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Structural and functional comparisons of pH-sensitive liposomes composed of phosphatidylethanolamine and three different diacylsuccinylglycerols

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The titratable, double-chain amphiphiles 1,2-dipalmitoyl-*sn*-3-succinylglycerol (1,2-DPSG), 1,2-dioleoyl-*sn*-3-succinylglycerol (1,2-DOSG) and 1,3-dipalmitoylsuccinylglycerol (1,3-DPSG) have been used in combination with phosphatidylethanolamine (PE) to form pH-sensitive liposomes. The effect of the compounds on dielaidoyl PE bilayer stabilization was examined by differential scanning calorimetry. Only 1,2-DPSG showed bilayer stabilization activity; whereas the other two are destabilizers at pH 7.4. All three amphiphiles became strong destabilizers at pH 5.0. The ability of the amphiphiles to stabilize DOPE liposomes was examined by light scattering and calcein entrapment. In general, 1,2-DPSG is the most potent stabilizer of PE bilayers while 1,3-DPSG is the weakest liposome stabilizer. All three compounds can be combined with DOPE to generate liposomes which are stable at neutral and basic pH. At weakly acidic pH, the liposomes are leaky and exhibit extensive lipid mixing, with protons and calcium showing synergistic effects on lipid mixing. DOPE/1,2-DPSG liposomes are stable in human plasma and remain acid-sensitive even after prolonged plasma incubation. Immunoliposomes prepared from either DOPE/1,2-DPSG or DOPE/1,2-DOSG can deliver diphtheria toxin A fragment to the cytoplasm of cultured cells in a process which involves endocytosis of the liposomes. Immunoliposomes prepared with 1,2-DPSG are more effective drug carriers than those prepared with 1,2-DOSG. These results indicate that the bilayer- and, hence the liposome-stabilization activity of the diacylsuccinylglycerol depends on the structure of the compounds. The potential drug delivery activity of the pH-sensitive liposomes composed of these lipids is discussed.

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Abbreviations: PE, phosphatidylethanolamine; DOPE, dioleoylphosphatidylethanolamine; DEPE, dielaidoylphosphatidylethanolamine; TPE, PE prepared from transphosphatidylation of egg phosphatidylcholine; DOPC, dioleoylphosphatidylcholine; *N*-NBD-DOPE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-DOPE; *N*-Rh-TPE, *N*-(lissamine rhodamine B-sulfonyl)-TPE; 1,2-DPSG, 1,2-dipalmitoyl-*sn*-3-succinylglycerol; 1,2-DOSG, 1,2-dioleoyl-*sn*-3-succinylglycerol; 1,3-DPSG, 1,3-dipalmitoyl-2-succinylglycerol; DTA, diphtheria toxin fragment A; DSC, differential scanning calorimetry; SUV, small unilamellar vesicles; LUV, large unilamellar vesicles; PBS, phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 5.5 mM KH_2PO_4, 1.12 mM Na_2HPO_4); DPPG, dipalmitoylphosphatidylglycerol; DOPG, dioleoylphosphatidylglycerol; IC_{50}, concentration of DTA which yields 50% inhibition of protein synthesis; T_H, peak temperature of $L_\alpha \rightarrow H_{II}$ phase transition endotherm.

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Introduction

pH-sensitive liposomes are stable at neutral pH and become leaky and/or fusion competent under acidic conditions [1–4]. Liposomes of this type have been shown to be effective, non-toxic vehicles for delivery of cytotoxic drugs [5], protein toxins [6] and plasmid DNA [7–9] to cultured cells. The mechanism of delivery involves liposome uptake by the cellular endocytic pathway and exposure of the liposomes to the mildly acidic pH of the endosomes. The liposomes then fuse with or destabilize the endosome membrane and release their contents into the cell cytoplasm [6,7]. While effective for in vitro delivery to cultured cells, their instability in bodily fluids has hampered the in vivo usage of pH-sensitive liposomes [10].

Previous formulations of pH-sensitive liposomes combined phosphatidylethanolamine (PE) with single-chain amphiphiles such as fatty acids [3] or *N*-acyl

amino acids [2]. In the case of fatty acid-containing pH-sensitive liposomes, serum instability arises following the rapid transfer of liposomal fatty acid to serum proteins [11]. The loss of stabilizing fatty acid also leads to bilayer defects which promote the insertion of proteins into the liposome bilayer [11]. In order to improve the serum stability, a high level of cholesterol has to be included in the liposome membranes [8,11]. However, inclusion of cholesterol seriously compromises the fusion activity of the liposomes [2]. Thus, the production of cholesterol-free liposomes which remain stable in plasma and retain pH sensitivity is desirable. Recently, pH-sensitive liposomes composed of DEPE and a series of synthetic double-chain amphiphiles were prepared and characterized by Leventis et al. [12]. These liposomes were shown to be less leaky in serum than TPE/oleic acid liposomes [12]. One of the double chain amphiphiles used by Leventis et al. is dioleoylsuccinylglycerol which, when mixed with DEPE, exhibits synergistic liposome fusion effect induced by proton and calcium ions [12].

In the present study, we have extended the work of Leventis et al. and we have described some important structural and functional properties of pH-sensitive liposomes prepared from diacylsuccinylglycerols and PE, including a comparison of the 1,2-diacyl and 1,3-diacyl isomers. The plasma stability of these pH-sensitive liposomes is also investigated, as well as the ability of the liposomes to mediate cytoplasmic delivery of DTA to cultured cells. These studies have shed some light on the structural basis of the liposome stabilization activity of the double-chain titratable amphiphiles, which should be useful information in the design of new drug delivery systems.

