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I am submitting herewith a dissertation written by Ana Maria Tari entitled "Studies on Bilayer Liposomes Composed of 1,2-Diacyl-3-succinylglycerol". I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biochemistry.

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STUDIES ON BILAYER LIPOSOMES COMPOSED OF 1,2-DIACYL-3-SUCCINYLGLYCEROL

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Ana Maria Tari

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DEDICATION

This thesis is dedicated to my parents Mr. Madeu Babaji Tari and Mrs. Maria Fatima Ng Tari who have given me invaluable educational opportunities.

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ABSTRACT

Bilayer liposomes composed of 1,2-diacyl-3-succinylglycerol (DASG) were prepared. These liposomes destabilize upon incubation with acid and/or divalent cations. Differential scanning calorimetry showed that at pH 7.4, the chain-melting temperature (Tm) of 1,2-dipalmitoyl-3-succinylglycerol (DPSG) was 60.4°C and increased with decreasing pH (Tm = 57.0°C and 62.7°C at pH 8.9 and 6.7, respectively). At pH 6.5, extensive phase separation was observed as the chain-melting peak split into three peaks. At pH 7.4, the Tm of DPSG also increased with increasing divalent cation concentrations. Under identical conditions, Mg^{2+} was more effective than Ca^{2+} in driving up the Tm. By further increasing the divalent cation concentration, DASG bilayers underwent a complete lamellar to hexagonal phase transition. The acid- and divalent cation-induced destabilization mechanisms were different. The former destabilization involved extensive phase separation while the latter destabilization involved a lamellar to hexagonal phase transition. Divalent cations acted synergistically with protons in destabilizing DASG bilayers. In the presence of divalent cations, phase separation of DPSG bilayers could be observed even at pH 6.8. Freeze fracture electron micrographs of 1,2-dioleoyl-3-succinylglycerol (DOSG) liposomes revealed that under acidic conditions, different final structures were formed by the

divalent cations as hexagonal phase and cubic phase structures were observed in the presence of Ca^{2+} and Mg^{2+}, respectively. DOSG immunoliposomes were efficient in cytoplasmic delivery. At 37°C DOSG liposomes, between 120 - 180 nm in diameter, were partially stable in plasma and partially acid-sensitive even after plasma incubation. Injected DOSG liposomes were rapidly removed from the circulation and taken up by the liver. Serum albumin was the major serum protein associated with DOSG liposomes. The association was dependent on divalent cations. The uptake of DOSG liposomes by differentiated THP-1 macrophages increased in the presence of serum albumin and divalent cations. We propose that DOSG liposomes mainly accumulate in the liver due to their preferential association with serum albumin, via an interaction of divalent cations. Thus, serum albumin is a divalent cation dependent, liver specific opsonin for DOSG liposomes.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

FIGURE PAGE

FIGURE PAGE

LIST OF ABBREVIATIONS

Chol,	cholesterol
DASG,	1,2-diacyl-3-succinylglycerol
DOC,	deoxycholate
DOPE,	1,2-dioleoyl-3-phosphatidylethanolamine
DOPS,	1,2-dioleoyl-3-phosphatidylserine
DOSG,	1,2-dioleoyl-3-succinylglycerol
DPSG,	1,2-dipalmitoyl-3-succinylglycerol
DTA,	A-chain of diphtheria toxin
EDTA,	ethylenediaminetetraacetic acid
ePC,	egg phosphatidylcholine
G_{M1},	monosialoganglioside 1b
[^{3}H]CE,	hexadecyl [^{3}H]cholestanyl ether
H_{II},	hexagonal phase
Hepes,	N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid
Hepps,	N-2-hydroxyethylpiperazine-N'-2-propane sulfonic acid
IgG,	immunoglobulin G
Lα,	liquid crystalline phase
Lß,	gel phase
MLV,	multilamellar vesicles
N-NBD-PE,	N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-PE
N-Rh-PE,	N-(lissamine rhodamine B-sulfonyl)-PE
OA,	oleic acid

PA, phosphatidic acid

PAGE, polyacrylamide gel electrophoresis

PBS, phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4 and 1 mM Na_2HPO_4)

PC, phosphatidylcholine

PE, phosphatidylethanolamine

PEG-PE, N-(monomethoxypolyethyleneglycol-succinyl)-dioleoylphosphatidylethanolamine

PG, phosphatidylglycerol

PHC, palmitoyl homocysteine

Pipes, piperazine-N,N'-bis(2-ethane-sulfonic acid)

PMA, phorbol 12-myristate 13-acetate

PS, phosphatidylserine

RES, reticuloendothelial system

REV, reverse-phase evaporation vesicles

SDS, sodium dodecyl sulfate

SUV, small unilamellar vesicles

TBS, tris buffered saline (30 mM Tris, 150 mM NaCl)

Tris, tris (hydroxymethyl) aminomethane

PART I

GENERAL INTRODUCTION

Introduction

Liposomes are microscopic particles comprised of alternating lipid bilayers and aqueous compartments arranged in a concentric manner. Depending on the preparative method, multilamellar (multi-layered) or unilamellar (single-layered) liposomes are obtained. Multilamellar liposomes are formed spontaneously when a lipid film is dispersed in excess aqueous solution. They are heterogenous in size (0.2 - 10 μm) and lamellarity. When multilamellar vesicles are subjected to reverse-phase evaporation, extrusion or sonication, unilamellar liposomes are formed.

Liposomes can entrap both hydrophilic and lipophilic drugs. Hydrophilic drugs are entrapped in the aqueous compartment of the liposomes by hydrating the lipid film in the presence of aqueous solutions containing water-soluble drugs. Lipophilic drugs are entrapped when the drug and the lipids are mixed together before the lipid film formation.

Liposomes have great potential to deliver drugs and biologically active molecules to cells due to these three properties. One is its ease in preparation. Two is its great drug loading capacity. Three is its biodegradable and non-toxic properties.

Liposomes and endocytosis:

Liposomes and immunoliposomes (liposomes bearing antibody specific to cell antigen) (1) are mainly taken up by cells

via the endocytic pathway (1,2). Endocytosis is the cellular process by which extracellular macromolecules or fluids are internalized. Endocytosis serves an important role in the uptake of nutrients and in the internalization of receptor-bound ligands, such as hormones, growth factors, lipoproteins and antibodies.

After the immunoliposome binds to antigenic cell-surface receptor(s), the resulting complex migrates to clathrin coated pits which are internalized by the plasma membrane to form coated vesicles (3). The coated vesicles are then uncoated, possibly by an uncoating ATPase (3), and the uncoated vesicles either coalesce to form a larger vacuole (4) or fuse with a pre-existing early endosome (5). The internalized materials are delivered to the acidic endosomes and eventually to the lysosomes (3). The lysosome is the end of the endocytic route and is the main intracellular digestive and degradative compartment (3). Methotrexate, but not cytosine arabinoside (ara C), could be delivered by liposomes, since ara C is rapidly inactivated in the lysosome (1,6). Thus, effective drug delivery requires that the liposomes be destabilized such that the encapsulated drug can escape. The site of liposome destabilization should ideally be the prelysosomal compartment, i.e. the endosomes. Phosphatidylethanolamine (PE) is ideal for drug delivery since PE-membranes are easily destabilized.

Lipid polymorphism:

Under physiological conditions, lipids such as PC, PS, PG or sphingomyelin form bilayers (Fig. I-1) (for a review, see 7). These lipids are well-hydrated because they bear large and/or charged headgroups. When the cross-sectional area of a bilayer lipid is examined, the areas of headgroup-water interface and the hydrocarbon zone are the same (7). Depending on the acyl chain length, these bilayer lipids may exist in one of these phases: (a) $L\beta$, a gel phase in which the acyl chains are untilted and ordered, (b) $L\beta'$, a gel phase in which the acyl chains are tilted and ordered and (c) $L\alpha$, a liquid crystalline phase in which the acyl chains are disordered or fluid (8). Shorter acyl chains favor the $L\alpha$ phase. At room temperature, C12-PC is in the $L\alpha$ phase while C16- and C20-PC are in the $L\beta'$ and $L\beta$ phases, respectively. By increasing the temperature, longer chain PC's can be driven into the $L\alpha$ phase. The transition order is as follows:

$$L\beta \xrightarrow{Tp} L\beta' \xrightarrow{Tm} L\alpha$$

where Tp and Tm indicate pretransition and chain melting transition, respectively.

Not all lipids can form bilayers under physiological conditions (7-9). PE and in the presence of excess Ca^{2+}, cardiolipin and phosphatidic acid have a high tendency to adopt the hexagonal phase. The hexagonal phase consists of lipid cylinders in which the lipid headgroups are oriented to

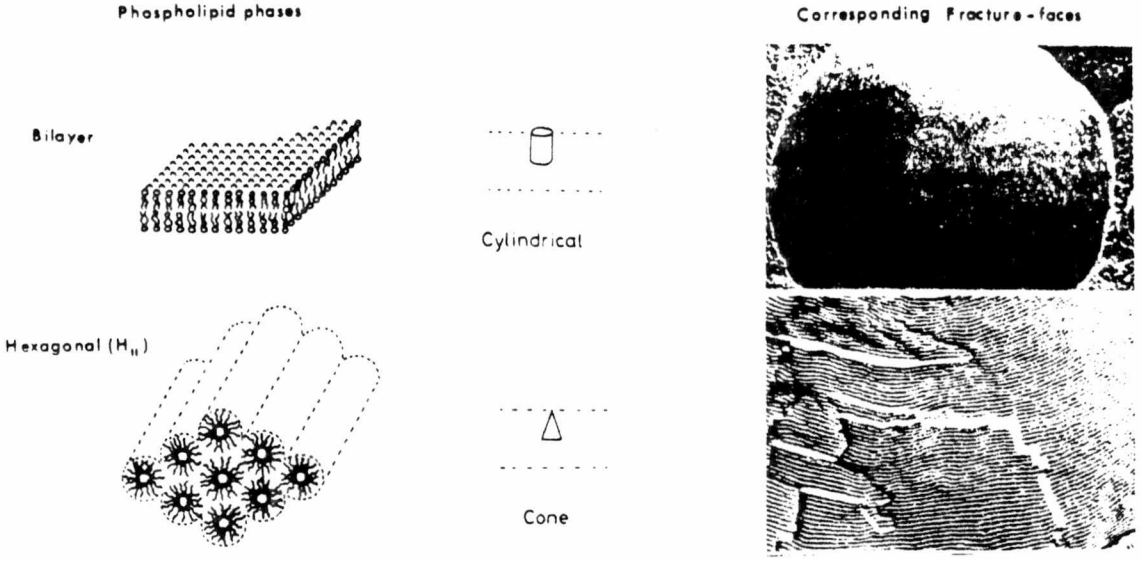


Fig. I-1. Polymorphic phases and the corresponding dynamic molecular shapes of component lipids. Freeze fracture faces (right column) are observed for these structures.

form a hydrophilic core (Fig. I-1). The headgroups of these hexagonal-phase forming lipids are small and/or uncharged. These lipids are less hydrated than the bilayer-phase forming lipids. When the cross-sectional area of PE is examined, the area of the headgroup-water interface is smaller than that of the hydrocarbon zone. The acyl chains tend to splay. The dynamic shape theory describes the PE molecule as "cone" shaped and the PC molecule as "cylinder" shaped (9,10).

Lipid headgroups, which are ionized and repel one another and are in the Lα phase, can be made to go into the hexagonal (H_{II}) phase (7-9). The Lα-H_{II} phase transition is dependent on temperature, hydration, ionic strength, pH and acyl chain length and unsaturation (7,8). The Lα-H_{II} phase transition can be detected by ^{31}P-nuclear magnetic resonance (nmr) (9), X-ray diffraction (11), differential scanning calorimetry (12) and visualized by freeze fracture electron microscopy (13). NMR studies have shown that the acyl chains of PE are more disordered in the H_{II} phase than in the Lα phase (14).

Three different parameters have been used to predict the polymorphic phase behavior of lipids. One is the "dynamic shape" of a molecule (9). A lipid is more likely to undergo the Lα-H_{II} transition when the hydrocarbon chains tend to splay or when the lipid molecule is becoming more "cone" shaped. Two is v/al, where v is the hydrocarbon chain volume, a is the headgroup area and l is the effective acyl chain length (10). Three is Ro, the "intrinsic radius of curvature" which

represents the tendency of a lipid monolayer to bend (15). Small Ro lipid systems exhibit low temperature $L\alpha$-H_{II} transitions, and large Ro lipid systems are stable as $L\alpha$ phases.

Both the dynamic shape theory and *v/al* values predict that dioleoyl-PE can undergo $L\alpha$-H_{II} transition at a lower temperature than stearoyl-PE. This is due to the fact that the effect of the introduction of *cis* double bonds in the hydrocarbon chains results in kinks in the chains, thereby (i) causing the chains to splay more widely and becoming a more "cone-shaped" molecule, and (ii) increasing the overall *v/al* value by increasing the hydrocarbon chain volume "v". The $L\alpha$-H_{II} transition for distearoyl-PE (16) and dioleoyl-PE (17) are 101°C and 10°C, respectively.

Increase in temperature stabilizes the H_{II} phase (7,8). *Gauche* rotamer states of saturated bonds introduce hydrocarbon disorder and may cause the chains to splay, thus more "cone" shaped. Each *gauche* rotamer consumes 0.5 kcal/mol (17). By increasing the temperature, more *gauche* rotamers occur. This is consistent with the fact that the H_{II} phase always occurs at a temperature above the L_{α} phase.

The *v/al* value suggests that the addition of a small fraction of long chain lipids can stabilize the H_{II} phase of PE (19,20). This is due to the fact that long chain hydrocarbon increases "v" by filling in the void space between the H_{II} tubes or cylinders, i.e. the space near the hexagonal lattice.

This minimizes the hydrocarbon packing energy and the overall free energy of the H_{II} phase (15). The longer chain amphiphiles may also reduce the "intrinsic radius of curvature", Ro, of the H_{II} tubes (15,20). At low concentration, C18-PG is more effective than C14- or C16-PG in stabilizing the H_{II} phase of dielaidoyl-PE (20).

The effective headgroup area is determined by the steric size (16,21) and may be influenced by lateral forces, such as electrostatic repulsion (22-24) or hydrogen bonding (21,25) which affect headgroup interactions. The headgroup area of a PE molecule is small since there is extensive hydrogen bonding between the amine of PE and the phosphate of an adjacent PE headgroup. An increase in the effective size of the headgroup stabilizes the Lα phase by decreasing the *v/al* value as well as increasing Ro. This can be done by N-methylation (26) or deprotonation of the amine group of the PE headgroup by increasing the hydration pH to 9.5 (27). Both procedures reduce hydrogen bonding between PE molecules. Deprotonation of the amine group even causes PE to be negatively charged. These data suggest that the "hydration force" of PE is increased with an increase in headgroup size.

For unsaturated PE with short chains, nonlamellar cubic phases have been observed between Lα and H_{II} phases (16). The cubic phase is most stable near the Lα-H_{II} transition region. Such a phase may reflect the necessity to accommodate tight headgroup packing along with an increase in chain packing, yet

where the shorter chains are insufficient in filling the hydrocarbon volume of the H_{II} phase. The cubic phase was found only under conditions where an isotropic ^{31}P-NMR resonance occurred. Lipidic particles representing the cubic phase could also be observed with freeze fracture electron microscopy (11,27).

Long chain unsaturated PE, such as DOPE, has a high tendency to go into the H_{II} phase. The ease with which the $L\alpha$-H_{II} transition occurs suggests that a relatively small energy barrier separates these phases. Epand (12) found that the energy involved in the $L\alpha$-H_{II} transition for DOPE is between 100 - 200 cal/mol. DOPE could be induced into cubic phase structures by repeated, rapid thermal cycling of the system (28). The difficulty involved in settling the system into or out of the cubic phase suggests that the corresponding free energy is defined by relatively large kinetic barriers. Methylated-DOPE has a higher Ro than DOPE and is more readily to go into the cubic phase (26). This suggests that lipids with intermediate Ro values are likely to form cubic phases (26).

Ellens et al. (27) found that liposome fusion occurred only under the circumstances in which cubic phases were found. Cubic phase represents an intermediate condition of the lipid dispersions between the $L\alpha$ phase, where no fusion occurs, and the H_{II} phase, where rapid lysis occurs.

During the thermotropic $L\alpha$-H_{II} phase transition, vesicles

are brought together through close apposition as the temperature increases. Inverted micellar intermediates (IMI) are formed between two apposed vesicles at temperatures slightly lower than the $L\alpha$-H_{II} transition temperature (T_h) (29). This causes the outer monolayers of the membranes to become continous. IMI can revert to planar bilayer structures or to another bilayer structure, the interlamellar attachment (ILA). ILA allows the fusion between two vesicles. Siegel (29) suggested that ILA in multilamellar arrays should assemble into a structure resembling the cubic phase. When the temperature is greater than T_h, IMI aggregates into H_{II} precursors (29).

pH-sensitive liposomes:

DOPE undergoes an L_α-H_{II} phase transition between 8-10°C at pH 7.4 (12,30). At physiological pH and temperature, the equilibrium phase of DOPE is the H_{II} phase. The bilayer phase of DOPE can be stabilized by the addition of a second lipid component such as PS (31), PG (20), PC (20), lyso PC (20,32) or ganglioside G_{M1} (33). Proteins, such as glycophorin (34) and fatty acid derivatized antibody (35) can also stabilize DOPE bilayers. The main driving force of the bilayer stabilization depends on the ability of the stabilizer to increase the hydration of PE molecules and to decrease the intermolecular hydrogen bonding between the adjacent PE molecules. This prevents close apposition of the PE membranes,

which is the first step in H_{II} phase formation (27).

