomes stabilized by either mPEG-DSPE or mPEG-DTP-DSPE. Such a loss of pH sensitivity after stabilization of DOPE/CHEMS liposomes with mPEG-DSPE was not unexpected since bilayer-stabilizing properties of this compound are likely to be pH-independent, at least in the pH 4–7 range, where neither PEG chain solubility nor dissociation of the phosphate group in the phosphodiester residue (pK_a 2.12) are affected. However, during incubation of DOPE/CHEMS/mPEG-DTP-DSPE (80:20:3) liposomes with DTT, their pH sensitivity was gradually restored (Fig. 4). This process was slower than the destabilization of DOPE/mPEG-DTP-DSPE liposomes under similar conditions. The efficiency of dye release from DOPE/CHEMS/mPEG-DTP-DSPE vesicles at pH 5.5 approached that of DOPE/CHEMS liposomes only after 4 h incubation with DTT. Such a difference can be explained by the presence of residual *N*-mercaptopropionyl residues on the surface of the liposomes after the loss of mPEG chains which, in synergy with CHEMS, might have an additional stabilizing effect on the DOPE bilayer. Overall, however, while the incorporation of 2.9 mol% PEG-phospholipid in the mixture of DOPE and CHEMS produced liposomes that lost their pH sensitivity, this property was restored by the cleavage of the polymer chains from the liposomal surface.

We have illustrated here the design of lipid vesicles sterically stabilized by PEG surface-grafted via chemically cleavable bond. While the utility of this system in vivo may require further optimization in linkage chemistry and/or lipid formulation, several features of heretofore unknown liposomes with detachable PEG chains were clearly demonstrated. Thus the use of cleavable PEG-lipids may provide SSL with the capacity for membrane fusion and rapid drug release specifically triggered by the detachment of the polymer. Fine-tuning of the linkage chemistry will allow this to occur in response to either natural environment at the target site or to external stimuli. In conclusion, lipid vesicles containing labile PEG-lipids open the opportunity for a number of new applications in further refinement of liposomal drug delivery.

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References

[1] Woodle, M.C. and Lasic, D.D. (1992) Biochim. Biophys. Acta 1113, 171–199.
[2] Lasic, D. and Martin, F. (Eds.) (1995) Stealth® Liposomes. CRC Press, Boca Raton, FL.
[3] Wu, N.Z., Da, D., Rudoll, T.L., Needham, D., Whorton, A.R. and Dewhirst, M. (1993) Cancer Res. 53, 3763–3770.
[4] Papahadjopoulos, D., Allen, T.M., Gabizon, A., Mayhew, E., Matthay, K., Huang, S.K., Lee, K.-D., Woodle, M.C., Lasic, D.D., Redemann, C. and Martin, F.J. (1991) Proc. Natl. Acad. Sci. USA 88, 11460–11464.
[5] Park, J.W., Hong, K., Carter, P., Asgari, H., L.Y., G., Keller, G.A., Wirth, C., Shalabi, R., Kotts, C., Wood, W.I. and Papahadjopoulos, D. (1995) Proc. Natl. Acad. Sci. USA 92, 1327–1331.
[6] Allen, T.M., Brandeis, E., Hansen, C.B., Kao, G.Y. and Zalipsky, S. (1995) Biochim. Biophys. Acta 1237, 99–108.
[7] Lee, R.J. and Low, P.S. (1995) Biochim. Biophys. Acta 1233, 134–144.
[8] Holland, J.W., Hui, C., Cullis, P.R. and Madden, T.D. (1996) Biochemistry 35, 2618–2624.
[9] Zalipsky, S. (1995) in: Stealth® Liposomes (Lasic, D. and Martin, F., Editor), CRC Press, Boca Raton, FL. pp. 93–102.
[10] Allen, T.M., Hong, K. and Papahadgopoulos, D. (1990) Biochemistry 29, 2976–2985.
[11] Zalipsky, S. (1993) Bioconjugate Chem. 4, 296–299.
[12] Zalipsky, S., Gilon, C. and Zilkha, A. (1983) Eur. Polym. J. 19, 1177–1183.
[13] Zalipsky, S., Brandeis, E., Newman, M.S. and Woodle, M.C. (1994) FEBS Lett. 353, 71–74.
[14] Lomant, A.J. and Fairbanks, G. (1976) J. Mol. Biol. 104, 243–261.
[15] Lai, M.Z., Vail, W.J. and Szoka, F.C. (1985) Biochemistry 24, 1654–1661.
[16] Chu, C.J., Dijksta, J., Lai, M.Z., Hong, K. and Szoka, F.C. (1990) Pharm. Res. 7, 824–834.
[17] Olson, F., Hunt, C.A., Szoka, F.C. and Papahadjopoulos, D. (1979) Biochim. Biophys. Acta 557, 9–23.
[18] Hope, M.J., Bally, M.B., Webb, G. and Cullis, P.R. (1985) Biochim. Biophys. Acta 812, 55–65.
[19] Morrison, W.R. (1964) Anal. Biochem. 7, 281–284.
[20] Daleke, D.L., Hong, K. and Papahadjopoulos, D. (1990) Biochim. Biophys. Acta 1024, 352–366.
[21] Struck, D.K., Hoekstra, D. and Pageno, R.E. (1985) Biochemistry 24, 3099–3106.
[22] Connor, J., Yatvin, M.B. and Huang, L. (1984) Proc. Natl. Acad. Sci. USA 81, 1715–1718.
[23] Litzinger, D.C. and Huang, L. (1992) Biochim. Biophys. Acta 1113, 201–227.
[24] Boggs, J.M. (1980) Can. J. Biochem. 58, 755–770.
[25] Mayhew, E., Rustum, Y., Szoka, F. and Papahadgopoulos, D. (1979) Cancer Treatment Rep. 63, 1923–1928.
[26] Holland, J.W., Cullis, P.R. and Madden, T.D. (1996) Biochemistry 35, 2610–2617.
[27] Litzinger, D.C. and Huang, L. (1992) Biochim. Biophys. Acta 1127, 249–254.
[28] Wilschut, J. (1991) in Membrane Fusion (Hoekstra, D. and Wilschut, J., Eds.) pp. 89–126. Marcel Dekker, New York.
[29] Dusgunes, N., Straubinger, R.M., Baldwin, P.A., Friend, D.S. and Papahadjopoulos, D. (1985) Biochemistry 24, 3091–3098.
[30] Collins, D., Litzinger, D.C. and Huang, L. (1990) Biochim. Biophys. Acta 1025, 234–242.