Materials and Methods

Materials

DOPE, DEPE, DOPC, *N*-NBD-DOPE and *N*-Rh-TPE were purchased from Avanti Polar Lipids (Birmingham, AL). 1,2-Dipalmitoyl-*sn*-glycerol, 1,2-dioleoyl-*sn*-glycerol, 1,3-dipalmitoylglycerol and succinate anhydride were purchased from Sigma Chemical Co. (St. Louis, MO). All solvents were re-distilled before use. Lipid purity was evaluated by thin layer chromatography using I_2 vapor detection. Phospholipids were quantitated by phosphate assay [13] and non phospholipids quantitated by chromate assay [14].

Synthesis of double-chain amphiphiles

Diacylglycerol (either 1,2-dipalmitoyl, 1,2-dioleoyl, or 1,3-dipalmitoyl) was combined with succinate anhydride and triethylamine in $CHCl_3$ at a mol ratio of 1 : 1 : 0.1 (diacylglycerol/ succinate anhydride/ triethylamine). The mixture was incubated at 37 °C for 16 h and then subjected to water extraction to remove hydrophilic contaminants and triethylamine. Diacylsuccinylglycerols were purified by preparative thin-layer chromatography using hexanes/ethyl ether/methanol/glacial acetic acid (70 : 30 : 5 : 1, v/v) as the solvent system. Diacylsuccinylglycerols had an R_f in this solvent system of approx. 0.18 while diacylglycerols exhibited R_f values of approx. 0.5. The identities of the compounds were verified by infrared spectroscopy using a Perkin-Elmer 1430 Ratio Recording Infrared Spectrophotometer.

Acylated antibody preparation

Anti-H2k^k antibody from the murine hybridoma cell line 11-4.1 was purified, labeled with ^{125}I and derivatized with the *N*-hydroxysuccinimide ester of palmitic acid as described by Huang et al. [15].

Preparation of diphtheria toxin fragment A

DTA was prepared from intact toxin (List Biological Laboratories, Campbell, CA, Lot No. DT-08) by the method of Cumber et al. [16]. The DTA was then labeled with ^{131}I using Iodogen (Pierce Chemical Co., Rockford, IL).

Differential scanning calorimetry

Samples for DSC were mixed and dried from $CHCl_3$ with N_2 gas. The dried lipid films were desiccated for 0.5 h under vacuum and then suspended in either PBS (pH 7.4) or 0.1 M citrate/citric acid buffer (pH 5.0) at a lipid concentration of 5 mM. The samples were incubated for 6 h at 45 °C then cooled to 25 °C and incubated at this temperature for 16 h before being loaded into the calorimeter. The samples were scanned from 25 °C to 85 °C at 20 deg/h using a Microcal MC2 calorimeter.

Liposome preparation

(1) Small unilamellar vesicles (SUV). Various combinations of phospholipid and diacylsuccinylglycerols were combined and dried from $CHCl_3$ with N_2 gas. The dried lipid films were vacuum desiccated and suspended in either PBS (pH 8.0) or 50 mM calcein, PBS (pH 8.0). The samples were then incubated overnight at 4 °C. Upon hydration, the sample pH was found to decrease and was adjusted to pH 8.0 prior to sonication. The samples were sonicated for 10 min in a bath-type sonicator (Laboratory Supplies, Hicksville, NY) followed by a 10 min resting period during which the pH of the samples was measured and adjusted to pH 8.0, if necessary. The samples were then sonicated for an additional 10 min. After 2 h incubation at room temperature, liposomes containing calcein were separated from unentrapped calcein by passage over a Bio-Gel A 1.5 m column equilibrated with PBS (pH 7.6) which was made isoosmotic to the calcein solution by addition of NaCl. To evaluate lipid recovery after chromatography, a trace

amount of hexadecyl [^{3}H]cholestanyl ether (0.8 μCi/mmol lipid) was included in the samples prior to $CHCl_3$ evaporation.

(ii) Large unilamellar vesicles (LUV). Liposomes of the indicated composition were prepared by a reverse phase evaporation method [17] with modifications [18,19]. Briefly, $CHCl_3$ solutions of DOPE and either 1,2-DOSG or 1,2-DPSG were combined at a 4:1 (mol/mol) ratio and the $CHCl_3$ evaporated under N_2 gas. The samples were desiccated and hydrated with either PBS (pH 8.0) or 50 mM calcein in PBS buffer (pH 8.0) at 4°C. The mixture was then sonicated and the pH adjusted to pH 8.0, since acidification occurred during hydration. Diethyl ether was added to the samples at a 3:1 (v/v) ratio and the samples sonicated 30 s to form a stable emulsion. DTA-containing liposomes were prepared by adding DTA (50 μg/10 μmol lipid) plus a trace amount of [^{131}I]DTA prior to diethyl ether addition. The diethyl ether was removed from emulsions by rotary evaporation under reduced pressure until liquified. The liposomes were then extruded five times through two stacked Nuclepore filters (0.1 μm). Calcein-containing liposomes were fractionated over a Bio-Gel A 1.5 m column as described above.