When liposomes or immunoliposomes bind to the cell surface, they are normally endocytosed and routed to the cellular degradative compartment, lysosomes (1,2). pH-sensitive liposomes have been developed to overcome the lysosomal degradation problem (36-38). At neutral or basic pH, pH-sensitive liposomes are stable and non-fusogenic. The basic components of the pH-sensitive liposomes are DOPE stabilized by lipids with titratable acidic headgroups, such as OA (37), PHC (36), cholesteryl hemisuccinate (38) and diacylsuccinylglycerol (DASG) (39,40). At pH 7.4, these lipids are negatively charged and provide an electrostatic repulsion force preventing the close apposition of the PE membranes. H_{II} phase formation is prevented. Under acidic conditions, the negative charge is neutralized. This decreases the interfacial hydration and allows close apposition of the membranes. DOPE liposomes become destabilized under these conditions.

When pH-sensitive liposomes and immunoliposomes enter into the acidic endosomal compartments, protonation of the amphiphile leads to liposomal destabilization and fusion with the endosomal membrane (36-40). In some cases, destabilization of the fused endosome membrane may also occur (41). This causes the liposomal contents to be released into the cytosol.

pH-sensitive immunoliposomes composed of DOPE/OA or DOPE/PHC were used to deliver calcein (42,43), an aqueous self-quenching fluorescent dye, to mouse L929 cells. Diffuse

fluorescence was observed within the cell cytoplasm. By direct measurement of calcein concentration, it was found that approximately 10^3 liposomes/cell had released calcein into the cell cytoplasm. This corresponds to release by approximately 50% of all liposomes taken up by the cells, and an intracellular calcein concentration of 50 μM (42).

The ability to target calcein to L929 cells has obvious potential for delivery of cytotoxic agents, nucleic acids and protein molecules to selected cells. pH-sensitive immunoliposomes composed of DOPE/OA could deliver ara C (43), plasmid DNA (44) and diphtheria toxin A fragment (DTA) (45) efficiently. Ara C is lysosome-sensitive. Both DTA and plasmid DNA cannot translocate across lipid membranes. Since pH-sensitive immunoliposomes are capable of delivering these molecules, the release of encapsulated contents is most likely at a prelysosomal site. The probable site of release is the endosome. The delivery likely involves the rupture or the fusion of endosomal membranes with destabilized liposomes.

The destabilization mechanism of pH-sensitive liposomes involves the formation of hexagonal and related non-bilayer phases (27,40,46). The inclusion of lipids, such as OA (37) and PHC (36), in DOPE bilayers can induce the formation of lipidic particles, i.e. the cubic phase. The ability of pH-sensitive liposomes to undergo fusion is probably related to the formation of the cubic phase.

Divalent cation effect:

The binding of divalent cations to anionic lipids has been well established (47-52). The binding neutralizes the negative charge of the lipids, reduces the interfacial hydration of the the lipids and induces bilayer destabilization. In the presence of excess Ca^{2+}, cardiolipin (47,48) or PA (49) bilayers can be driven into the H_{II} phase. Ca^{2+} can also induce the fusion of PS vesicles (50). In mixed systems of PC/PS or PC/PA (51), phase separation was observed when Ca^{2+} was in excess. It was suggested that Ca^{2+} induced segregation of individual lipids in separate domains (52) or segregation of different structures (51) within the vesicle membranes.

The behavior of pH-sensitive liposomes can also be influenced by divalent cations. It has been demonstrated that Ca^{2+} in the range of 2-3 mM and Mg^{2+} in the range of 3-4 mM can induce fusion between liposomes composed of DOPE/OA (37). Ca^{2+} and Mg^{2+} can also induce fusion of DOPE/DPSG vesicles at neutral pH. Similar to protons, the divalent cations neutralize the negative charged stabilizer, which reduces the interbilayer hydration and permits close apposition of the bilayers. In addition, both Ca^{2+} and Mg^{2+} cause a "bridging" effect between adjacent liposomes, as has been observed with the PS/Ca^{2+} system (50).

Divalent cations act synergistically with protons in

inducing fusion. Divalent cations allow fusion of liposomes at higher pHs than in the absence of divalent cations (39,40,46). In the presence of 2 mM Ca^{2+}, the pH_{50} of lipid mixing of DOPE/OA liposomes is shifted from 6.9 to 7.5 (46). The combination of a low pH and 2 mM Ca^{2+} induces more rapid lipid mixing and content mixing between liposomes composed of DOPE/DPSG than either treatment alone.

It has been suggested that protons and divalent cations induce destabilization and fusion through different mechanisms. The result of protonation of OA under acidic conditions is fusion and destabilization and a general transition of the system to H_{II} phase (46). However, the final structures produced by Ca^{2+} at neutral pH were lamellar sheets with many lipidic particles (46). Associated with the lamellar sheets were regions of H_{II} phase (46). There was a concomitant reduction of H_{II} phase structures of DOPE/OA vesicles at low pH and 2 mM Ca^{2+} when compared with DOPE/OA vesicles at low pH but in the absence of Ca^{2+}.

The effects of divalent cations on pH-sensitive liposomes may be an important consideration in the use of pH-sensitive liposomes as drug delivery vehicles. Both Ca^{2+} and Mg^{2+} are important components of biological fluids and tissue culture media, and are likely to be constituents in the lumen of the endosome.

Stability of liposomes in plasma:

The therapeutic use of liposomes as drug carriers *in vivo* requires the liposomes to remain stable in the circulation before reaching the target cells. Studies of stability of liposomes are usually done by incubating liposomes with plasma *in vitro*. Aqueous fluorescent markers or radioactive solutes are entrapped in the liposomes. After incubation with plasma, the percentage of entrapped marker molecules remaining inside the liposomes indicates the stability of liposomes.

Destabilization of liposomes was observed when there was a net transfer of liposomal lipids to plasma components (53). These plasma components are mainly lipoproteins and apolipoproteins. After the incubation of high density lipoprotein with liposomes entrapping [^{125}I]albumin, release of entrapped [^{125}I]albumin was found (54). The incubation of liposomes with apolipoprotein A-I or E resulted in the release of entrapped carboxyfluorescein (55). Other proteins, such as complement proteins (56), serum albumin (57) and C-reactive protein (58,59), have also been found to induce liposome destabilization.

Large pH-sensitive liposomes (d > 300 nm) composed of DOPE and single chain amphiphiles, such as OA and PHC, are not stable in plasma. Single chain amphiphiles easily dissociate from the liposome membranes (39, 60). The dissociation leads to destabilization of the liposomes and the formation of bilayer defects. Serum proteins may then insert into the

liposomes through these bilayer defects. The insertion of serum proteins causes small DOPE/OA liposomes (d < 200 nm) to become pH-insensitive (61).

pH-sensitive liposomes composed of DOPE and double-chain amphiphiles are developed. Double-chain amphiphiles are harder to remove from liposomes than single-chain amphiphiles (39,40). DOPE/DOSG and DOPE/DPSG liposomes are stable in plasma. The liposomes remained pH-sensitive though the pH_{50} was shifted from 5.3 to 4.2 after incubation with plasma (40). This effect may be due to insertion or adsorption of serum proteins to the pH-sensitive liposomes, which may prevent close apposition of the liposome membranes.

Biodistribution of liposomes:

Intravenously injected liposomes are rapidly removed from the circulation in a biphasic behavior, with an initial rapid rate followed by a slow rate of elimination (for a review, see 60). Liposomes are mainly taken up by the phagocytic cells of the reticuloendothelial system (RES), particularly the liver Kupffer cells and the splenic macrophages (for reviews, see 53,62,63). The rate of liposome uptake by the RES can be modified by injected liposome size and dosage, and the surface characteristics of the liposomes.

Large liposomes have a higher clearance rate than small liposomes. The circulation half-life ($t_{1/2}$) of large liposomes (~600 nm in diameter) was 5 min while the $t_{1/2}$ of small

liposomes (~30 nm in diameter) was greater than 50 min (64). High doses lead to longer circulation time of liposomes. When the lipid dose of PC/Chol (1:1, m/m) liposomes increased from 0.4 to 40 mg/kg, the $t_{1/2}$ increased from 20 min to 3 h (64).

Liposomes composed of negatively charged lipids are cleared more rapidly than those composed of neutral lipids (65). The circulation time of liposomes is extended when molecules such as ganglioside G_{M1} (66) or amphiphatic polyethyleneglycol-PE (PEG-PE) conjugates (67) are incorporated into PC/Chol liposomes. Other negatively charged lipids with a bulky headgroup (68), for example sulfatides and phosphatidylinositol, show a similar but smaller effect in prolonging the circulation time of liposomes. Liposomes composed of saturated PC are cleared more slowly than those composed of unsaturated PC (68).

The biodistribution of pH-sensitive liposomes has been examined. DOPE/OA liposomes, labeled with [^{125}I] tyraminyl-inulin as an aqueous content marker and hexadecyl [^{3}H] cholestanyl ether as a lipid marker, were injected intravenously into Balb/c mice (60). Injected small DOPE/OA liposomes ($d < 200$ nm) were rapidly removed from the circulation and accumulated in liver. Large DOPE/OA liposomes ($d > 200$ nm) accumulated in the lung. These liposomes likely aggregated in the circulation due to a concomitant increase in size, accumulated in the lung capillary bed as a result of embolism (69). The aqueous content marker dissociated from the

pH-sensitive immunoliposomes, accumulated in the kidneys and was subsequently excreted. This further indicates rapid destabilization of DOPE/OA liposomes *in vivo*, probably as a result of OA extraction by albumin.

The behavior of DOPE/DPSG vesicles was also studied. DOPE/DPSG liposomes were also rapidly removed from the circulation and taken up by the RES. When 5 mol % ganglioside G_{M1} was included in the liposome composition, the circulation time of the vesicles was prolonged as the RES uptake was reduced (70). These liposomes even retained partial pH-sensitivity. At pH 4, 65% calcein was released from the vesicles when incubated with plasma. Thus, a relatively plasma-stable liposome which retains partial pH-sensitivity and avoids RES uptake has been successfully developed. However, targeting these vesicles is a problem.

Monoclonal 34A antibody, which specifically binds to a surface glycoprotein gp 112 on the pulmonary endothelial cell surface, was incorporated into DOPE/DPSG and DOPE/DPSG/G_{M1} (75:20:5, m/m) vesicles. 34A-immunoliposomes composed of DOPE/DPSG and DOPE/DPSG/G_{M1} revealed lower levels of lung accumulation (< 10% of injected dose) than 34A-immunoliposomes composed of ePC/Chol, approximately 53% of injected dose, (71).

Uptake of liposomes by the reticuloendothelial system (RES):

The mechanism(s) by which liposomes are recognized and removed from the circulation is unknown. It is believed that the phagocytic cells of the RES possess cell surface receptors which recognize the blood molecule coating the liposomes. Such molecule is named "opsonin". The inclusion of G_{M1} or PEG-PE in liposome composition can prolong the circulation time of liposomes. It has been suggested that these amphiphiles lower the uptake of liposomes by the RES via these two mechanisms: (a) by increasing the hydrophilicity of the liposomes thus reducing nonspecific interactions of liposomes with RES cells and (b) by providing steric barriers thus reducing specific interactions of liposomes with RES cells (67,72).

The identity of the opsonin is not clear. However, the opsonin appears to be organ-specific (73). Liver-specific opsonin is a heat-stable macromolecule which on heating or on freezing and thawing exhibits enhanced opsonin activity (74). Serum contains a dialyzable factor that can inhibit the liver-specific opsonin activity. Spleen-specific opsonin is a heat-labile macromolecule which is sensitive to freezing and thawing and requires a dialyzable serum cofactor for its optimum opsonin activity (74).

IgG (75) and complement C3 (76) may play a role in the opsonization of liposomes. When liposomes were coated with IgG, a five-fold increase in the uptake of coated liposomes as compared with non-coated liposomes was observed (75). This is

due to the attachment of the Fc portion of the IgG molecule to the liposomes, which is recognized by specific Fc receptors on the phagocytic cells (77). Liposomes coated with complement components are taken up more readily by culture cells (76). Stable convertases can be assembled on PG-containing vesicles and lead to C3 activation and C3b deposition (78). Receptors specific for complement C3 can be found on the surface of phagocytic cells (79). However, the interactions of C3 fragments with liposomes are not clearly understood.

Fibronectin receptor belongs to a large family of cell membrane glycoproteins known as "integrins" (80). These glycoproteins promote several cell/cell and cell/matrix adhesion processes and recognize the amino acid sequence RGD (arginine-glycine-aspartate) in the extracellular matrix ligands. Fibronectin can bind to liposomes of various composition. Increased uptake of liposomes was observed when the liposomes were coated with fibronectin (81). The interaction of fibronectin with phagocytic cells is mediated by specific cell surface integrins (82).

Apolipoproteins can be transferred to liposomal membranes and may serve as opsonins. Apolipoproteins E-containing liposomes compete effectively with β-very low density protein for binding to apolipoprotein E receptors on macrophages (83). Apolipoproteins have been suggested to play a role in the uptake of liposomes by hepatocytes via apolipoprotein B or apolipoprotein E receptors (84).

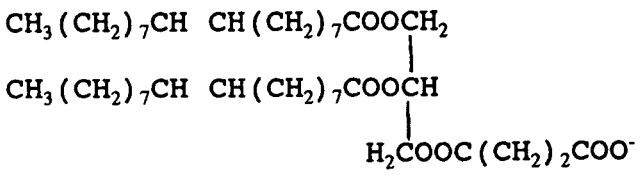
Anti-phospholipid antibodies have also been implicated to be opsonins (85). They bind to various anionic phospholipids, PS, PA, phosphatidylinositol or cardiolipin, and not to net neutral PC or PE. The binding requires a cofactor, apolipoprotein H (86). It has been suggested that in normal human serum the anti-phospholipid antibodies are masked and that apolipoprotein H may play a positive regulatory role (87).

In addition to the opsonin receptor, the scavenger receptor may play a role in the uptake of liposomes (88). Scavenger receptors, mediating the uptake of acetylated low density lipoprotein (LDL), are found on macrophages (89,90). Various ligands of scavenger receptors involving acetylated LDL, dextran sulfate or fucoidan compete effectively for the binding of PS-containing vesicles to the scavenger receptor (88). On the other hand, PS-containing vesicles can also compete effectively for the binding of acetylated LDL to the receptor (88). These studies indicate that acidic phospholipids are recognized by the scavenger receptors located on the surface on macrophages.

Scope of the Project described in this Discussion

Earlier work with the double-chain synthetic amphiphile DASG showed that these amphiphiles could be used to prepare single-component pH-sensitive liposomes. Fig. I-2 shows the structures of DASG. This is an exciting finding as no single-

DOSG



DPSG

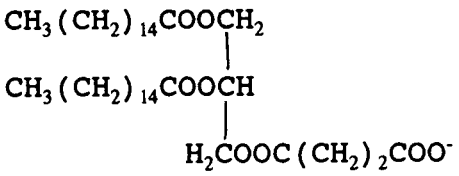


Fig. I-2. Structures of 1,2-dioleoyl-3-succinylglycerol (DOSG) and 1,2-dipalmitoyl-3-succinylglycerol (DPSG).

component pH-sensitive liposome has ever been developed. These prompted us to investigate the fusion and the cytoplasmic delivery efficiencies of DASG vesicles. We then studied the acid-induced destabilization mechanisms of these vesicles. At pH 7.4 or above, DASG is negatively charged and may interact with divalent cations. Thus, we have also studied the interactions of divalent cations with DASG vesicles. To apply DASG liposomes as drug delivery vehicles *in vivo*, it was necessary to examine its biodistribution behavior. This has then led us to use DOSG liposomes to purify potential serum "opsonins" and to study the effect of these opsonins on liposome uptake.

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PART II

ACID INDUCED BILAYER DESTABILIZATION OF LIPOSOMES COMPOSED OF 1,2-DIACYL-3-SUCCINYLGLYCEROL

Abstract

Bilayer liposomes were prepared by using pure DOSG (1,2-dioleoyl-3-succinylglycerol) or DPSG (1,2-dipalmitoyl-3-succinylglycerol) at pH 7.4 or above. These liposomes undergo destabilization upon incubation with acid. When calcein was used as an entrapped aqueous marker, half maximal content leakage was observed between pH 5.8 - 6.3. Differential scanning calorimetry showed that at pH 7.4, the chain-melting temperature (Tm) of DPSG was 60.4°C, and increased with decreasing pH (Tm = 57.0°C and 62.7°C at pH 8.9 and 6.7, respectively). Below pH 6.7, extensive phase separation occurred as the major chain melting peak split into three peaks. These three peaks coalesced into one peak below pH 5. Freeze fracture electron micrographs of DOSG liposomes at pH 4 showed the formation of non-bilayer as well as hexagonal phase structures. There was a size-dependency on the plasma stability of DOSG liposomes. DOSG liposomes that were smaller in size were more stable in plasma than the larger ones. After incubation with plasma, DOSG liposomes became less acid-sensitive. DOSG immunoliposomes entrapping diphtheria toxin A chain were used as a model for cytoplasmic delivery of the novel pH-sensitive liposomes. The delivery activity was comparable to that of the conventional pH-sensitive liposomes containing unsaturated phosphatidylethanolamine. Our data indicate that the mechanism of liposome destabilization involves extensive bilayer phase separation as well as the

formation of non-bilayer structures.