Immunoliposome preparation

LUV were prepared as described above except palmitoyl anti-H2K^k antibody in PBS (pH 8.0), 0.15% deoxycholate was added at a lipid/antibody ratio of 10:1 (w/w) to the liquified sample after rotary evaporation. To facilitate antibody incorporation, diethyl ether was added to the vortexing suspension to a final concentration of 2% (v/v) [19]. The liposome/antibody mixture was then dialyzed for 24 h against 12 l of PBS (pH 7.8) to remove diethyl ether and deoxycholate. The samples were then extruded as described above. Liposomes and immunoliposomes containing DTA were fractionated over a Bio-Gel A 0.5 m column to remove unencapsulated DTA.

Light scattering of liposomes

Sonicated dispersions of various compositions were diluted to a final lipid concentration of 20 μM with PBS (pH 8.0). Light scattering at 90° was measured in a Perkin-Elmer LS5 spectrofluorometer using an excitation and emission wavelength of 660 nm.

Calcein entrapment

The degree of calcein entrapment in liposomes was measured by the self-quenching of calcein fluorescence. The percent quenching was calculated from

$$\% \text{ quenching} = (1 - (F_0/F_T)) \cdot 100$$

where F_0 and F_T are the fluorescence intensities (λ_{ex} = 490 nm; λ_{em} = 520 nm) before and after addition of deoxycholate (final concentration 0.2%). The quenching of the samples was also recorded periodically over 21 days storage at 25°C.

Acid-induced leakage

SUV or LUV containing calcein were diluted to a final lipid concentration of 50 μM in PBS (pH 7.8) and assayed for acid-induced calcein release as described previously [2].

Acid-induced lipid mixing

Labeled SUV or LUV containing 1 mol% *N*-NBD-DOPE and 0.5 mol% *N*-Rh-TPE were incubated with a 3-fold excess of unlabeled liposomes of the same composition. Acid induced lipid mixing was assayed as described by Connor et al. [2]. To examine the effect of Ca^{2+} on lipid mixing, $CaCl_2$ was added to the sample cuvette prior to liposome addition.

Plasma-induced leakage

SUV or LUV of various compositions were diluted 10-fold into normal human plasma (Fort Sanders Regional Medical Center, Knoxville, TN) or PBS which had been prewarmed to 37°C and incubated at 37°C throughout the assay. At various times after liposome addition, 10-μl aliquots of the mixtures were removed and assayed for calcein release using

$$\% \text{ Release} = 100(F - F_0)/(F_T - F_0)$$

where F_0 is the initial calcein fluorescence, F is the fluorescence at various times of incubation in plasma or PBS and F_T is the total fluorescence after addition of deoxycholate (0.2%).

In some experiments, calcein-containing SUV which had been incubated in plasma for 3 h at 37°C were separated from excess plasma components by fractionation over a Bio-Gel A 1.5m column equilibrated with PBS (pH 8.0). The samples were then assayed for acid-induced leakage as described above.

Cytotoxicity assay

The delivery of DTA to murine L929 cells by liposomes or immunoliposomes was evaluated by the incorporation of [^{3}H]leucine into cellular proteins as described [6], except the cells were harvested onto glass fiber filters using an automated cell harvester (Cambridge Technology, Watertown, MA).

Results

Calorimetric studies

It is known that titratable amphiphiles can modulate the physical properties of PE. We have investigated the thermotrophic phase behavior of DEPE and various amounts of diacylsuccinylglycerols by differential scan-

ning calorimetry. For ease of comparison, we have plotted T_H, the temperature at the peak of the bilayer-to-hexagonal phase transition endotherm, vs. mol% of diacylsuccinylglycerol. Fig. 1 shows that 1,2-DPSG is a weak stabilizer of the DEPE L_α bilayer phase at pH 7.4, showing a small increase of T_H only at 16 mol%. Both 1,2-DOSG and 1,3-DPSG destabilize the DEPE bilayer phase by decreasing the temperature of the bilayer-to-hexagonal phase transition. When mixtures of DEPE and either 1,2-DPSG or 1,2-DOSG are suspended in pH 5.0 buffer, both amphiphiles decrease the T_H (Fig. 1). Thus, 1,2-DPSG is a bilayer stabilizer at pH 7.4, but becomes a destabilizer at a mildly acidic pH. 1,3-DPSG also decreases the temperature of this transition at low pH (data not shown).

DEPE is useful for physical studies such as calorimetry since evaluation of both the chain melting temperature and the hexagonal phase transition temperature is convenient. However, DEPE is not useful for studies which involve cultured cells since experiments must be carried out at temperatures near the chain melting temperature (37 °C) of the lipid. For this reason, all the experiments described below use DOPE rather than DEPE.

Stabilization of DOPE liposomes

Modifications of the bilayer-to-hexagonal phase transition of DOPE by diacylsuccinylglycerols is difficult to measure by DSC, because the transition involves a small enthalpy change which is also critically dependent on the thermal history of the sample [20]. We have thus studied the stabilization of DOPE liposomes by optical methods. The formation of stable liposomes was monitored by light scattering at 660 nm. To determine the amounts of 1,2-DPSG or 1,3-DPSG needed to form stable, small liposomes, sonicated dispersions of DOPE

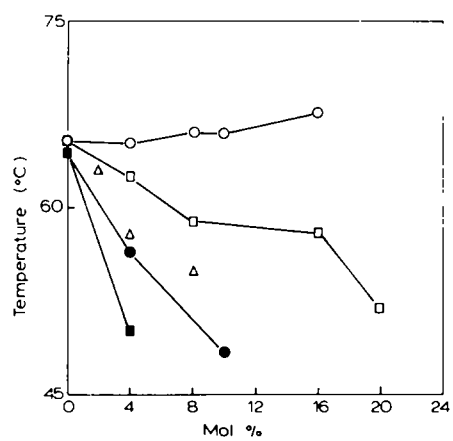

Fig. 1. Effect of diacylsuccinylglycerols on the $L_\alpha \rightarrow H_{II}$ phase transition of DEPE. The peak temperatures of transition, T_H, were determined by differential scanning calorimetry (scan rate 20 deg/h) and plotted vs. mol% diacylsuccinylglycerol. Open symbols pH 7.4; closed symbols, pH 5.0. DEPE containing 1,2-DPSG (○, ●), 1,2-DOSG (□, ■), 1,3-DPSG (△).

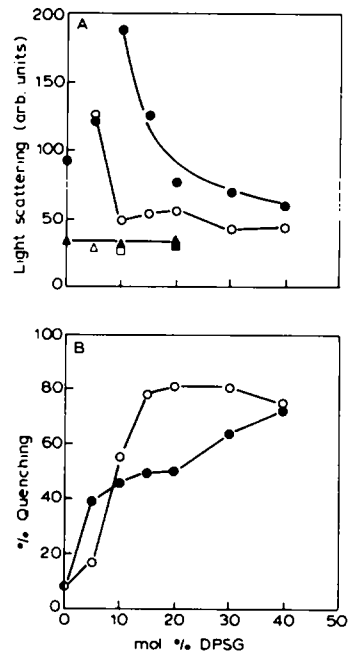

Fig. 2. Stabilization of SUV composed of DOPE and various amounts of DPSG. (A) Effect of DPSG on light scattering. 90° light scattering was monitored as a function of mol% DPSG in DOPE with 1,3-DPSG (●) or 1,2-DPSG (○) and in DOPC with 1,3-DPSG (▲) or 1,2-DPSG (△). (B) Effect of DPSG on calcein entrapment. Fluorescence quenching was evaluated as a function of DPSG concentration for sonicated dispersions of DOPE with 1,2-DPSG (○) or 1,3-DPSG (●).

and various mol% of the amphiphiles were prepared. When stable sonicated vesicles (SUV) were formed, the turbidity was low resulting in low light scattering. At concentrations of 20 mol% or above, SUVs of 1,3-DPSG and DOPE could be formed (Fig. 2A). At 1,3-DPSG concentrations between 10 and 20 mol% the turbidity of the samples was high, indicating the presence of non-uniform, aggregated particles. Below 10 mol% 1,3-DPSG, large precipitates formed and the light scattering was again low (Fig. 2A). DOPE/1,2-DPSG SUV could be prepared at amphiphile concentrations of greater than 10 mol% (Fig. 2A). Between 5 and 10 mol% 1,2-DSPG the samples were quite turbid and the samples aggregated at 1,2-DPSG concentrations below 5 mol%. DOPC formed small vesicles regardless of the concentration of either 1,2-DPSG or 1,3-DPSG (Fig. 2A). In all cases, DOPE/1,3-DPSG samples were more turbid than DOPE/1,2-DPSG samples. This indicates that samples containing 1,3-DPSG were larger and/or more aggregated than those containing 1,2-DPSG. These results show that 1,2-DPSG is a stronger DOPE liposome stabilizer than the 1,3-DPSG.

We also examined the ability of sonicated dispersions of DOPE/DPSG (1,2-DPSG or 1,3-DPSG) to encapsulate calcein. When neither 1,3-DPSG or 1,2-DPSG are included in the samples, no calcein entrapment is obtained as indicated by the low quenching (Fig. 2B). As the amount of 1,2-DPSG in DOPE is increased to 10

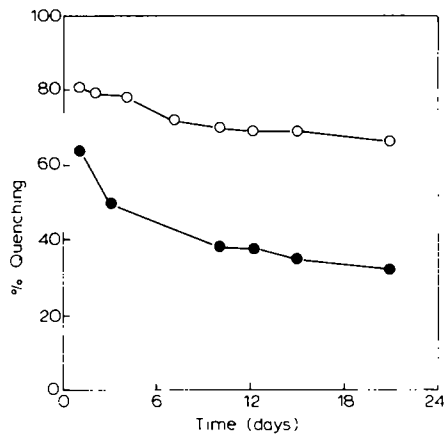

Fig. 3. Long term stability of SUV composed of DOPE and DPSG. Fluorescence quenching of entrapped calcein was measured over 3 weeks storage for DOPE/1,2-DPSG (4:1, mol/mol) liposomes (○) and DOPE/1,3-DPSG (7:3, mol/mol) liposomes (●).

mol%, the quenching of calcein increases to approx. 50%. At and above 15 mol% 1,2-DPSG, maximal quenching (approx. 80%) for this calcein concentration is observed (Fig. 2B). The addition of 5 mol% 1,3-DPSG leads to an increase in the concentration of encapsulated calcein and an increase in the % quenching of the samples (Fig. 2B). Between 10 and 20 mol% 1,3-DPSG, no great increases in trapping are noted. At 30 and 40 mol% 1,3-DPSG, the percent quenching increases and equals the amount of calcein entrapped by 1,2-DPSG-containing liposomes at 40 mol% 1,3-DPSG (Fig. 2B). These results again indicate that 1,2-DPSG has a higher bilayer stabilization activity than the 1,3-DPSG.