Introduction

Cytoplasmic delivery of bioactive molecules is an important goal of the targeted drug delivery. This is particularly the case for macromolecules such as enzymes, antibodies and nucleic acids because they do not easily penetrate the cellular membranes. Fusogenic, pH-sensitive liposomes have been developed for this purpose because of their enhanced cytoplasmic delivery activity (1). This class of liposomes is stable and non-fusogenic at neutral pH but becomes destabilized and fusogenic at a mildly acidic pH, such as pH 5-6 (2-4). pH-sensitive liposomes, similar to all other liposomes types, are internalized by cells via an endocytic mechanism (5,6). These liposomes release their entrapped contents into the cytoplasm when they encounter the acidic environment in the endocytic compartment and destabilize (1-4, 7). Antitumor drugs (8) fluorescent dyes (9), toxin (7) and DNA (10,11) have been successfully delivered by this class of liposomes.

The basic design of pH-sensitive liposomes takes advantage of the high tendency of unsaturated PE to form the reverse hexagonal phase at the physiological temperature, pH and ionic strength (12). Stable bilayer liposomes, however, can be prepared by mixing the unsaturated PE with a weakly acidic amphiphile such as fatty acids (4,13), fatty acyl amino

acid (2), cholesterol hemisuccinate (3) and acid conjugates of PE (14). DASG was first described by Leventis et al. (15) as a double-chain acidic amphiphile which can stabilize the Lα phase of unsaturated PE. When DASG is protonated at acidic pH, a rapid destabilization of the PE bilayer occurs primarily due to a phase transition to the non-bilayer phases (3,4,15). It has been shown by us that liposomes composed of DOPE and DASG are an effective cytoplasmic delivery vehicle for DTA (16). Furthermore, the delivery event takes place at 20-25 min after the liposomes are endocytosed, indicating that the site of cytoplasmic release is the late endosome or lysosome compartment (17).

We report here that stable bilayer liposomes can be prepared with an aqueous dispersion of pure DASG, i.e. no unsaturated PE is required. Stabilities of these liposomes at different pH values have been studied with liposomes of the dioleoyl form of DASG by the entrapped content leakage. The thermal phase transition of DPSG is also studied with differential scanning calorimetry. These studies have shed light on the mechanism of the acid-induced bilayer destabilization of DASG. Furthermore, we have shown that DOSG liposomes can deliver a protein toxin to target cells and have compared this cytoplasmic delivery activity with that of the conventional pH-sensitive liposomes composed of primarily unsaturated PE. A preliminary account of the project has been reported previously (18,19).

Materials and Methods

Materials:

DOSG and DPSG were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). The purity of the lipids was confirmed by thin-layer chromatography using hexane:ethylether:methanol:acetic acid (70:30:5:1, v/v) as a developing solvent system. Other lipids were also purchased from Avanti. Calcein and DOC were purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification. [^{3}H]leucine was purchased from New England Nuclear (Boston, MA). Ricin was isolated from castor beans by the method of Nicholson and Blaustein (20). Anti-H2K^{k} antibody (11-4.1) was purified, labeled with ^{125}I, and derivatized with N-hydroxysuccinimide ester of palmitic acid, as described by Huang et al. (21). DTA was prepared from intact toxin by the method of Gill and Dinius (22), and labeled with ^{131}I by the iodobead methods according to the manufacturer's protocol (Pierce Co., Rockford, IL).

Liposome preparation:

Liposomes were prepared by two different methods. Method 1 involved the preparation of SUV. A thin film of solvent-free lipids was vacuum desiccated for at least 30 min. A trace amount of hexadecyl [^{3}H]cholestanyl ether was added to the lipids to monitor lipid concentration. The lipids were suspended in PBS, containing 1 mM EDTA with or without 50 mM

calcein, at a final concentration of 10 μmol/ml for 2-4 h before a 20-min sonication in a bath sonicator (Laboratory Supplies, Hicksville, NY). The pH of the suspension was maintained between 7.8 - 8.2 by adding 1 N NaOH. The sonicate was then incubated overnight for equilibration at room temperature. Untrapped calcein was removed by gel filtration of the liposome suspension on a Bio-Gel A-0.5m column. Calcein-containing liposomes were further passed over a Sepharose 4B column to obtain different pools of size of SUV. The size of liposomes was measured by dynamic laser light scattering using a Coulter N4SD instrument.

Method 2 involved the preparation of reverse-phase evaporation vesicles (REV) (23). Solvent-free DOSG was vacuum desiccated and suspended in PBS containing 0.2 mg/ml DTA. A trace amount of hexadecyl [^{3}H]cholestanyl ether was added as a radioactive lipid marker. The lipid suspension was sonicated at room temperature for 10 min with a bath sonicator. The pH of the sonicate was maintained between pH 8.0 - 8.5 by adding 1 N NaOH. Ethylether was then added to the aqueous phase (3:1, v/v). The mixture was sonicated for 30 sec to form a stable emulsion which was rotary evaporated at room temperature until all of the organic phase was removed. The resulting REV were incubated in a fume hood for 1 h to remove any residual organic solvent. PBS was added to the REV suspension to bring the final lipid concentration to 10 mM. Palmitoyl antibody was then added to the liposomes with a lipid-to-protein ratio of

10:1 (w/w) to allow the incorporation of antibody to liposomes (7). Untrapped DTA was separated from liposomes by gel filtration using a Bio-Gel A-0.5m column. The liposomes were then extruded through a 0.2 μm polycarbonate filter (Nucleopore Co.) to obtain size uniformity.

Pretreatment of liposomes with plasma:

Two hundred fifty μl of calcein-containing SUV were mixed with the same volume of plasma and incubated for 1 h at 37°C before passing through a Bio-Gel A-1.5m column to separate free plasma components from liposomes. The liposomal fractions were pooled and acid-sensitivity of liposomes was then determined.

Content leakage of liposomes:

Calcein-containing SUV was prepared as described above. A LS-5 Perkin-Elmer spectrophotometer was used for the fluorescence assay. The excitation and emission wavelengths were 490 nm and 520 nm, respectively. The excitation and emission slit widths were 5 nm and 3 nm, respectively. Five μl of liposomes were added to a cuvette containing 2 ml of PBS (pH 7.4). After the relative fluorescence was measured, 0 - 20 μl of 0.5 N HCl was added to achieve the desired pH. After continuous stirring at room temperature for 2 min, appropriate amounts of 0.5 N NaOH were added to bring the pH back up to 7.4. The relative fluorescence was again measured. DOC was

then added, at a final concentration of 0.15%, to disrupt the liposomes. Percent release was calculated from the following formula:

% Release = [(F-Fo)/(Ft-Fo)] X 100 where

Fo = fluorescence intensity of liposomes before acidification,

F = fluorescence intensity after acid incubation and

Ft = fluorescence intensity after DOC addition.

Stability of liposomes in plasma:

Calcein-containing SUV, ranging from 60 - 260 nm in diameter, was used to determine the stability of liposomes in plasma. Fifty μl of liposomes containing 50 nmol of lipid were added to 450 μl of serum pre-warmed to 37°C. Ten μl of the mixture were used for the fluorescence measurement at different incubation time-points. DOC was then added. Percent release of liposomal calcein was calculated as above, except

Fo = fluorescence intensity of liposomes in PBS at time zero,

F = fluorescence intensity at different time-points and

Ft = fluorescence intensity after DOC addition.

Freeze fracture electron microscopy:

A thin film of DOSG lipids was suspended at 1 μmol/ml in 20 mM Pipes or 20 mM succinic acid buffer containing 1 mM EDTA. The lipids were kept initially at pH 7.4 and extruded

through 0.2 μm polycarbonate filters. The pH of the liposome suspension was then lowered to pH 6.0 or 4.0 by adding 1 N HCl. The freeze fracture procedure was done as described previously (24). The replicas were viewed on a Philips 300 electron microscope at magnifications of 7,000 to 27,000.

Differential scanning calorimetry:

After vacuum desiccation, solvent-free lipids were suspended at 5 μmol/ml in one of these buffers: (i) 20 mM Pipes with 1 mM EDTA, (ii) 20 mM Hepes with 1 mM EDTA, (iii) 20 mM Hepps with 1 mM EDTA or (iv) 20 mM succinic acid with 1 mM EDTA. The initial pH of all buffers was pH 7.4. DOSG and DPSG were hydrated, and vortexed occasionally, for 3 h at room temperature and 67°C, respectively. Various amounts of 1 N HCl or 1 N NaOH were added to the lipid mixture to achieve the desired pH. Lipids were vortexed and further incubated at the appropriate temperature and desired pH for an additional h before incubation at room temperature for overnight equilibration.

The equilibrated lipids were loaded into a Microcal MC-2 scanning calorimeter (Amherst, MA) and scanned from 20 to 80°C at a scan rate of 30°C/h. The excess heat capacity was recorded as a function of temperature. The peak transition temperature was determined by an IBM PC computer interfaced with the instrument.

Cytotoxicity assay:

The cytotoxicity assay was done as described previously (7), except after a 6 h [^{3}H]leucine incubation, cells were washed, trypsinized and harvested with a PHD cell harvester (Cambridge Technology). The filter remnants were processed for scintillation counting as described (7). All experiments were done in 5 duplicates.

Results

Acid-induced bilayer destabilization of DOSG liposomes:

When DOSG vesicles were sonicated in the presence of 50 mM calcein, pH 7.4, and chromatographed on Bio-Gel A-0.5m to remove unentrapped calcein, it was found that the vesicles could entrap calcein with a fluorescence quenching of 71-78% which indicates that the calcein concentration in the vesicles was approximately 50 mM (25). The vesicles maintained this level of fluorescence quenching even after a prolonged storage in PBS, pH 7.4 at 4°C for 2-3 weeks. However, the entrapped calcein rapidly leaked out if the medium pH was lowered by adding acid (Fig. II-1, closed symbol). Fifty percent of calcein release was observed at pH 5.8-6.3. Sonicated DPSG vesicles which were prepared by full hydration at 67°C could also entrap calcein with 72-75% fluorescence quenching. The release of calcein from DPSG vesicles at acidic pH was similar to that of the DOSG vesicles (data not shown).

Bilayer destabilization at acidic pH was also measured

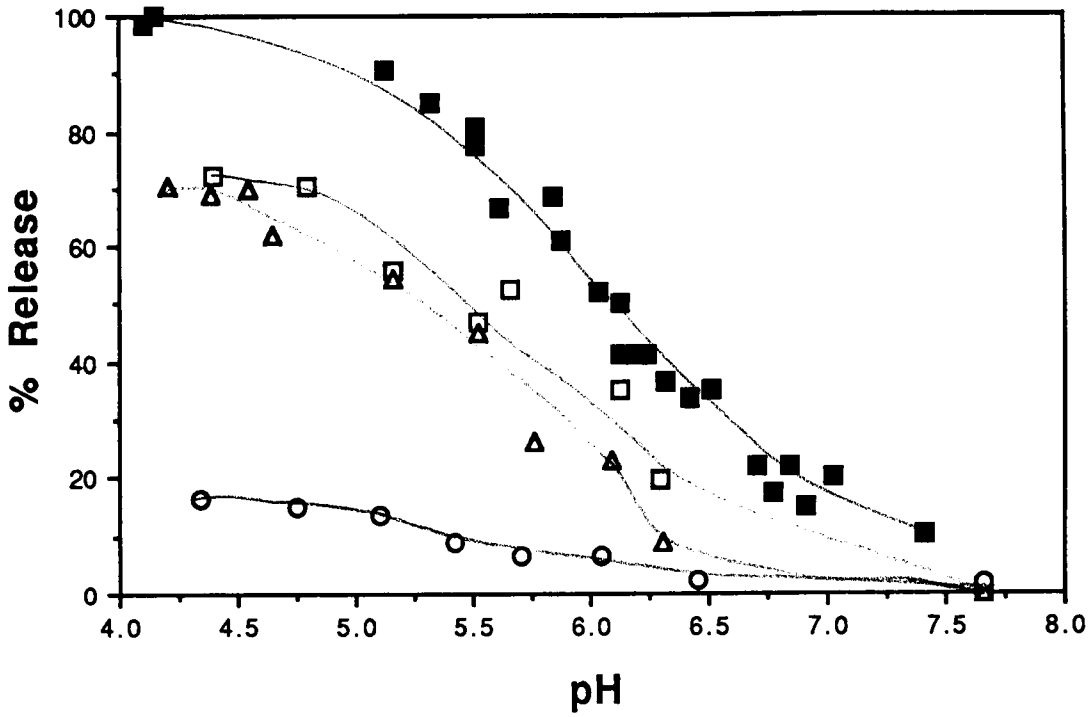

Figure II-1. Effect of acidification on calcein-release from SUV composed of DOSG. DOSG liposomes were 183±32 nm (□,■), 126±21 nm (△) and 62±8 nm (○) in diameter. Closed and open symbols represent DOSG liposomes before and after pretreatment with plasma, respectively.

with a lipid mixing assay, using N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-PE and N-(lissamine rhodamine B-sulfonyl)-PE (26). Acid-induced lipid mixing of DOSG vesicles showed a maximal mixing at approximately 60% at pH < 5, with the half-maximal mixing at about pH 5.8 (data not shown).

Thermal phase transition of DPSG dispersions:

The phase behavior of DASG dispersions prepared as MLV was studied by differential scanning calorimetry. No endo- or exothermic transitions could be detected for DOSG dispersions between 0 and 90°C at pH 7.4 or lower, indicating no thermal phase changes occurred with this lipid in the temperature range tested. Fig. II-2 shows heating scans of DPSG dispersions equilibrated at different pH values. At pH 7.4, two endothermic peaks appeared, one at 43.3°C and another at 60.4°C. In a separate experiment, using a trace amount of diphenylhexatriene incorporated into DPSG dispersion, the transition at 60.4°C was accompanied with a large decrease in the fluorescence polarization of the incorporated diphenylhexatriene (data not shown), indicating that this transition was the chain-melting transition. This result also implies that the transition at 42.3°C is a pretransition.

Enthalpies of the transitions are listed in Table II-1. At lower pH values, there was a progressive increase of the transition temperature of the chain-melting peak (Fig. II-2). For example, the main transition at pH 6.7 occurred at 62.7°C,

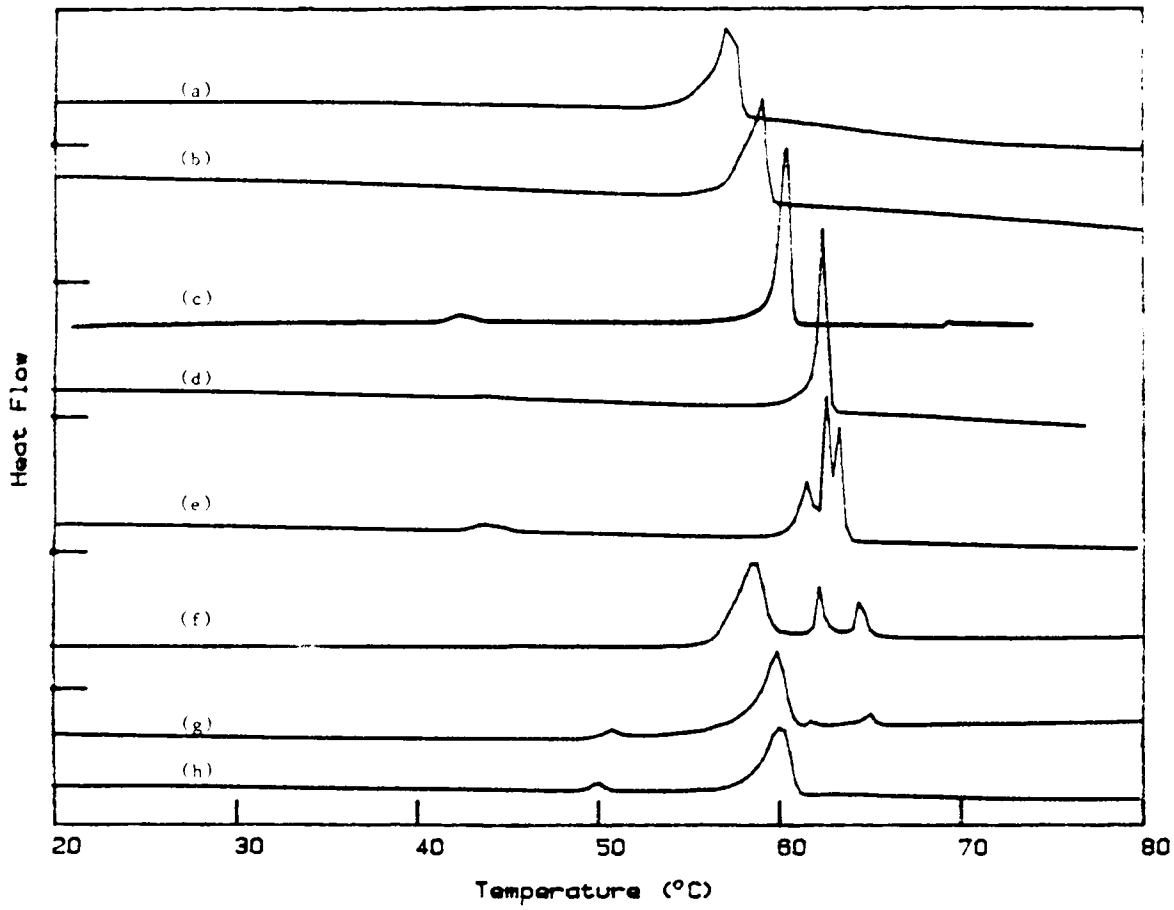

Figure II-2. Representative differential scanning calorimetry scans of DPSG dispersions at (a) pH 8.9, (b) 8.2, (c) 7.4, (d) 6.8, (e) 6.5, (f) 5.4, (g) 5.0 and (h) 4.6.