For comparison of the long-term stability of SUV prepared using DOPE and either 20 mol% 1,2-DPSG or 30 mol% 1,3-DPSG, we evaluated the quenching of calcein as a function of storage time at 25°C (Fig. 3). For 1,3-DPSG stabilized SUV, significant loss of contents occurs within the first three days of incubation and continues to decline with time up to 21 days (Fig. 3). SUV prepared from 1,2-DPSG and DOPE are much more stable upon storage with very little leakage of calcein occurring over a 3-week period (Fig. 3).

Acid-induced liposome destabilization

SUV composed of DOPE and 30 mol% 1,3-DPSG are acid sensitive (Fig. 4A) as are SUV composed of DOPE and 20 mol% 1,2-DPSG (Fig. 4B). As the pH of the buffer is decreased, calcein leakage from DOPE/1,3-DPSG (7:3, mol/mol) increases. The pH at which 50% release occurs (pH_{50}) for this liposome composition is 4.6 (Fig. 4A). By contrast, DOPE/1,2-DPSG (4:1, mol/mol) SUV exhibit a pH_{50} of 5.3 (Fig. 4B).

SUV composed of DOPE/1,2-DPSG (4:1, mol/mol) also undergo mixing of membrane components under acidic conditions (Fig. 5). For SUV of this composition, in the absence of Ca^{2+}, no significant lipid mixing is noted until the media pH is below 5.5 (Fig. 5). Maximal lipid mixing occurs at pH ≈ 4.0 and the pH_{50} for lipid mixing in the absence of Ca^{2+} is 4.8 (Fig. 5). The presence of Ca^{2+} at 2 mM can modulate the acid sensitivity of DOPE/1,2-DPSG (4:1, mol/mol) liposomes (Fig. 5). Significant lipid mixing occurs even at pH values as high as 7.0. When Ca^{2+} is included in the buffer, the pH_{50} is 6.0 (Fig. 5). LUV of this lipid composition gave very similar results, except that higher Ca^{2+} concentrations were needed to give an equivalent effect on pH_{50} (data not shown).

Plasma stability

In comparison to previous formulations of pH-sensitive liposomes prepared as LUV by the reverse-phase evaporation method, DOPE/1,2-DPSG (4:1, mol/mol) and DOPE/1,2-DOSG (4:1, mol/mol) liposomes are much more stable in human plasma (Fig. 6). In agreement with earlier results [10], LUV composed of DOPE/OA (4:1, mol/mol) leak rapidly when exposed to human plasma at 37°C (Fig. 6). This rapid release seems to involve the removal of OA from the liposome membrane and insertion of plasma proteins and lipids into the liposome membrane [11]. Significant leakage from DOPE/OA liposomes also occurs in PBS when the temperature is elevated to 37°C (Fig. 6). Liposomes prepared from DOPE and either 1,2-DPSG or 1,2-DOSG (both at 20 mol%) are quite stable in human

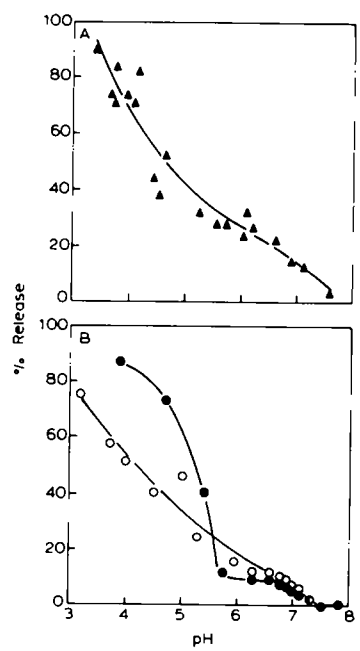

Fig. 4. (A) Acid-induced leakage from SUV composed of DOPE/1,3-DPSG (7:3, mol/mol). Calcein release was monitoired at different pH values as described in Materials and Methods. (B) Effect of human plasma pre-incubation of acid sensitivity of SUV composed of DOPE/1,2-DPSG (4:1, mol/mol). Liposomes were assayed for acid-induced leakage of calcein either before (●) or after (○) 3 h incubation at 37°C in the presence of 90% human plasma.

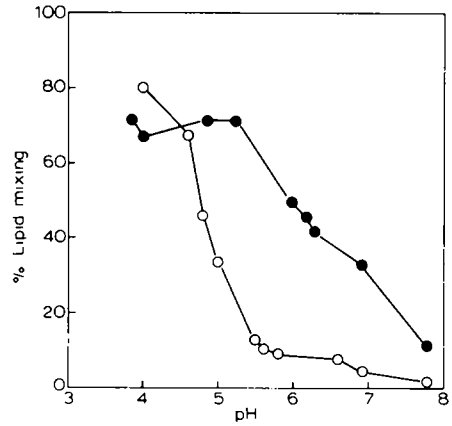

Fig. 5. Acid-induced membrane mixing for SUV composed of DOPE/1,2-DPSG (4:1, mol/mol). Lipid mixing was monitored as a function of pH in the absence (○) or presence (●) of 2 mM Ca^{2+}.

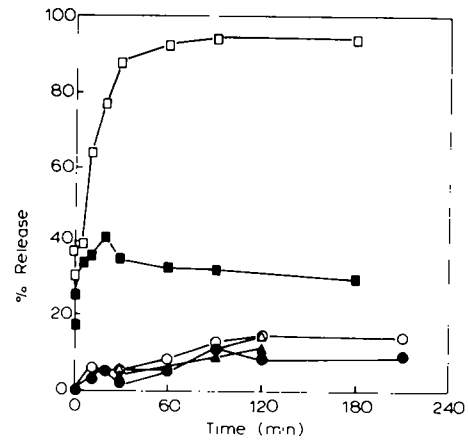

Fig. 6. Plasma stability of pH-sensitive liposomes. pH-sensitive liposomes prepared as LUV of various compositions were assayed for calcein release due to incubation in 90% plasma (open symbols) or PBS (closed symbols). DOPE/OA (4:1, mol/mol) (□, ■), DOPE/1,2-DOSG (4:1, mol/mol) (△, ▲) or DOPE/1,2-DPSG (4:1, mol/mol) (○, ●) liposomes.

plasma at 37°C. After 3 h in plasma at 37°C only 15% release was recorded for liposomes composed of DOPE/1,2-DPSG (4:1, mol/mol) (Fig. 6). Even after overnight incubation in human plasma at 37°C DOPE/1,2-DPSG and DOPE/1,2-DOSG liposomes exhibited less than 20% release (data not shown).

In some experiments, we recovered liposomes which had been incubated in plasma for 3 h at 37°C and examined their pH-sensitivity. As shown in Fig. 4B, SUV composed of DOPE/1,2-DPSG (4:1, mol/mol) which had not been exposed to plasma exhibited a pH_{50} of 5.3. Liposomes preincubated in human plasma showed a shift in pH_{50} to lower pH values, with a pH_{50} of approx. 4.2 (Fig. 4B). This effect may be due to the insertion of proteins into the liposome bilayer [11] which may either prevent close apposition of liposomes upon collision or inhibit leakage from the liposomes.

Cytoplasmic delivery to cultured cells

We have previously shown that liposomes bearing fatty acid derivatized antibody ('immunoliposomes') can be taken up efficiently by the cellular endocytic pathway [18,21]. Once endocytosed, the liposomes encounter the acidic pH of the endosomes. If the pH-sensitive immunoliposomes are used, liposome-endosome fusion and/or endosome destabilization can occur with release of the liposome contents into the cytoplasm [5–9].

To test the drug delivery ability of 1,2-DPSG and 1,2-DOSG stabilized pH-sensitive liposomes, we used DTA as a marker for cytoplasmic delivery [6]. We have previously shown that DTA delivery only occurs when a liposome-endosome fusion and/or endosome destabilization event occurs, since DTA cannot cross lipid membranes by itself. Thus, free DTA is non-toxic to cultured cells. However, if DTA is introduced into the cell cytoplasm, protein synthesis inhibition occurs due to ADP-ribosylation of eukaryotic EF-2 [22,23].

We encapsulated DTA in LUV composed of either DOPE/1,2-DPSG or DOPE/1,2-DOSG (both at 4:1,

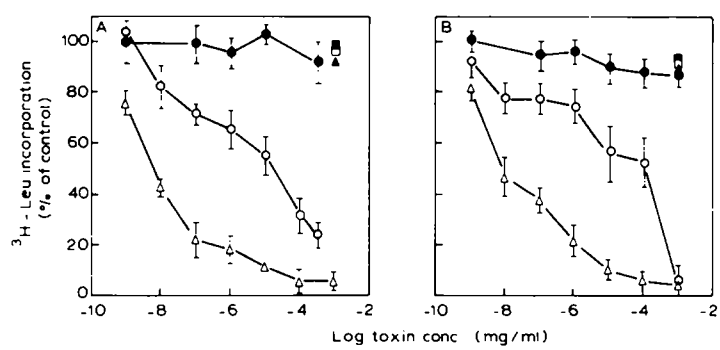

Fig. 7. DTA delivery by DOPE/1,2-DPSG and DOPE/1,2-DOSG immunoliposomes. Immunoliposomes and liposomes containing DTA (prepared by the reverse-phase evaporation method) were incubated with L929 cells and protein synthesis assayed as described in Materials and Methods. (A) DTA delivery by DOPE/1,2-DPSG (4:1, mol/mol) liposomes. ○, DOPE/1,2-DPSG immunoliposomes containing DTA; ●, DOPE/1,2-DPSG liposomes containing DTA; ■, DOPE/1,2-DPSG immunoliposomes containing DTA in the presence of 10-fold excess of free antibody; □, empty DOPE/1,2-DPSG immunoliposomes; ▲, free DTA; and △, ricin. (B) DTA delivery by DOPE/1,2-DOSG (4:1, mol/mol) liposomes. ○, DOPE/1,2-DOSG immunoliposomes containing DTA; ●, DOPE/1,2-DOSG liposomes containing DTA; ■, DOPE/1,2-DOSG immunoliposomes containing DTA in the presence of 10-fold excess of free antibody; □, empty DOPE/1,2-DOSG immunoliposomes; ▲, free DTA; and △, ricin.