Table II-1. DSC data of Tp, Tm and ΔH of DPSG dispersions at different pH values.[a]

pH	Tp (°C)	Tm (°C)		ΔH (kcal/mol)		
8.9	---	57.0 ± 0.1		7.6 ± 0.2		
8.4	---	58.6 ± 0.1		7.3 ± 0.2		
8.2	---	58.1 ± 0		7.6 ± 0.2		
8.0	---	59.4 ± 0.1		6.7 ± 0.1		
7.6	---	59.9 ± 0.1		6.3 ± 0.7		
7.4	42.3 ± 0	60.4 ± 0.1		7.1 ± 0.3		
7.2	---	61.3 ± 0.1		6.4 ± 0.2		
7.0	---	61.8 ± 0.1		6.2 ± 0.2		
6.8	43.7	62.4 ± 0		6.7 ± 0.1		
6.7	44.1 ± 0.4	62.7 ± 0		7.4 ± 0.1		
6.5	44.1 ± 0.1	61.3 ± 0.2	62.7 ± 0	63.3 ± 0.1	2.3 ± 0.3	3.5 ± 0.4	1.4 ± 0.1
6.3	44.2	59.5 ± 0.1	62.5 ± 0.1	63.8 ± 0.1	1.3 ± 0.4	2.7 ± 0.1	1.7 ± 0.3
6.1	44.4 ± 0.1	58.2 ± 0	62.5 ± 0.1	64.2 ± 0.1	2.3 ± 0.1	4.0 ± 0.1	1.4 ± 0.8
5.7	44.3 ± 0	58.7 ± 0	62.3 ± 0	63.9 ± 0.1	3.4 ± 0.4	1.3 ± 0.2	0.9 ± 0.6
5.4	45.1 ± 0.3	58.5 ± 0.2	62.1 ± 0.2	64.4 ± 0.1	7.4 ± 0.4	1.1 ± 0.4	0.9 ± 0.2
5.2	45.5 ± 0.3	59.2 ± 0.2	61.9 ± 0.1	64.6 ± 0	8.4 ± 0.6	0.2 ± 0	0.2 ± 0
5.0	50.7 ± 0.2	59.1 ± 0.1	61.2 ± 0.1	64.5 ± 0.1	7.3 ± 0.5	0.3 ± 0.1	0.3 ± 0.1
4.6	50.0	---	60.1 ± 0.2	---	---	6.9 ± 0.2	---

[a]ΔH values are matched with corresponding values of Tm. Data are average values of two independent experiments ± error.

approximately 2.3°C above the chain-melting temperature at pH 7.4. Below pH 6.7 the main transition split into 3 peaks. For instance, at pH 6.5, only one of those peaks remained at 62.7°C (Fig. II-2) while the other two peaks appeared at 61.3°C and 63.3°C. This evidence of phase separation could also be observed at other acidic pH values. Splitting of the chain-melting transition peak into 3 peaks did not involve a change of the total transition enthalpy. At pH 7.4, ΔH = 7.1 kcal/mol. At acidic pH values, the average sum of ΔH = 7.4 kcal/mol. This phase separation event is likely the result of different DPSG domains which experience different degrees of protonation. Further decreasing the pH to 5.4, the peak at the lowest temperature ($\sim$ 59°C) became the dominant peak. The sum of ΔH still remained relatively constant. Thus, the lower temperature peak had increased its ΔH at the expense of the other two peaks. At even more acidic pH values, e.g. pH 4.6, only one peak (at 60.1°C) was observed; indicating that only a single dominant structure existed at this condition.

Fig. II-2 also indicated that the main transition at pH > 7.4 occurred at lower temperatures than 60.4°C. In fact, the chain-melting temperature, Tm, could be titrated between pH 6.7 and 8.9 as shown in Fig. II-3. The apparent pKa for the transition was approximately 7.8.

At pH 7.4 or above, the pre-transition peak was rather difficult to detect since it would not readily appear unless the DPSG dispersions had been equilibrated at room temperature

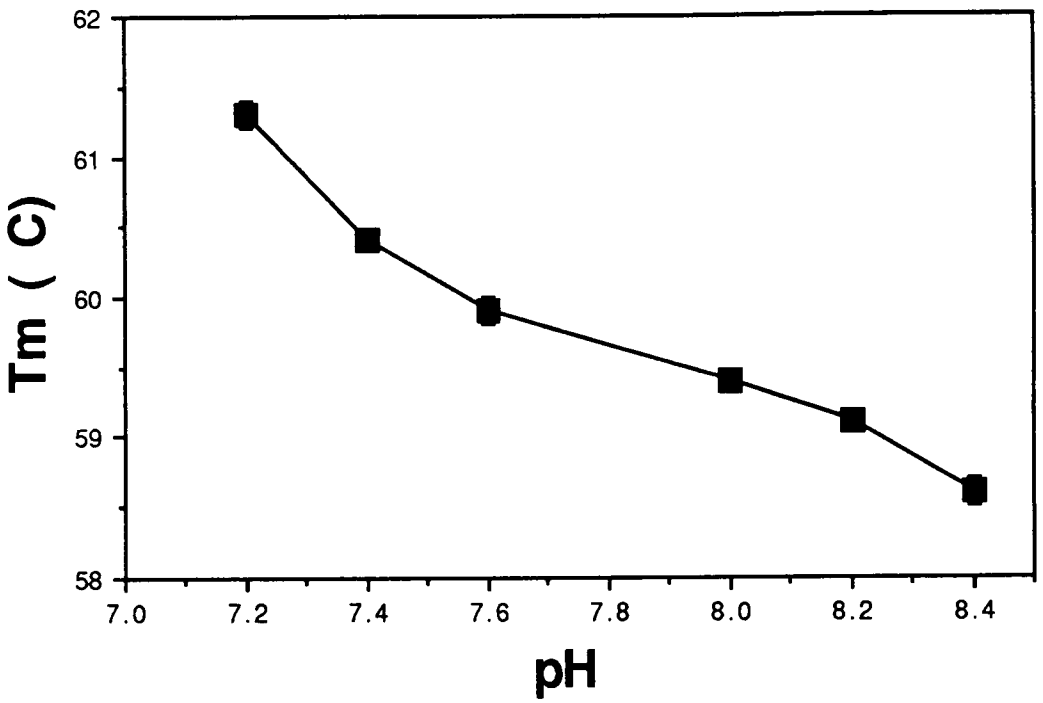

Figure II-3. Dependence of Tm of DPSG dispersions on pH. Data are average values of two independent experiments ± error.

for at least 39 h before scanning. At acidic pH values (< pH 6.8) the pre-transition peak could be readily observed.

Freeze fracture electron microscopy of DOSG liposomes:

The polymorphic phase behavior of DOSG liposomes at different pH was also studied by freeze fracture electron microscopy. As shown in Fig. II-4A, DOSG liposomes at pH 7.4 formed unilamellar vesicles of approximately 0.2 μm in diameter. Under acidic conditions, DOSG liposomes were found to fuse (Fig. II-4B-D). This fusion product exhibited morphological characteristics of non-bilayer phases. In certain specimens, striations resembling hexagonal phase tubes (Fig. II-4B and C) and cross-fractures of tubes (Fig. II-4D) were found along with the non-bilayer phases. However, hexagonal phase was not the predominant structure. Aggregation and fusion were more extensive at pH 4.0 as compared to pH 6.0. The structures at pH 4.0 also appeared to be more tightly packed than at pH 6.0.

Effects of plasma on DOSG liposomes:

A. Plasma stability of DOSG liposomes

For the use of liposomes as an in vivo drug delivery vehicle, the liposomes must be stable in the blood. We have examined the stability of DOSG liposomes in 90% normal human plasma at 37°C by measuring the release of the entrapped calcein. As shown in Fig. II-5, the stability of DOSG

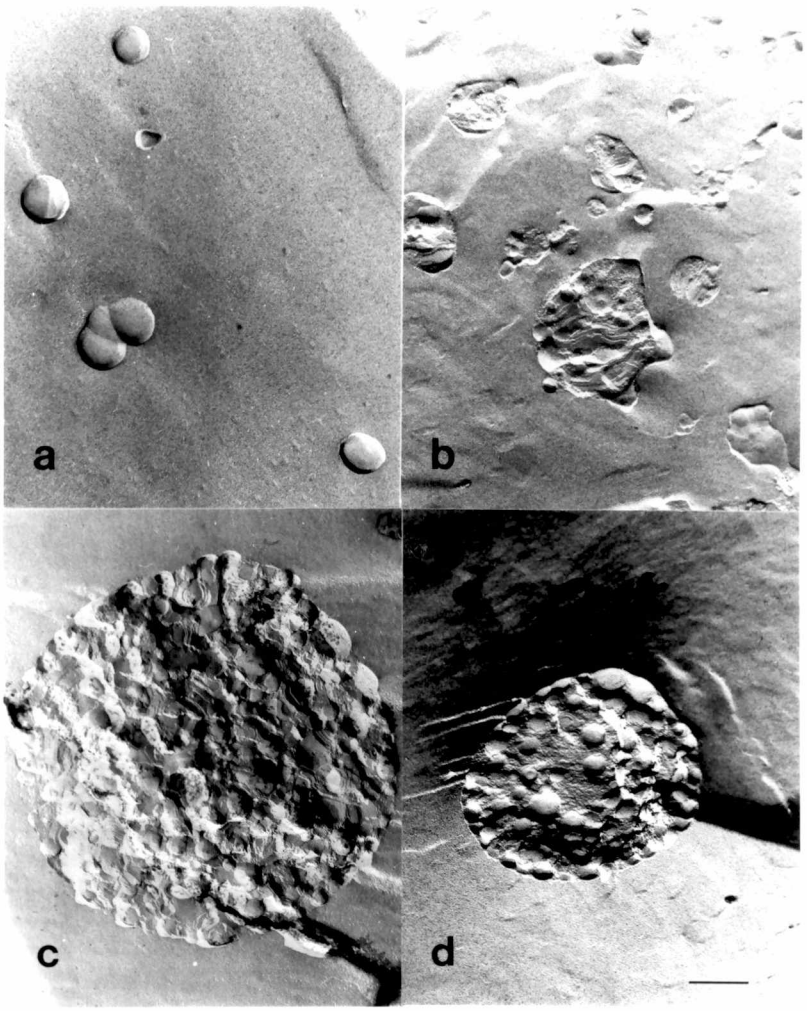

Figure II-4. Freeze fracture electron micrographs of DOSG at (a) pH 7.4, (b) 6.0 and (c,d) 4.0. Bar = 0.2 μm.

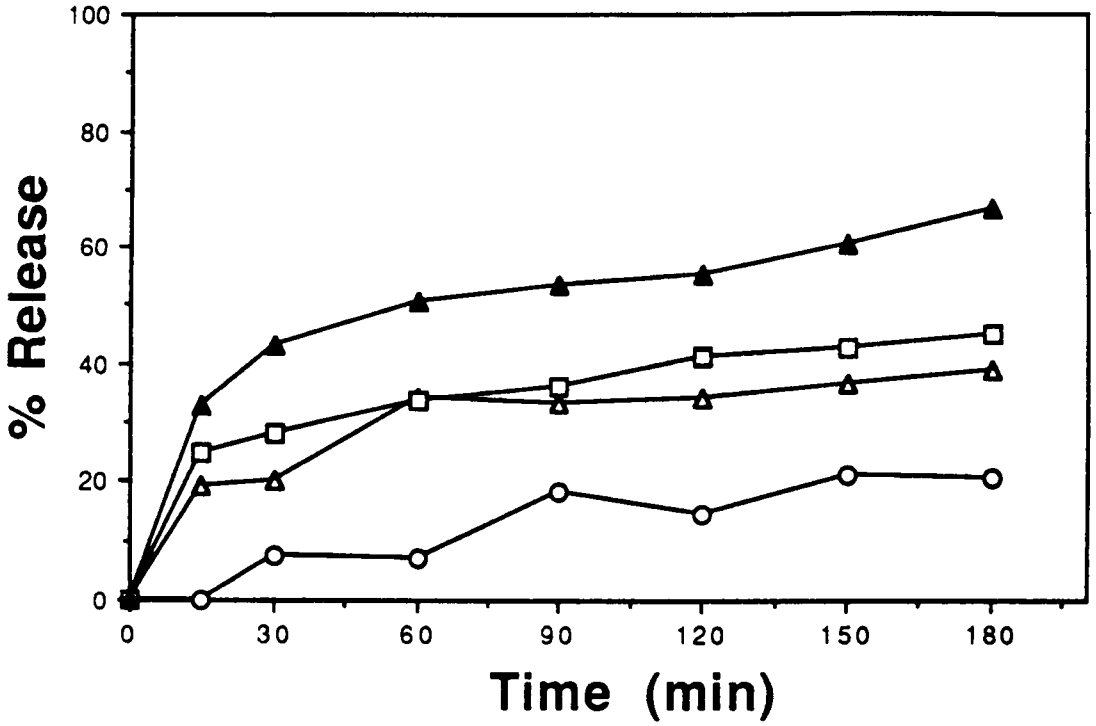

Figure II-5. Plasma stability of DOSG liposomes at 37°C. Calcein release was measured from DOSG liposomes of approximately 256±44 nm (▲), 183±32 nm (□), 126±21 nm (△) and 62±8 nm (○) in diameter.

liposomes in plasma was size-dependent. For instance after incubation in plasma for 3 h, DOSG liposomes of 256±44 nm in diameter were not stable in plasma since they released 70% entrapped calcein. DOSG liposomes ranging between 126-183 nm in diameter were partially stable in plasma, releasing 30-40% entrapped calcein after incubation in plasma for 3 h. However, DOSG liposomes of 62±8 nm in diameter were stable in plasma, releasing only 20% entrapped calcein at the end of the 3-h plasma incubation. These data showed that DOSG liposomes that were smaller in size were more stable in plasma than DOSG liposomes that were larger in size. This size-dependent stabilization effect had been observed with DOPE/oleic acid liposome system and had been related to apolipoprotein A1 (36).

B. Acid-sensitivity of DOSG liposomes after incubation with plasma

It has been widely documented that plasma components are found to associate with liposomes upon exposing liposomes to plasma (27). This association may affect the acid-sensitivity of pH-sensitive liposomes, such as DOSG liposomes, after incubation with plasma.

The release of entrapped calcein, under acidic conditions, from SUV composed of DOSG before incubation with plasma was shown in Fig. II-1 (closed symbol). Percent of calcein release from DOSG liposomes, prepared at various sizes ranging from 62-180 nm in diameter, was very similar to each

other. After incubation with plasma, the acid-sensitivity of DOSG liposomes was reduced (Fig. II-1, open symbols). The degree of reduction in acid-sensitivity of DOSG liposomes was size-dependent. For example at pH 4.5, DOSG liposomes, ranging between 126-183 nm in diameter, were found to release 70% entrapped calcein while DOSG liposomes of 62±8 nm in diameter released < 20% entrapped calcein.

Cytoplasmic delivery of diphtheria toxin A chain (DTA) by the DOSG immunoliposomes:

The fact that DASG vesicles undergo an acid-induced destabilization, similar to what has been observed for liposomes composed of unsaturated PE and an acidic amphiphile (16), had prompted us to examine the cytoplasmic delivery activity of DASG vesicles. DTA was used as an entrapped content marker, because it exhibits a potent inhibitory activity of protein synthesis once it gains access to the cellular cytoplasm (28,29). Palmitoyl anti-H2K^{k} was incorporated into liposome surface to enhance the binding of liposomes with the mouse L929 cells. There was a dose-dependent inhibition of protein synthesis of the L929 cells. The cellular protein synthesis in mouse L929 cells which was assayed as [^{3}H]leucine incorporation was measured as a function of the toxin concentration (Table II-2). Control treatments which included empty DOSG liposomes with and without antibody, empty liposomes or immunoliposomes with free

Table II-2. Cytotoxicity of DTA entrapped in pH-sensitive immunoliposomes composed of different lipid compositions.[a]

DTA concentration (mg/ml)	[^{3}H]Leucine Incorportaion, % Control ± s.d.[b]		
	DOSG	DOPE/DOSG (8:2, m/m)[c]	DOPE/DPSG (8:2, m/m)[c]
10^{-8}	80.0 ± 7.9	70.9 ± 7.8	65.3 ± 7.0
10^{-7}	67.2 ± 9.6	66.4 ± 11.0	54.7 ± 8.0
10^{-6}	41.0 ± 12.1	53.4 ± 10.0	31.5 ± 6.8

[a]Immunoliposomes containing DTA preprared as described in Experimental Procedures were added to mouse L929 cells. The cytotoxicity was assayed by [^{3}H]leucine incorporation into cellular proteins.

[b]Data expressed as ^{3}H-cpm incorporated as % of control cells which did not recevie any DTA.

[c]Data taken from Ref. 16.

DTA added outside all showed no inhibition at the concentration range used (data not shown). The cytoplasmic delivery activity of DOSG immunoliposomes was comparable to that of the DOPE:DOSG and DOPE:DPSG (both 4:1, m/m) immunoliposomes (Table II-2). We conclude that immunoliposomes composed of DOSG exhibit cytoplasmic delivery activity.

Discussion

Aqueous dispersions of both DOSG and DPSG at neutral pH have been prepared. These DOSG liposomes have been found to be able to entrap a H_2O-soluble fluorescent dye, calcein. While the vesicles of DOSG and DPSG are stable at pH 7.4, they undergo a rapid destabilization reaction at acidic pH. The entrapped calcein leaks out (Fig. II-1). As shown by freeze fracture electron micrographs (Fig. II-4B-D), DOSG vesicles undergo fusion under acidic conditions.