mol/mol) and evaluated the ability of liposomes or immunoliposomes to deliver DTA to the cytoplasm of L929 cells. As shown in Fig. 7A, empty DOPE/1,2-DPSG immunoliposomes are non-toxic to L929 cells as is free DTA. DTA containing DOPE/1,2-DPSG liposomes without antibody are also non-toxic due to a lack of sufficient binding and internalization of the liposomes. DOPE/1,2-DPSG immunoliposomes containing DTA showed a dramatic increase in toxicity over free DTA. The IC_{50} of DOPE/1,2-DPSG immunoliposomes containing DTA was approx. $2 \cdot 10^{-5}$ mg/ml. The toxicity of DOPE/1,2-DPSG immunoliposomes containing DTA could be blocked by preincubation of the cells with a 10-fold excess of free antibody (Fig. 7A). This indicates that specific binding of the liposomes to the cells is required for DTA delivery. The toxicity of DOPE/1,2-DPSG immunoliposomes containing DTA could also be blocked by 10 mM NH_4Cl (data not shown) which raises the endosome pH. This result shows that DTA delivery also requires acidification of the endocytic compartment. DTA delivery by DOPE/1,2-DOSG liposomes and immunoliposomes is qualitatively similar (Fig. 7B). A major difference is that the IC_{50} for this composition is higher (approx. $2 \cdot 10^{-4}$ mg/ml DTA) than for DOPE/1,2-DPSG immunoliposomes containing DTA (Fig. 7A), indicating that the DOPE/1,2-DPSG immunoliposome is a more effective delivery vehicle.

Discussion

In this study we have presented some important properties of pH-sensitive liposomes prepared using PE and diacylglycerol-based double-chain amphiphiles. Similar to previous formulations of pH-sensitive liposomes, the liposomes described here are stable at neutral pH and become destabilized at mildly acidic pH. Unlike fatty acid-stabilized pH-sensitive liposomes, liposomes prepared with DOPE and diacylsuccinylglycerols are stable in plasma. Leventis et al. [12] have previously described the synthesis and properties of 1,2-DOSG. They also characterized the pH-sensitivity of liposomes composed of DEPE/1,2-DOSG (7 : 3, mol/mol) [12]. In the present study, we have examined two additional saturated isomers of diacylsuccinylglycerol, i.e., 1,2-DPSG and 1,3-DPSG.

The propensity of PE to adopt non-lamellar structures [24] prompted us to examine the effect of diacylsuccinylglycerols on the thermotrophic phase behavior of DEPE. Other titratable amphiphiles, such as cholesteryl hemisuccinate (CHEMS) [25] and certain double-chain amphiphiles [12] have been shown to stabilize the PE bilayer phase when charged, and to destabilize the bilayer phase and to promote hexagonal phase formation when protonated. Only one of the compounds examined, 1,2-DPSG, slightly increased the T_H of DEPE at pH 7.4 at low mol%. The other two diacylsuccinylglycerols (pH 7.4) decrease the T_H of DEPE at all mole fractions examined. In this regard the compounds behave like diacylglycerols [26–28]. Above 16 mol%, 1,2-DPSG and 1,3-DPSG abolished the $L_\alpha \rightarrow H_{II}$ phase transition of DEPE at pH 7.4 (data not shown). This result suggests that a high mol% (≥ 20 mol%) of the amphiphiles may be required to provide sufficient hydration and/or interbilayer charge repulsions in order to stabilize DEPE bilayers. This result is consistent with recent studies by Tari and Huang [29] examining the effects of phosphatidylglycerols(PG) on DEPE bilayer stabilization. Several PG species are bilayer destabilizers at low mol%; whereas they become stabilizers at higher mol%. When the pH of the medium is decreased to pH 5.0, 1,2-DPSG decreases the T_H as does 1,2-DOSG. Under these conditions, the succinyl group of the amphiphiles is protonated. This leads to a reduction in interbilayer repulsions and a loss of head group hydration. Protonated 1,2-DPSG and 1,2-DOSG probably behave as diacylglycerols and promote hexagonal phase formation [26–28]. 1,2-DOSG destabilizes the bilayer phase of DEPE at pH 7.4 while 1,2-DPSG does not. This result is also consistent with the results of Tari and Huang [29] in that saturated phosphatidylglycerols (DMPG and DPPG) were more potent stabilizers of DEPE bilayers than DOPG. This effect is probably due to the greater volume occupied by the kinked 18 : 1 acyl chains in the dioleoyl species. This greater acyl chain volume increases the stability of H_{II} phase since the DOPG and 1,2-DOSG can pack better into hexagonal phase [24,30]. The increased chain length of 18 : 1 vs. 16 : 0 may also act to stabilize the H_{II} phase [30].

An interesting result of our studies with the 1,2- and 1,3-isomers of DPSG is that 1,2-DPSG has higher bilayer stabilization activity than 1,3-DPSG in both DEPE and DOPE. For DEPE, deprotonated 1,3-DPSG decreases the T_H at all mole fractions examined while 1,2-DPSG had the opposite effect. In both our light scattering and calcein encapsulation studies, 1,2-DPSG was a more potent stabilizer of DOPE bilayers than was 1,3-DPSG. In the light scattering studies, sonicated dispersions of DOPE/1,3-DPSG always scattered more light than did DOPE/1,2-DPSG samples. This indicates that the DOPE/1,3-DPSG samples were more aggregated and/or larger than their 1,2-DPSG-containing counterparts. These results can be understood on the basis of stabilizer acyl chain volume on PE bilayer stabilization activity. The two acyl chains of the 1,3-isomer are separated by the number 2 carbon of the glycerol backbone; whereas the two chains of the 1,2-isomer are positioned directly next to each other. We speculate that the acyl chains of the 1,3-isomer occupy a larger volume than those of the 1,2-isomer because of the higher degree of motional freedom. This may allow

the 1,3-isomer to pack better into H_{II} phase than the 1,2-isomer [30]. 1,3-DPSG can stabilize DOPE into liposomes; however, DOPE/1,3-DPSG liposomes exhibit more leakage over time than DOPE/1,2-DSPG liposomes (Fig. 3). We suggest that DOPE/1,3-DPSG liposomes may not be thermodynamically stable bilayers and may merely represent kinetic products of sonication, whose equilibrium destiny is a leaky non-bilayer phase.