The mechanism of acid-induced destabilization of DASG liposomes was studied with differential scanning calorimetry. DPSG dispersions showed a large endothermic (ΔH = 7.1 kcal/mol), chain-melting transition at 60.4°C (Table II-I). At pH $\leq$ 6.5, this peak splits into multiple endothermic peaks, which is indicative of phase separation (Fig. II-2). In fact, in the pH range of 6.7 to 8.9, the temperature of the main transition (Tm) can be titrated with pH with an apparent pKa of 7.8 (Fig. II-3). At pH 7.4, a significant portion of DPSG is already protonated. It is known that lipids in the Lα phase

are more hydrated than those in the Lß phase (30). Protonation of DASG would reduce the level of interfacial hydration because of the loss of the negative charge. Hence, a partially protonated DASG bilayer would be more in favor of the Lß phase than a less protonated one. This is probably why the Tm of the main transition increases with reducing pH (Fig. II-2). After a substantial fraction of DASG is protonated at even more acidic pH (pH $\leq$ 6.5), neutral, protonated species do not mix with the ionized species and phase separation occurs. But as the acidity increases, more and more DPSG becomes protonated until all the DPSG is protonated. Thus only one predominant structure exists, for example at pH 4.6, and no phase separation can be observed. The morphology of this predominant structure had been viewed by freeze fracture electron microscopy. The structure adopted the characteristics of amorphous non-bilayer phases similar to what had been observed with α-tocopherol hemisuccinate at pH 3.5 (24).

It is well established that phase-separated bilayer structures are prone to fuse with each other, probably at the site of packing defects of the phase boundaries (31,32). For example, liposomes composed of phosphatidylserine undergo rapid fusion and destabilization reactions in the presence of divalent cations which induce extensive phase separation of this acidic lipid (33-35).

pH-sensitive liposomes composed of unsaturated PE and a weakly acidic amphiphile are excellent cytoplasmic delivery

vehicles (2-4, 7-11, 16). We have decided to examine the cytoplasmic delivery activity of the DOSG liposomes in a manner similar to what we have previously observed for the DOPE:DASG (4:1, mol/mol) liposomes (16). The data in Table II-2 clearly indicated that DTA entrapped in the DOSG immunoliposomes was delivered to the L929 cells, and the observed activity was similar to that of the DOPE:DPSG and DOPE:DOSG (both 4:1, mol/mol) immunoliposomes (Table II-2).

To use DOSG liposomes for drug delivery studies, DOSG liposomes ranging between 120-180 nm in diameter should be used. DOSG liposomes of this size are partially stable in human plasma at 37°C (Fig. II-5) and retain their acid-sensitivity (Fig. II-1). Since they also show cytoplasmic delivery activity (Table II-2), their use in the drug delivery studies warrants further exploration.

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PART III

INTERACTIONS OF BILAYER COMPOSED OF 1,2-DIACYL-3-SUCCINYLGLYCEROL WITH DIVALENT CATIONS

Abstract

The effects of divalent cations, such as Ca^{2+} and Mg^{2+}, on the destabilization of bilayers composed of 1,2-diacyl-3-succinylglycerol (DASG) have been studied. Under limiting conditions in which the molar ratio of divalent cation to lipid was ≤ 1, Mg^{2+} was more effective than Ca^{2+} in inducing the aggregation and lipid mixing of liposomes composed of 1,2-dioleoyl-3-succinylglycerol (DOSG). Differential scanning calorimetry studies of bilayers composed of 1,2-dipalmitoyl-3-succinylglycerol (DPSG) showed that Mg^{2+} was more effective than Ca^{2+} in increasing the chain-melting temperature (Tm) of the main transition of DPSG. In the presence of excess divalent cations in which the molar ratio of divalent cation to lipid was $>> 1$, X-ray diffraction and freeze fracture electron microscopy indicated that both Ca^{2+} and Mg^{2+} could induce a lamellar to hexagonal phase transition. Both divalent cations acted synergistically with protons in destabilizing DASG bilayers. As shown by differential scanning calorimetry, in the presence of divalent cations phase separation of DPSG bilayers could be observed at pH 6.8; whereas in the absence of divalent cations, phase separation of DPSG bilayers could not be observed until the pH was further reduced to 6.5. Freeze fracture electron micrographs revealed that under acidic conditions, hexagonal phase and cubic phase structures could be seen in the presence of Ca^{2+} and Mg^{2+}, respectively. Thus, the final structures formed in Ca^{2+} and Mg^{2+} appear to be

different in the presence of acid.

Introduction

Liposomes and immunoliposomes (1) show promising potential as drug delivery vehicles. They are normally taken up via the endocytic pathway and eventually delivered to the lysosomes, where liposomes and their contents are degraded (1,2). Acid-sensitive liposomes have been developed to overcome this problem (3-5). Acid-sensitive liposomes are stable at pH 7.4, and become destabilized in a mildly acidic environment, such as what is inside the endosomes. The destabilized liposomes fuse with the endosome membranes and discharge at least a part of their contents into the cytoplasm. Conventional acid-sensitive liposomes are composed of unsaturated PE and a weakly acidic amphiphile (3-6).

We have previously reported that liposomes composed of pure 1,2-diacyl-3-succinylglycerol are acid-sensitive (7,8). DASG liposomes showed a half-maximal release of the entrapped calcein between pH 5.8-6.3 (7,8). DOSG liposomes are active in cytoplasmic delivery as demonstrated by its ability to deliver the A chain of diphtheria toxin to mouse fibroblasts (7,8). Since the acid sensitivity is directly linked to the cytoplasmic delivery activity of the liposomes (6), destabilization of liposomes in the presence of acid is a biologically relevant event.

Ca^{2+} and Mg^{2+} are important components of biological

fluids and tissure culture media. These divalent cations have also been shown to destabilize liposomes containing anionic lipids, such as phosphatidylserine (9-12), phosphatidic acid (13) and cardiolipin (14-16). Since DASG is also an anionic lipid, Ca^{2+} and Mg^{2+} could also affect the stability of liposomes composed of DASG. Furthermore, acid-sensitive liposomes are often sensitive to divalent cations (17,18). Protons and divalent cations act synergistically to destabilize the acid-sensitive liposomes (18). In this report we describe a systematic study on the effect of Ca^{2+} and Mg^{2+} on the stability of DASG bilayers and the synergistic effect of proton. The results should be relevant to the delivery activity of this novel type of acid-sensitive liposomes.

Materials and Methods

Materials:

All lipids were purchased from Avanti Polar Lipids. The purity of DOSG and DPSG was confirmed by thin-layer chromatography using hexane: ethylether: methanol: acetic acid (70:30:5:1, v/v) as developing solvent. Pipes was obtained from Sigma Chemical Co.

Liposome preparation:

Solvent-free lipids were vacuum dessicated for at least 30 min. Lipids were suspended overnight in PBS, at a final concentration of 10 μmol/ml, pH 7.4. Large liposomes were then

extruded through 0.2 μm polycarbonate filters (Nucleopore Co.) to form SUV. The size of liposomes was determined by dynamic laser light scattering using a N4SD Particle Sizer (Coulter). The average diameter of SUV was 184±29 nm. Liposomes were used freshly for light scattering and lipid mixing experiments.

Light scattering measurement:

A LS-5 Perkin-Elmer spectrophotometer was used. The excitation and emission wavelengths were 600 nm and the excitation and emission slit widths were 3 nm. Two hundred μM of DOSG liposomes were added to a cuvette and were continously stirred for 1 min before any divalent cation was added. This is to ensure that aggregation or fusion events did not take place in the absence of divalent cation. Zero to 500 μM of divalent cations were then added. An increase in the size of DOSG liposomes due to aggregation or fusion would lead to an increase in light scattering signal. The aggregation of liposomes was monitored continuously for 10 min.

Lipid mixing assay:

Both unlabeled and labeled SUVs were prepared as described above, except that the labeled liposomes contained 1 mol % N-NBD-PE and 0.5 mol % N-Rh-PE. Unlabeled and labeled DOSG liposomes were mixed together in a molar ratio of 3 to 1 and percent of lipid mixing was monitored. Liposomes were stirred for 1 min to confirm that the liposomes do not undergo

fusion in the absence of divalent cations. Percent of lipid mixing was assayed as described (3) and monitored over a period of 10 min after the addition of divalent cations.

Differential scanning calorimetry:

After vacuum dessication, solvent free lipids were suspended at 5 μmol/ml in 20 mM Pipes buffer, pH 7.4. DPSG was hydrated and vortexed occasionally for 2 h at 67°C. Various amounts of $CaCl_2$ or $MgCl_2$ were then added to the lipid mixture. To some lipid samples, various amounts of 1N HCl were also added so that the final pH of the mixture was 6.8. Lipids were vortexed and further incubated for 2 additional h at 67°C before incubation at room temperature for overnight equilibration. The equilibrated lipids were loaded into a Microcal MC-2 scanning calorimeter (Amherst, MA) and scanned from 20 to 80°C at a scan rate of 30°C/h. The excess heat capacity was recorded as a function of temperature. The peak transition temperature was determined by an IBM PC computer interfaced with the instrument.

X-ray diffraction:

The hydrated lipids were mounted into X-ray sample holders at the equilibrating temperature. Each sample was combined with some powdered teflon as an X-ray calibration standard and then sealed between mica windows 1 mm apart. X-ray diffraction was used to characterize the structures formed

by the various lipid mixtures. The $CuK_{\alpha 1}$ line (λ = 1.540 Å) was isolated using a bent quartz crystal monochromator, and diffraction patterns were recorded photographically using Guinier X-ray cameras operating in vacuo. Temperature was controlled with thermoelectric elements to approximately ±0.5°C.

A lamellar phase is characterized by a series of three to five X-ray spacings in the ratios of the unit cell dimension, d_{lam} of 1, 1/2, 1/3, 1/4, etc. A hexagonal phase is characterized by X-ray spacings bearing ratios to the dimension of the first order, d_{hex} of 1, $1/\sqrt{3}$, $1/\sqrt{4}$, $1/\sqrt{7}$, $1/\sqrt{9}$, $1/\sqrt{11}$, etc.

Freeze fracture electron microscopy:

After vacuum dessication, solvent-free DOSG lipids were suspended in 20 mM Pipes buffer, pH 7.4. The lipids were extruded through 0.2 μm polycarbonate filters. $CaCl_2$ or $MgCl_2$ were then added to the liposomes at a 10-fold molar excess. To some samples, 1 N HCl was also added so that the final pH was 6.0. Freeze fracture was done as described (17) and the replicas were viewed with a Philip 300 electron microscope at magnifications of 7,000 to 27,000.

Results

Destabilization of DOSG liposomes by divalent cations:

It is known that destabilization of liposome bilayers can cause aggregation and/or fusion of the liposomes (10-13). We have used light scattering and lipid mixing methods to monitor these aggregation and fusion events, respectively.

Within 1 min of the addition of divalent cations, the increase in light scattering signal had already reached steady-state (data not shown). Under identical conditions, Mg^{2+} was found to be more effective than Ca^{2+} in inducing the aggregation of DOSG liposomes (Fig. III-1). For example, the level of light scattering induced by Mg^{2+} at 300 μM was approximately 10 fold greater than what was induced by Ca^{2+} at the same concentration.

Bilayer destabilization was also studied by a lipid mixing assay (19). Maximal % of lipid mixing occured within 1 min of the addition of divalent cations. Under identical conditions, Mg^{2+} was again found to be more effective than Ca^{2+} in inducing the fusion of DOSG liposomes (Fig. III-1). For example, 70% lipid mixing was seen with 500 μM of Mg^{2+} while only 40% lipid mixing was seen with 500 μM of Ca^{2+}.

Thermal phase transitions of DPSG dispersions at pH 7.4:

A. By differential scanning calorimetry

An endothermic scan of DPSG dispersions at pH 7.4 in the absence of divalent cation showed that the chain-melting

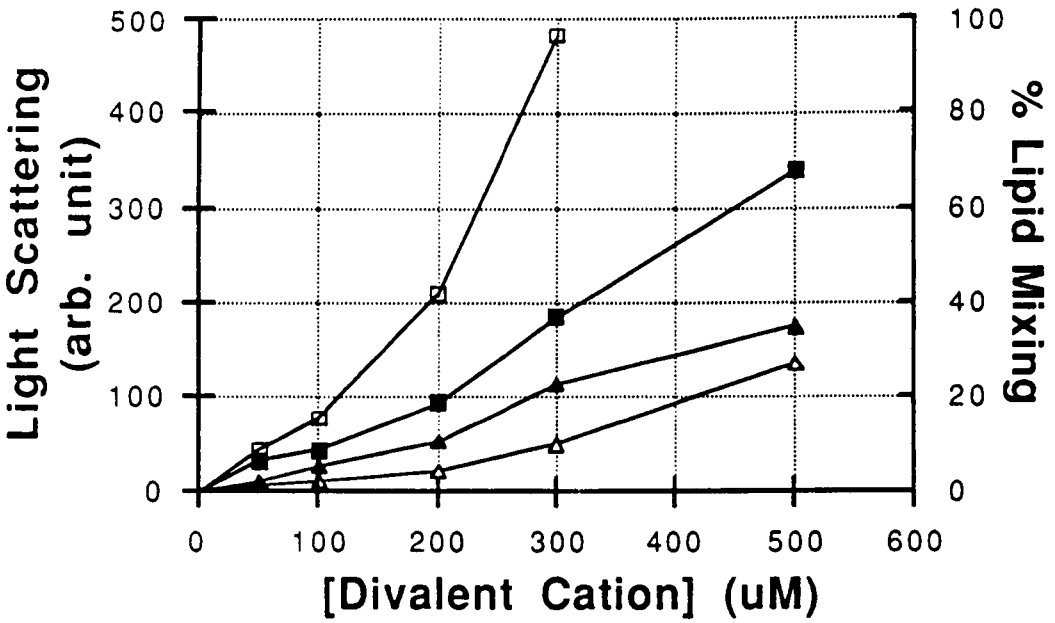

Figure III-1. Effects of divalent cations on light scattering and lipid mixing of DOSG liposomes. Addition of Ca^{2+} (△, ▲) and Mg^{2+} (□,■). Open and closed symbols represent light scattering and lipid mixing experiments, respectively.

temperature (Tm) of DPSG dispersion was 60.4°C and the enthalpy change (ΔH) of the chain-melting transition was 7.1 kcal/mol (part II). Fig. III-2 shows the effects of divalent cations on the thermal phase transitions of DPSG dispersions at pH 7.4. Tm of DPSG increased as the concentration of divalent cations increased. Under identical conditions, Mg^{2+} was more effective than Ca^{2+} in driving up the Tm. When 400 μM of divalent cations were added, the Tm of DPSG was approximately 63.5°C in the presence of Ca^{2+} and 67.2°C in the presence of Mg^{2+} (Table III-1). Although divalent cations could increase the Tm of DPSG, the ΔH remained approximately the same over the range of the divalent cation concentrations used.

The pre-transition temperature (Tp) of DPSG at pH 7.4 in the absence of divalent cations was 42.3°C (part II). Tp also increased as the concentration of divalent cations increased. Both Ca^{2+} and Mg^{2+} could increase the Tp of DPSG to approximately the same degree. When 300 μM of divalent cations were used, the Tp was 48.2 and 47.8°C in the presence of Ca^{2+} and Mg^{2+}, respectively.

Higher concentrations of divalent cations were added to DPSG dispersions. However sample aggregation was so great that reproducible loading of the sample into the calorimeter was technically difficult. Therefore, we have decided to use X-ray diffraction and freeze fracture electron microscopy to study the effects of excess divalent cations on DASG bilayers.

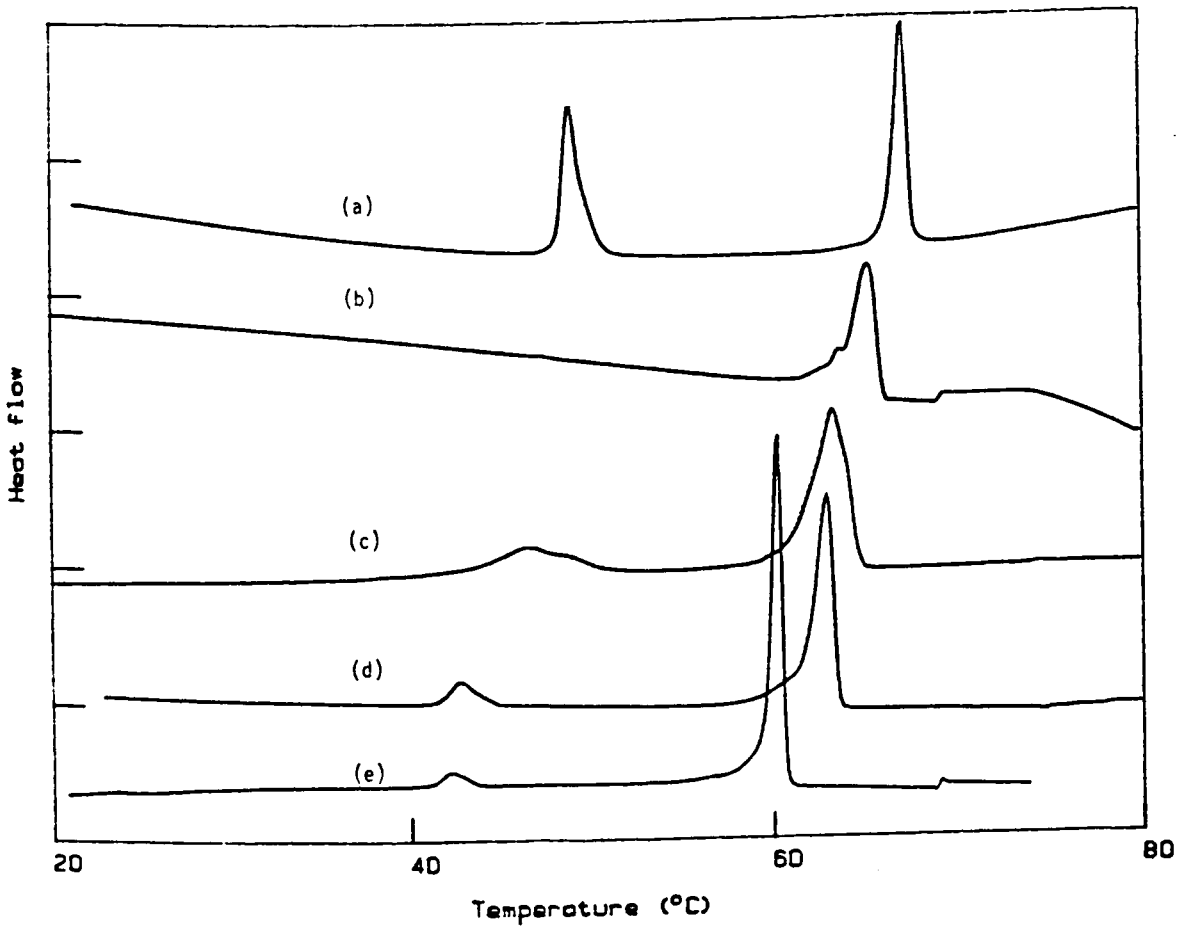

Fig. III-2. Representative differential scanning calorimetry scans of DPSG dispersions at pH 7.4 in the presence of (a) 400 μM Mg^{2+}, (b) 200 μM Mg^{2+}, (c) 400 μM Ca^{2+}, (d) 200 μM Ca^{2+} and (e) in the absence of divalent cations.

Table III-1. DSC data of Tp, Tm and ΔH of DPSG dispersions in the presence of divalent cation at pH 7.4.[a]

Divalent cation[b]	(μM)	Tp (°C)	Tm (°C)	ΔH (kcal/mol)
Ca^{2+}	0	42.3	60.4±0.1	7.1±0.3
	100	---	61.8±0.1	6.8±0.1
	200	42.8	62.8±0.2	6.5±0.4
	300	48.2	63.5±0.1	6.8±0.2
	400	---	63.5±0.1	7.6±0.5
Mg^{2+}	0	42.3	60.4±0.1	7.1±0.3
	100	43.3	63.2±0.2	6.8±0.3
	200	46.9	65.1±0.1	6.7±0.8
	300	47.8	66.1±0.1	7.4±0.1
	400	48.8	67.2±0.1	6.4±0.5

[a]ΔH values are matched with corresponding values of Tm. Data are average values of two independent experiments ± error.

[b]Divalent cations concentrations were limiting. Molar ratio of divalent cation to DPSG was < 1.

B. By X-ray diffraction

$MgCl_2$ was added to DPSG at a three-fold molar excess. X-ray diffraction was performed at various temperatures (Fig. III-3). At 20°C, DPSG existed as lamellar structures with frozen chains and a repeat spacing of 56.3 Å (Table III-2). A high degree of chain order of DPSG was shown by many diffraction lines in the hydrocabon chain region. Between 50 - 60°C, DPSG was still in a lamellar phase. However the repeat spacing of the bilayers had increased to 61.8 Å. Also DPSG bilayers had become less crystalline, as only a single diffraction line was obtained in the hydrocarbon chain region. This increase in repeat spacing is probably due to an increase in hydration. Thus, DPSG dispersions probably undergo a pre-transition at somewhere below 50°C.

At 60°C or above, DPSG dispersions underwent a lamellar to hexagonal phase transition of repeat spacing 51.8 Å and fluid or "melted" hydrocarbon chains. Under identical conditions, the effect of Ca^{2+} on DPSG was similar to that of Mg^{2+}. The lattice repeat spacings of DPSG in the presence of Ca^{2+} at 20°C, 50°C and 70°C are shown in Table III-2. The hexagonal spacing in particular is very small.

DOSG dispersions with divalent cations form a hexagonal phase at 20°C. The repeat spacing of DOSG dispersions in the presence of Ca^{2+} and Mg^{2+} were similar to those of DPSG dispersions at 70°C (data not shown).

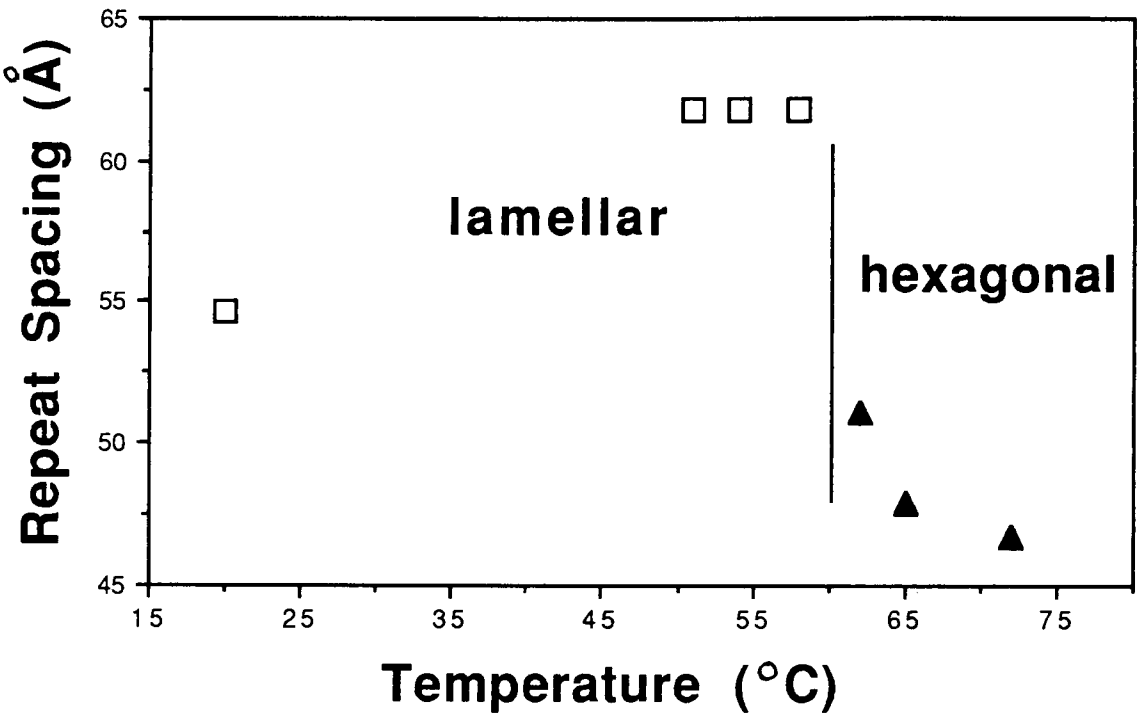

Figure III-3. X-ray diffraction measurement of the repeat spacings of DPSG bilayers in the presence of 3-fold molar excess of divalent cations at various temperatures. Lamellar (□) and hexagonal (▲) repeat spacings.

Table III-2. X-ray diffraction data of DPSG dispersions in the presence of divalent cations at different temperature.[a]

Cation	temperature (°C)	X-ray phase	Repeat spacing (Å)	Condition of chains
Ca^{2+}	20	lamellar	55.3	frozen -many lines
	50	lamellar	52.1	frozen -1 line
	70	hexagonal	36.0	melted
Mg^{2+}	20	lamellar	56.3	frozen -many lines
	50	lamellar	61.8	frozen -1 line
	70	hexagonal	51.8	melted

[a]Divalent cation concentrations were in excess. Molar ratio of divalent cation to DPSG was 3.

Freeze fracture electron microscopy of DOSG liposomes at pH 7.4:

At pH 7.4 and in the absence of divalent cations, DOSG vesicles were prepared as unilamellar liposomes (Fig. III-4A). But in the presence of ten-fold molar excess of divalent cations, DOSG vesicles had fused with each other and had undergone an hexagonal phase transition (Fig. III-4B and C).

Thermal phase transitions of DPSG dispersions at acidic pH:

To determine if there was any synergistic effect between H^+ and the divalent cations on the destabilization of DASG bilayers, we studied the thermal phase transitions of DPSG dispersions at acidic pH in the presence of divalent cations. DPSG was hydrated under the conditions described above. However when 0 - 400 μM of Ca^{2+} or Mg^{2+} were added to the lipid suspensions, 1 N HCl was also added so that the final pH of the lipid dispersion was 6.8.

As shown in Fig. III-5, the chain-melting temperature (Tm) of DPSG at pH 7.4 and 6.8 were 60.4 and 62.4°C, respectively. The increase of the Tm at acidic pH is probably due to the dehydration of DPSG bilayers as the headgroups of the lipids are protonated (part II). In the absence of divalent cations, phase separation of DPSG bilayers did not occur until the pH was reduced to 6.5 (part II). When divalent cations were added to the lipid sample, phase separation could be observed even at pH 6.8. With 100 μM of Mg^{2+}, the chain-

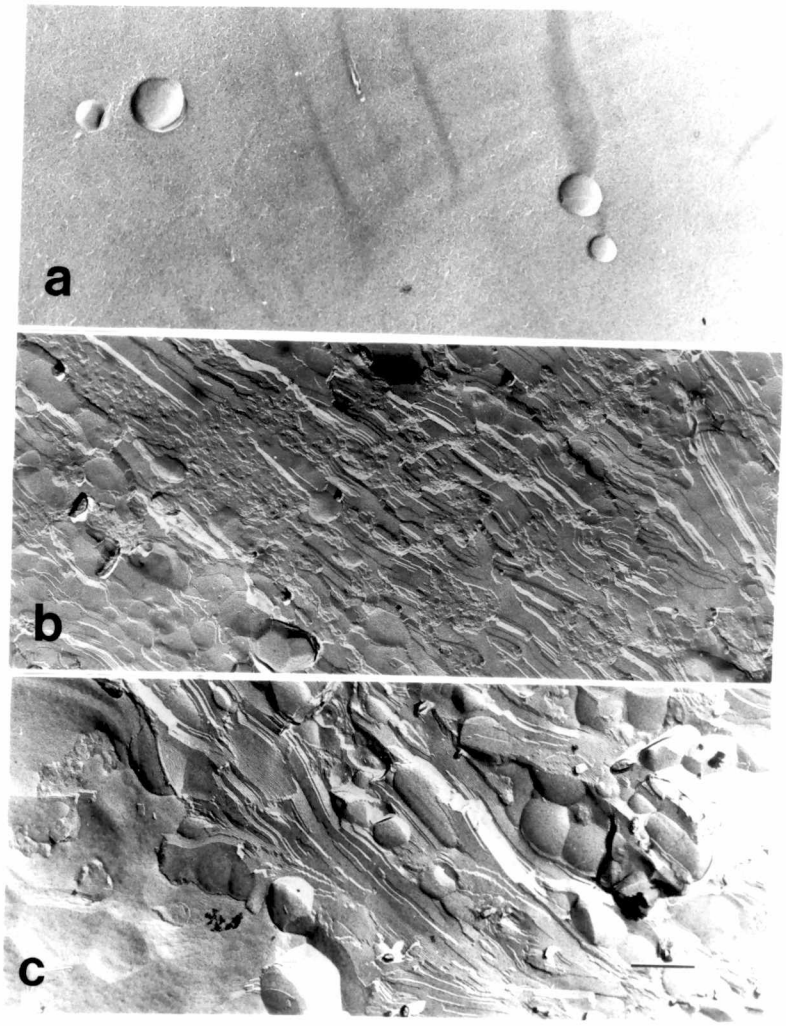

Figure III-4. Freeze fracture electron micrographs of DOSG liposomes at pH 7.4 in the absence of divalent cations (a) and in the presence of 10-fold molar excess of (b) Ca^{2+} or (c) Mg^{2+}. Bar = 0.2 μm.

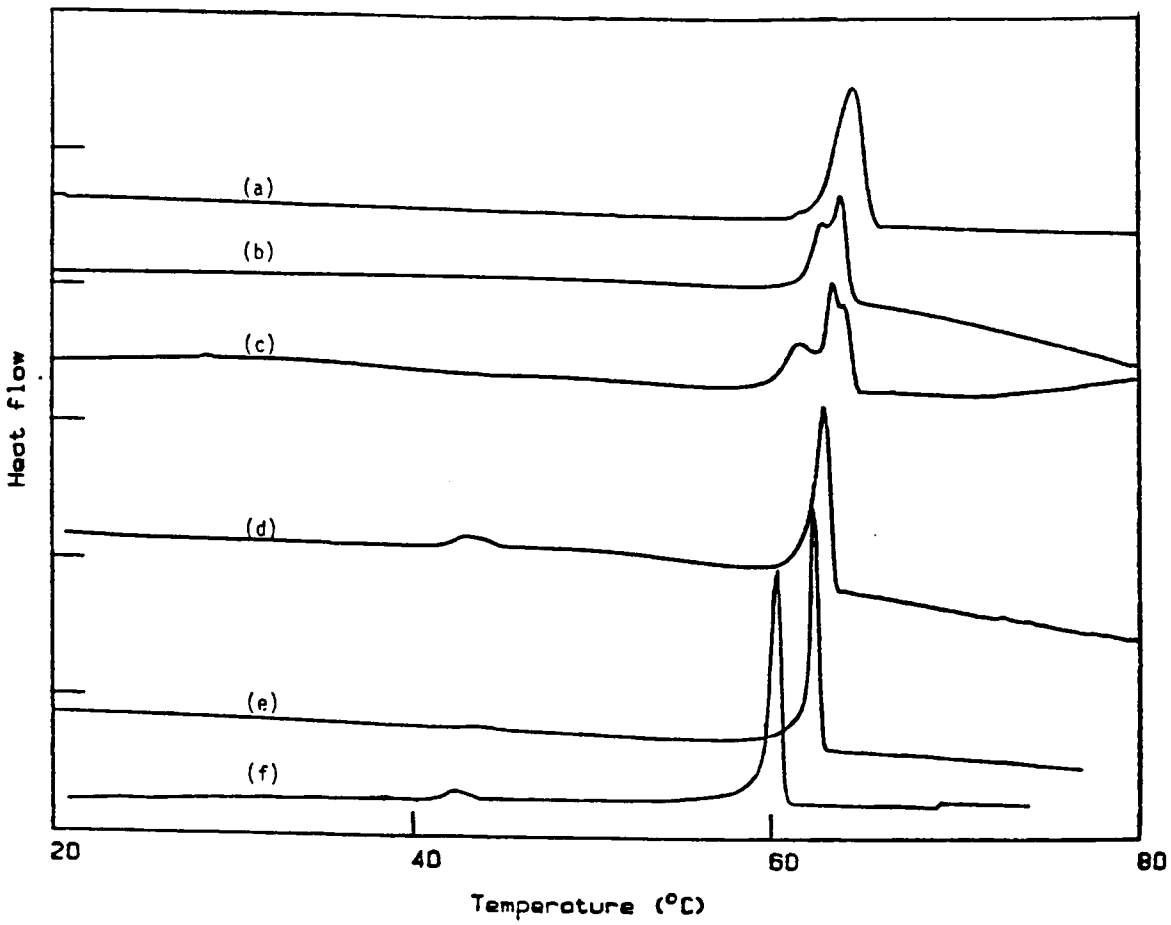

Figure III-5. Representative differential scanning calorimetry scans of DPSg dispersions in the presence of (a) 300 μM Mg^{2+}, (b) 100 μM Mg^{2+}, (c) 300 μM Ca^{2+}, (d) 100 μM Ca^{2+} and (e,f) in the absence of divalent cations. Scans (a-e) and (f) were done at pH 6.8 an d7.4, respectively.

melting peak split into two (Fig. III-5) and the peak temperatures were 62.8 and 63.9°C (Table III-3). However, with increasing concentrations of Mg^{2+}, the split peaks coalesced into a single peak, indicating the existence of a predominant phase. Phase separation was not observed with DPSG until 200 μM of Ca^{2+} was added. The chain-melting peak split into three peaks with the peak temperatures occurring at 61.9, 63.1 and 63.8°C. Increasing the concentration of Ca^{2+}, up to 400 μM, continued to cause the DPSG bilayers phase separated and the peak temperatures remained approximately the same.

The pre-transition temperature (Tp) had increased in the presence of the divalent cations. Tp was driven up to 47.6°C and 48.2°C with 400 μM of Mg^{2+} and Ca^{2+}, respectively.

Freeze fracture electron microscopy of DOSG liposomes at acidic pH:

DOSG liposomes were prepared initially at pH 7.4. Then 1 N HCl was added so that the final pH was 6.0. In the absence of divalent cations, DOSG liposomes fused with each other and existed in an amorphous, non-bilayer phase with some structures resembling hexagonal phase (Fig. III-6A). But in the presence of Ca^{2+} and Mg^{2+}, DOSG mainly existed as hexagonal phase structures (Fig. III-6B) and cubic phase structures (Fig. III-6C), respectively.