All the DOPE/diacylsuccinylglycerol combinations tested here are acid-sensitive. Leakage vs. pH curves show that DOPE/1,3-DPSG liposomes require more acidic conditions for release than do DOPE/1,2-DPSG liposomes (Figs. 4A, 4B). At the present time, the reason for this effect is unclear, although intermolecular H-bonding between the carboxyl group of 1,3-DPSG and the amine of the DOPE may be involved. It is known that DOPE can form extensive intermolecular H-bonds [31]. If the DOPE amine and the 1,3-DPSG carboxyl and H-bonded, the pK_a of the amine should be higher while that of the carboxyl should be lower than when no H-bonds form [32]. A lower pK_a for the 1,3-DPSG carboxyl would be reflected in a lower pH_{50} value for the DOPE/1,3-DPSG liposomes. Since 1,2-DPSG-containing liposomes have a higher pH_{50} value than liposomes containing 1,3-DPSG, we suggest that 1,2-DPSG does not form intermolecular H-bonds with DOPE as well as the 1,3-isomer. In fact, 1,2-DPSG may actually inhibit the intermolecular H-bonding among the DOPE molecules by increasing the interfacial hydration of the bilayers [32]. The acid sensitivity of DOPE/1,2-DPSG liposomes can also be modulated by low Ca^{2+} concentrations. Ca^{2+}-H^+ synergism shown in Fig. 5 is similar to those reported by Leventis et al. for the DEPE/1,2-DOSG liposomes [12]. Similar effects have also been observed for other pH-sensitive liposome formulations [3,12,25] and may be an important consideration in the use of pH-sensitive liposomes as drug delivery vehicles.

One of the most important properties of DOPE/1,2-DPSG liposomes is their stability in human plasma. In contrast with DOPE/OA liposomes, DOPE/1,2-DPSG liposomes are plasma-stable. OA has been shown to be rapidly removed from pH-sensitive liposomes by plasma proteins [11]. Recent studies from this laboratory have shown that 1,2-DPSG is also removed by the plasma proteins except that the rate of removal is somewhat lower than that of OA [33]. Thus, the mechanism of the plasma stability of the DOPE/1,2-DPSG liposomes is not clear. Plasma proteins have also been shown to insert into pH-sensitive liposome membranes [11]. Protein insertion and/or coating may be responsible for the plasma stability and the altered pH-sensitivity of DOPE/1,2-DPSG liposomes after plasma incubation (Fig. 4B). Although the pH_{50} of the liposomes is decreased after plasma incubation, the liposomes remain acid-sensitive in a biologically relevant range of pH and may be effective for in vivo drug delivery.

In vitro studies using cultured cells suggest that both DOPE/1,2-DPSG and DOPE/1,2-DOSG immunoliposomes are effective drug delivery vehicles. Delivery of the liposome contents to the cell cytoplasm occurs only when the liposomes fuse with and/or destabilize the cellular endosome [7]. Efficient delivery of DTA by both DOPE/1,2-DPSG and DOPE/1,2-DOSG liposomes requires that acylated antibody be included in the liposome membrane. This is due to the fact that very little uptake of liposomes of either composition occurs in the absence of antibody. The IC_{50} of DTA encapsulated in DOPE/1,2-DPSG immunoliposomes is lower than when DOPE/1,2-DOSG immunoliposomes are used. The reason for this difference is not due to different acid sensitivities, since DOPE/1,2-DPSG and DOPE/1,2-DOSG liposomes exhibit identical pH_{50} values (data not shown). The different IC_{50} values could be due to the lower stability of the DOPE/1,2-DOSG liposomes resulting in a possible loss of DTA from the liposomes before entering the cells.

By comparison with previous formulations of pH-sensitive liposomes, DOPE/1,2-DPSG and DOPE/1,2-DOSG liposomes require more acidic conditions to achieve the same level of destabilization. Thus, the site of intracellular delivery by these liposomes may be the endosomes of low pH, or the late endosomes [34]. This is to be compared with the DOPE liposomes containing either PHC or OA which show a higher pH_{50} than these liposomes [2]. One would predict that the DOPE/PHC or DOPE/OA liposomes deliver at the less acidic, or early endosomes. Indeed, recently we have demonstrated that the delivery kinetics of DOPE/PHC, DOPE/OA and DOPE/1,2-DPSG liposomes differ [35]. The kinetics of delivery by DOPE/PHC and DOPE/OA liposomes is consistent with fusion at the level of the early endosome, while DOPE/1,2-DPSG liposomes deliver at a later compartment, possibly the late endosome or lysosome. Thus, the pH-sensitive liposomes with different acid sensitivity may be useful for intracellular delivery at different endocytic compartments.

In summary, our results show that the ability of diacylsuccinylglycerol to produce pH-sensitive liposomes when combined with PE depends on the structure of the diacylsuccinylglycerol. The liposomes are plasma stable and remain pH-sensitive after prolonged plasma incubation. pH-sensitive liposomes prepared from DOPE and double chain amphiphiles are effective drug carriers in vitro. Since these liposome compositions can be further modified to allow prolonged residence time of the liposomes in circulation, they may also have potential as in vivo drug delivery vehicles [33].

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