Table III-3. DSC data of Tp, Tm and ΔH of DPSG dispersions in the presence of divalent cations at pH 6.8.[a]

Divalent cation[b]	(μM)	Tp (°C)	Tm (°C)	ΔH (kcal/mol)
Ca^{2+}	0	43.7	62.4	6.7±0.1
	100	43.1	63.0±0.1	6.2±0.3
	200	43.1	61.9±0.1, 63.1±0.1, 63.8±0.1	1.8±0.3, 1.5±0.1, 2.2±0.3
	300	----	61.8±0.1, 63.4, 64.2±0.2	2.6±0.5, 2.8±0.1, 2.2±0.1
	400	48.2	61.4±0.2, 63.4±0.2, 64.4±0.2	4.8±0.8, 2.3±0.2, 0.9±0.1
Mg^{2+}	0	43.7	62.4	6.7±0.1
	100	44.7	62.8±0.1, 63.9±0.1	3.1±0.3, 3.4±0.1
	200	46.3	63.8±0.2	5.8±0.3
	300	47.0	64.6±0.2	7.7±0.3
	400	47.6	64.6±0.1	7.5±0.9

[a]ΔH values are matched with corresponding values of Tm. Data are average values of two independent experiments ± error.

[b]Divalent cation concentrations were limiting. Molar ratio of divalent cation to DPSG was < 1.

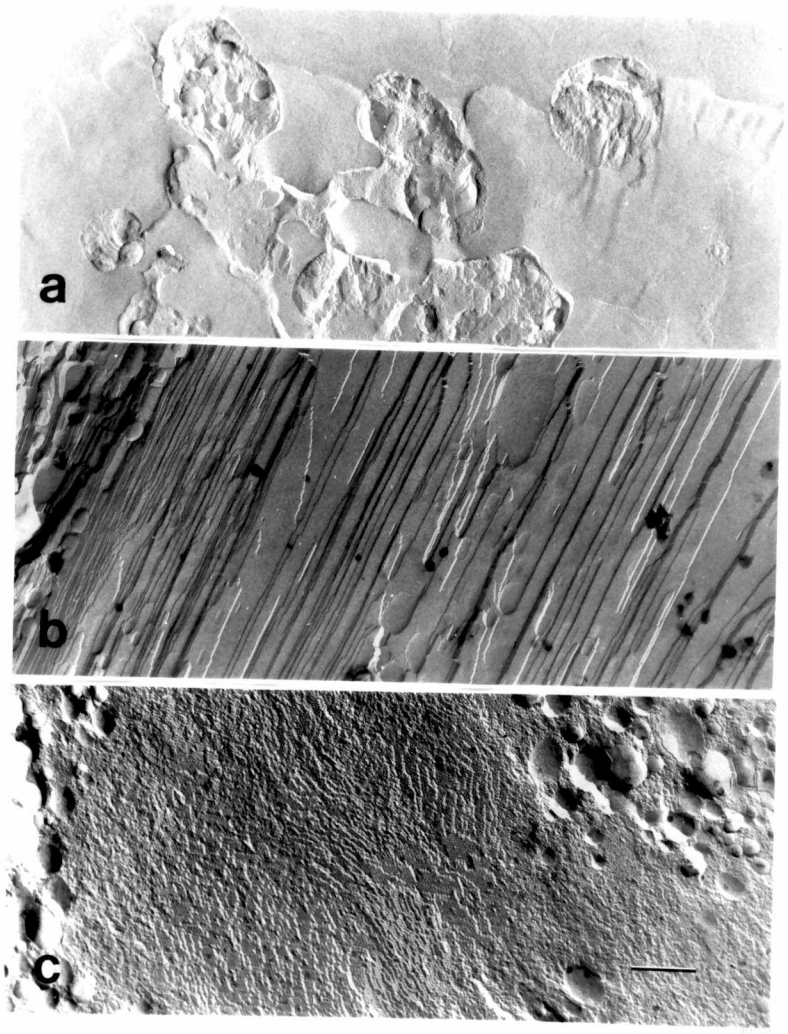

Figure III-6. Freeze fracture electron micrographs of DOSG liposomes at pH 6 in the absence of divalent cations (a) and in the presence of 10-fold molar excess of Ca^{2+} (b) or Mg^{2+} (c). Bar = 0.2 μm.

Discussion

Divalent cations, such as Ca^{2+} and Mg^{2+}, are known to induce the destabilization of liposome bilayers composed of anionic lipids (9-16). This destabilization process leads to the aggregation and/or fusion of liposomes. Ca^{2+} is more effective than Mg^{2+} in causing the aggregation and fusion of liposomes composed of phosphatidylserine (9-12) or phosphatidylglycerol (20-22). Ca^{2+} may enhance aggregation and fusion of liposomes by increasing intervesicular contact via a "bridging" effect between the anionic lipid molecules (10). Alternatively, Ca^{2+} causes dehydration of the bilayers and allow the vesicles to come in close apposition (9).

Divalent cations also induce the destabilization of bilayers composed of DASG. The aggregation and lipid mixing of DOSG liposomes were observed as a result of bilayer destabilization (Fig. III-1). However, Mg^{2+} was more effective than Ca^{2+} in destabilizing DOSG liposomes. Under limiting conditions in which the molar ratio of divalent cation to DASG was ≤ 1, the destabilization mechanism was probably due to dehydration of the DASG bilayers. Differential scanning calorimetric studies showed that both divalent cations could increase the chain-melting temperature (Tm) of DPSG (Fig. III-2, Table III-1). It is known that lipids in the Lα phase are more hydrated than those in the Lß phase (23). The binding of divalent cations to DASG would neutralize the negative charge of DASG and reduce the interfacial hydration of DASG

bilayers, thus stabilizing the Lß phase. This is probably why the Tm of the main transition increases as the concentration of divalent cations increases.

The final product of DASG bilayers destabilized by excess divalent cations (molar ratio of divalent cation to lipids was >> 1) was studied by X-ray diffraction and freeze fracture electron microscopy (Fig. III-3 & III-4, Table III-2). Hexagonal phase structures had been observed for both Ca^{2+} and Mg^{2+}. Thus when excess divalent cations were used, the destabilization mechanism of DASG bilayers led to the formation of hexagonal phase structures. The formation of hexagonal phase by Ca^{2+} but not by Mg^{2+} has also been reported for other anionic lipids, such as phosphatidylserine, phosphatidic acid and cardiolipin (9-13).

The main structural difference between DASG and other anionic phospholipids is that DASG does not contain a phosphate group while the other anionic lipids do. The neighbouring headgroups of DASG are probably closer to each other than the neighbouring headgroups of the other anionic lipids due to the lack of repulsive force between the negatively charged phosphate groups. Mg^{2+}, being a smaller hydrated cation, may bind more effectively than Ca^{2+} to the headgroups of the DASG lipid. This may explain why Mg^{2+} is more effective than Ca^{2+} in destabilizing the DASG bilayers.

The destabilization of DASG bilayers by acid had been reported by us (part II). The mechanism of acid-induced

destabilization involves extensive bilayer phase separation as well as the formation of non-bilayer phase structures (part II). Extensive phase separation was not observed until the pH was reduced to 6.5 (part II). Liposomes that have become phase separated are prone to fuse with each other, probably at the site of the phase boundaries with packing defects (24,25). Our data showed that there was a synergism in the destabilization of DASG bilayers by proton and divalent cations. By increasing the concentration of divalent cations, extensive phase separation could be readily observed even at pH 6.8 (Fig. III-5, Table III-3). Protons and divalent cations can neutralize the DASG bilayers. Under limiting conditions, DASG bilayers would only be partly neutralized. The neutralized species do not mix well with the ionic species and phase separation occurs. More neutralized species would be formed by increasing the concentration of divalent cations. This eventually led to the formation of hexagonal phase structures by Ca^{2+} (Fig. III-6B) and the formation of cubic phase structures by Mg^{2+} (Fig. III-6C). The fusion mechanism of liposomes has also been related to the existence of interlamellar attachments between unilamellar vesicles (26-29). Under identical conditions, interlamellar attachments assemble into cubic phase structures in multilamellar vesicles (27). The fact that Mg^{2+} can induce the fusion of DASG vesicles more effectively than Ca^{2+} may be related to its ability in the formation of cubic phase structures.

We have previously reported that DOSG liposomes are efficient in cytoplasmic delivery (part II). They are able to deliver the A fragment of diphtheria toxin to mouse fibroblasts (part II), probably by fusion with the endosome membranes. Since both Ca^{2+} and Mg^{2+} are present in the extracellular medium and probably also in the endosomes, our data suggest that these cations may play an important role in augmenting the membrane fusion and destabilization events in the endosomes.

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PART IV

PURIFICATION OF SERUM-BINDING PROTEINS: BINDING OF SERUM ALBUMIN TO LIPOSOMES COMPOSED OF 1,2-DIOLEOYL-3-SUCCINYLGLYCEROL

Abstract

Liposomes composed of 1,2-dioleoyl-3-succinylglycerol (DOSG) are rapidly removed from the blood circulation and accumulate in the liver at a high level. The circulation time of DOSG liposomes could not be prolonged even when G_{M1} was included in the liposome composition. Thus, similar to phsophatidylserine, DOSG must also be an opsonin attracting lipid. Using an affinity column with immobilized DOSG liposomes, serum albumin was found to be the major serum protein associated with DOSG liposomes. Binding of serum albumin with DOSG liposomes was significantly enhanced in the presence of physiological concentrations of Ca^{2+}, but not Mg^{2+}. We propose that DOSG liposomes accumulate mainly in the liver due to their preferential association with serum albumin via an interaction with Ca^{2+}. Thus, serum albumin could be a divalent cation dependent, liver specific opsonin for the DOSG liposomes.

Introduction

For liposomes to be optimally used as a drug delivery vehicle, it is necessary to design liposomes with controlled biodistribution and content release. One such type of liposomes with controlled content release activity is the acid-sensitive liposomes (1-3). These liposomes undergo a rapid bilayer destabilization and fusion when they encounter mildly acidic pH such as the pH in the endosomes. Thus

acid-sensitive liposomes are able to discharge their entrapped contents to the cytoplasm of target cells for the expression of biological activity (4-7).

Liposomes composed of a synthetic, weakly acidic amphiphile, 1,2-dioleoyl-3-succinylglycerol, have been reported by us (8,9). DOSG liposomes are acid-sensitive and efficient in cytoplasmic delivery of macromolecules, such as the A chain of diphtheria toxin, to the mouse fibroblasts (8,9). Furthermore, DOSG liposomes are partially stable in serum and retain the acid-sensitivity after incubation with serum (9). Thus DOSG liposomes might be useful for drug delivery. We describe here a biodistribution study of the intravenously injected DOSG liposomes in mice. Serum proteins which specifically bind to these liposomes have been purified by affinity chromatography. These proteins might be the putative opsonins which are responsible for the blood clearance of DOSG liposomes. The results have revealed several unusual properties of this novel liposome type.

Materials and Methods

Materials:

DOSG was custom synthesized by Avanti Polar Lipids. The purity of the lipids was confirmed by thin-layer chromatography using hexane: ethyl ether: methanol: acetic acid (70/30/5/1, v/v) as a developing solvent system. All other phospholipids were also purchased from Avanti. G_{M1} was

obtained from Calbiochem. ^{111}In and ^{125}I were obtained from New England Nuclear. Normal human serum, human serum albumin, cholesterol, Tris (Trizma Base) and EDTA were from Sigma Co. 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and diaminodipropylamine were obtained from Pierce Co. Synthesis and radiolabeling of distearylamide of diethylenetriaminepentaacetic acid with ^{111}In have been described in (10) and (11), respectively. [^{125}I]albumin (1.5 X 10^4 cpm/μg protein) was prepared using the Iodo-Gen (Pierce) method and purified by using a Bio-Gel P-4 (Bio-Rad) spin column. The synthesis of [^{14}C]DOSG, using [^{14}C]succinic anhydride as the starting material, was carried out as described in (12).

Liposome preparation:

Solvent-free lipids were vacuum desiccated for at least 30 min. The lipid film was suspended overnight in PBS or TBS at a final concentration of 4 μmol/ml. The pH of liposome suspension was kept between 7.6 - 7.8. Liposomes were then extruded through 0.2 μm polycarbonate filters (Nucleopore Co.) to obtain size uniformity which was estimated by using a Coulter N4SD sub-micron particle analyzer (Hialeah, Fl). Liposome size ranged between 180-203 nm in diameter.

Biodistribution studies:

Two hundred μg of liposomes labeled with ^{111}In were injected into Balb/c male mice (6-8 weeks old) via the tail vein. After a specified period of time, the mice were anesthetized and bled via the retroorbital sinus. Mice were then sacrificed by cervical dislocation. Blood and major internal organs were weighed and ^{111}In radioactivity was determined in a gamma counter. Weight of mouse blood was assumed to be 7.3% of the body weight (13). Data were calculated as percent of injected dose accumulated per organ.

Preparation of DOSG-affinity column:

DOSG liposomes were prepared as described above. The coupling reaction was carried out according to described (14). The reaction mixture was consisted of a 4% cross-linked agarose gel containing diaminodipropylamine, DOSG liposomes and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide. The molar ratio of diaminodipropylamine, DOSG and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide was 1:2:2. The mixture was kept at pH 7.0 and left shaking overnight at room temperature. The conjugated gel was extensively washed and used to pack 1 ml columns. [^{14}C]DOSG liposomes were used in the coupling procedure to determine the degree of coupling.

Protein elution using DOSG-affinity columns:

Seventy five μl of human serum were loaded to the column. Proteins were sequentially eluted by: (i) TBS pH 7.4, (ii) TBS containing 10 mM EDTA, (iii) TBS pH 11, and (iv) TBS containing 20 mM octylglucoside. Elution was done at 4°C and 0.8 ml/fraction was collected. The protein concentration of each fraction was determined by the Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, CA).

Resin binding with albumin:

Before the binding studies, purified human serum albumin was first dialyzed against 1 mM EDTA for 12 h and then re-dialyzed against distilled water twice for over 24 h at 4°C. The twice dialyzed serum albumin was radiolabeled with ^{125}I. Ten to fifty μg of [^{125}I]albumin was added to resin containing 1 μmol of bound DOSG liposomes. The resin was washed and equilibrated with TBS, TBS containing 3 mM Ca^{2+} or TBS containing 3 mM Mg^{2+}. The incubation of serum albumin with the resin was carried out in the above buffers at 4°C overnight. Unbound serum albumin was removed by repeated washing and centrifugation. The amount of serum albumin bound to the resin was determined by counting ^{125}I radioactivity.

SDS-PAGE:

Ten percent SDS-PAGE under reducing conditions was done according to the method of Laemmli (15).

Results

Biodistribution of DOSG liposomes:

Fig. IV-1 shows the biodistribution of DOSG liposomes compared with ePC/Chol (2/1, m/m) and DOPS liposomes. After 15 min of injection, 25, 40 and 8% of injected dose were found in circulation for liposomes containing DOSG, ePC/Chol and DOPS liposomes, respectively. DOPS liposomes cleared from the circulation most rapidly, followed by DOSG liposomes and then ePC/Chol liposomes. Liver accumulations of liposomes reciprocated the blood clearence. High level (approximately 80%) of injected DOPS liposomes were found in the liver at only 15 min postinjection. Liver accumulation of ePC/Chol and DOSG liposomes were slower; they reached approximately 70% at 3 h postinjection. In all three cases, the amount of liposomes found in the spleen was very low; less than 10% of liposomes were found in the spleen at 3 h postinjection.

These data showed that DOSG liposomes did not stay long in the circulation. They were cleared mainly by the liver much like other negatively charged liposomes previously studied (16,17). A similar biodistribution pattern was observed when higher dose (up to 1 mg/mouse) or larger liposomes (up to 300 nm in diameter) were used (data not shown). The amount of liposomes accumulated in the spleen never exceeded 5% in these experiments.

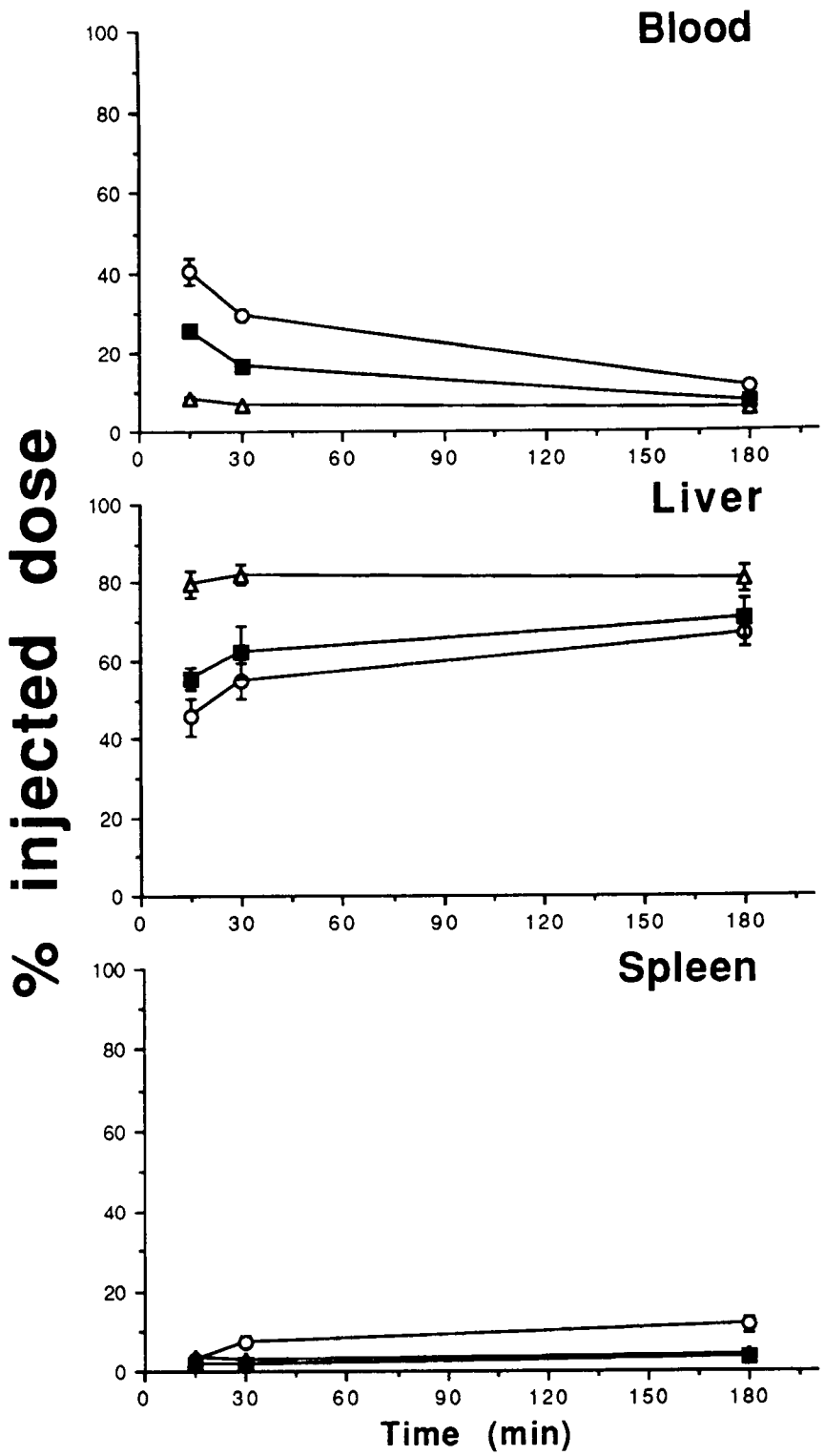

Figure IV-1. Biodistribution of liposomes in mouse. ePC/Chol (2/1, m/m) liposomes (○), DOPS liposomes (△) and DOSG liposomes (■). Data are mean ± s.d. (n=3).

Effect of G_{M1} incorporation on the biodistribution of DOSG liposomes:

It is known that the circulation time of some liposomes, such as those composed of ePC/Chol, can be increased upon incorporation of G_{M1} (18) or PEG-PE (10,19). Thus we also attempted to increase the circulation time of DOSG liposomes by incorporating G_{M1} into the liposome membranes. When 6.3 mol % of G_{M1} was incorporated, the circulation time of DOSG liposomes did not increase; identical biodistribution of liposomes was found at 1 h after injection (Fig. IV-2). The amount of DOSG liposomes accumulated in the liver also remained approximately the same. We had also included 6.3 mol % of PEG-PE (m.w. of polyethylene glycol = 5000) in DOSG liposomes. Again the circulation time of DOSG liposomes was not increased (data not shown). G_{M1} or PEG-PE incorporation increases the circulation time of liposomes except those composed mainly of PS (11,20). Again, DOSG and PS liposomes behaved quite similarly. Since PS is known to be an opsonin attracting lipid (20), DOSG might also be the same.

Opsonin purification using DOSG-affinity column:

Like DOPS liposomes, DOSG liposomes were mainly taken up by the liver. Since DOSG contains a simple carboxyl headgroup which can be easily conjugated to a solid support, we have decided to use DOSG liposomes as an affinity resin to see if any serum proteins might bind to these liposomes. If so, these

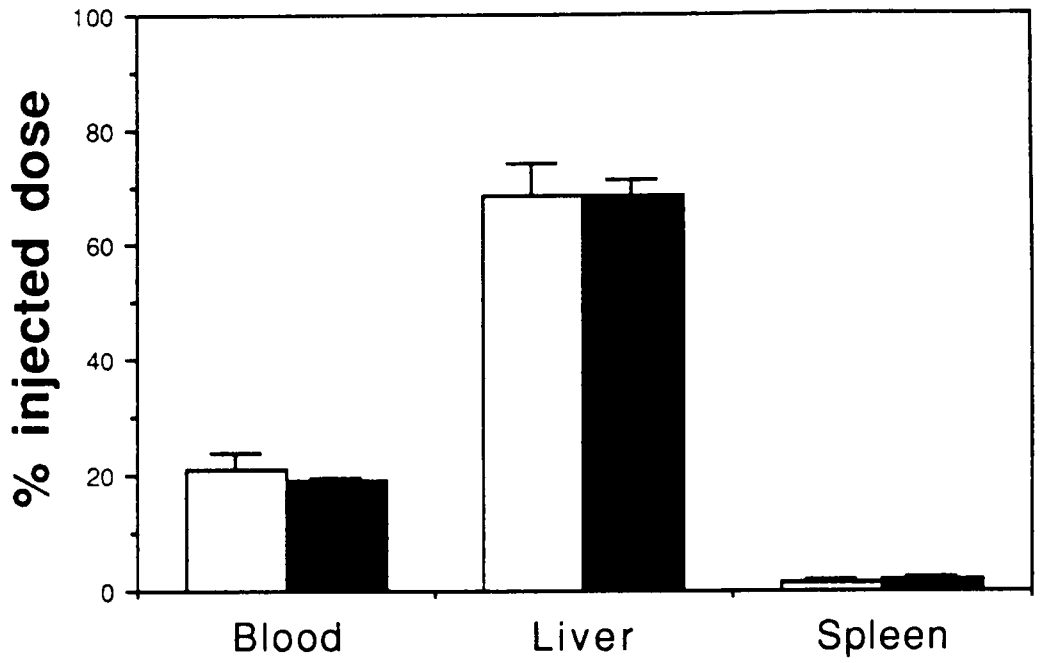

Figure IV-2. Effect of G_{M1} on the biodistribution of DOSG liposomes at 1 h after i.v. injection. DOSG liposomes (□) and DOSG/G_{M1} (93.7/6.3, m/m) liposomes (■). Data are mean ± s.d. (n=3).

proteins might be related to the liver-specific opsonins (21). We proceeded to couple DOSG liposomes with diaminodipropylamine. Under the conditions used, 60-70% of diaminodipropylamine was cross-linked with DOSG liposomes, as determined by using [^{14}C]DOSG.

Normal human serum was loaded onto the DOSG-affinity column and TBS, pH 7.5, was used to wash off the unbound proteins. When no more proteins were detected in the wash-off fractions, TBS containing EDTA was added (Fig. IV-3A). We previously reported that DOSG liposomes had a high affinity for Mg^{2+} and Ca^{2+} (22). We suspected that the putative opsonins might interact with DOSG liposomes via these divalent cations. If the divalent cations were removed by a chelating agent, such as EDTA, the interaction of the proteins with DOSG liposomes might be weakened. Indeed, the presence of EDTA in the elution buffer resulted in proteins eluted off the column (Fig. IV-3). We carried out further protein elution by treating the column with an alkaline solution (TBS, pH 11) and a detergent solution, octylglucoside (data not shown). Only very small amounts of protein were eluted by these solutions.

Analysis of eluted proteins by SDS-PAGE:

Fig. IV-4 shows a typical reducing SDS-PAGE profile of proteins eluted from the DOSG liposome affinity column. It can be seen that there was only one major protein eluted by TBS containing EDTA (Lane 4) and that the protein had the same

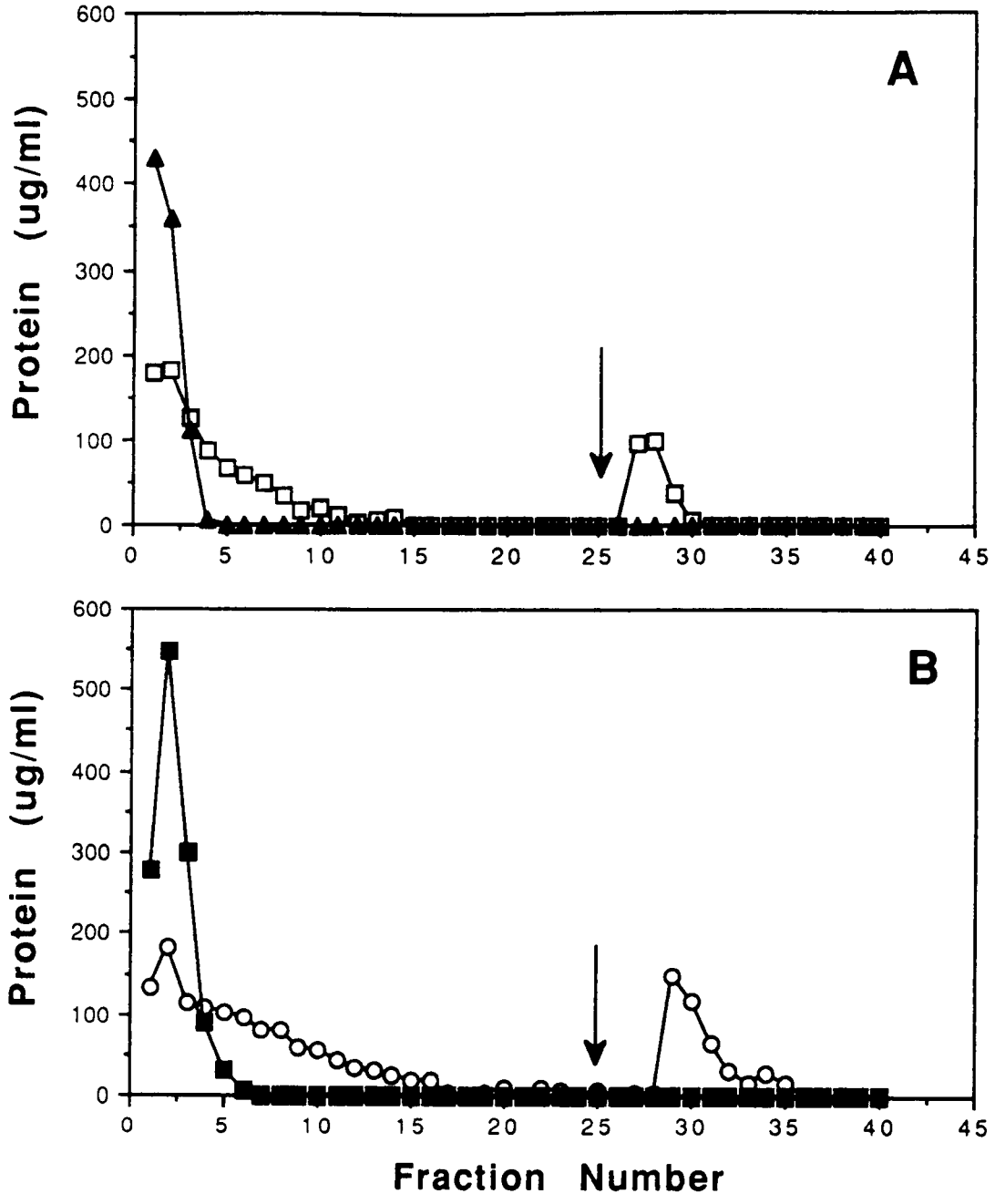

Figure IV-3. Protein elution profiles using DOSG-affinity columns. Not pretreated (□) and EDTA-pretreated (▲) columns in (A). Ca^{2+}- (○) and Mg^{2+}-pretreated (■) columns in (B). Arrows indicate change of elution buffer. See text for detail. Data are mean ± s.d. (n=3).

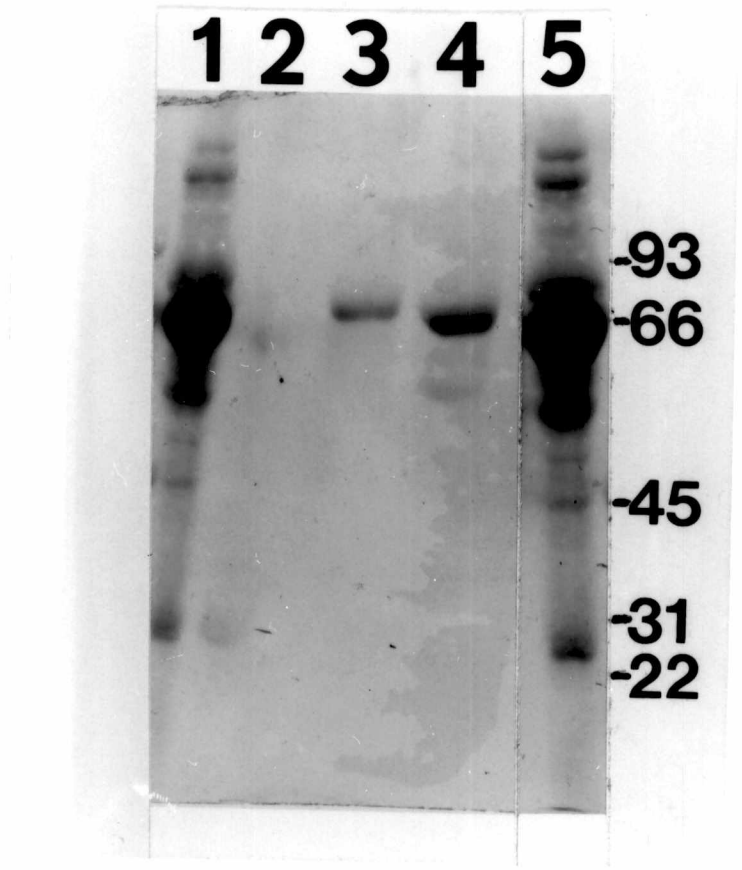

Figure IV-4. SDS-PAGE (reducing condition) analysis of human serum proteins eluted from DOSG-affinity column. Lane 1, unbound fraction; lane 2, empty lane; lane 3, human serum albumin standard; lane 4, EDTA-eluted raction and lane 5, whole human serum. Column was not pretreated as described in Fig. IV-3 legend.

migration mobility as the human serum albumin (Lane 3). We also performed a non-reducing SDS-PAGE analysis. The EDTA-eluted protein again showed the same migration mobility as the human serum albumin (data not shown). Thus we tentatively assign the EDTA-eluted protein being serum albumin. The protein eluted at high pH in small amounts was also serum albumin (data not shown).

Effects of divalent cations on protein elution:

Serum albumin was only eluted from the DOSG liposome affinity column in the presence of EDTA, indicating a possible role of divalent cations. The divalent cations were probably Ca^{2+} and Mg^{2+} as both serum albumin (23-25) and DOSG liposomes (22) interact strongly with these two divalent cations.

In order to understand the interaction of serum albumin with DOSG liposomes, we pre-treated our serum samples and the affinity columns with TBS containing 10 mM EDTA, 3 mM Ca^{2+} or 3 mM Mg^{2+}.

A. EDTA pre-treatment

EDTA (10 mM) was added to the serum sample before loaded on the column which was pre-washed with 5 ml TBS containing 10 mM EDTA. In order to keep the concentration of EDTA constant during the protein elution, TBS containing 10 mM EDTA was used as the first elution buffer. The column was then sequentially eluted with: (i) TBS, pH 7.5, (ii) TBS containing 10 mM EDTA and (iii) TBS, pH 11. After the unbound proteins were washed

off by the first EDTA containing buffer, no protein was detected with any of the subsequent elution buffers (Fig. IV-3A). This result indicates that by chelating the divalent cations, serum albumin was not able to bind with the DOSG liposomes. In order to explore which divalent cation was responsible for the observed effect, we have performed the following additional elution conditions.

B. Ca^{2+} pre-treatment

The pre-treatment of the serum sample and of the affinity column by Ca^{2+} was done identically to those described above, except that TBS containing 3 mM Ca^{2+} was used. The elution buffers were, sequentially: (i) TBS containing 3 mM Ca^{2+}, (ii) TBS containing 10 mM EDTA and (iii) TBS, pH 11. The protein elution profile (Fig. IV-3B) was similar to that of the untreated procedure (Fig. IV-3A). Serum albumin was again the major protein eluted with EDTA containing buffer. A very small amount of serum albumin was also eluted by TBS, pH 11.

C. Mg^{2+} pre-treatment

The pre-treatment and the order of the elution buffers were identical to that of Ca^{2+} experiment, except that TBS containing 3 mM Mg^{2+} was used. Here the elution profile (Fig. IV-3B) was similar to that of the EDTA-pretreatment (Fig. IV-3A). The amount of serum albumin eluted with the addition of the second buffer, i.e. TBS containing 10 mM EDTA, was significantly reduced.

These data taken together suggest that serum albumin

binding to DOSG liposomes requires the presence of divalent cation and the activity of Ca^{2+} was far greater than that of Mg^{2+}.

Resin-binding with serum albumin:

Binding of serum albumin to DOSG liposomes may require the presence of some other serum factor, besides Ca^{2+}. To determine if this is the case, purified human serum albumin was used to bind to the affinity resins with covalently attached DOSG liposomes. As shown in Fig. IV-5, purified serum albumin could bind to the DOSG-resin in a concentration dependent manner. The levels of serum albumin binding in the presence of Mg^{2+} and buffer only were quite similar. However, when Ca^{2+} was present, the binding of serum albumin increased by approximately two-fold. These data indicated that the binding of serum albumin to DOSG liposomes was mainly mediated by Ca^{2+} and not by Mg^{2+}. Furthermore, the binding did not require the presence of other serum components.

Discussion

Most liposomes introduced i.v. are eventually cleared from the blood by the phagocytic cells of the reticuloendothelial system (RES), particularly the liver Kupffer cells and the splenic macrophages [for reviews, see (24-26). The mechanism(s) by which liposomes are recognized and removed from the circulation by the phagocytic cells

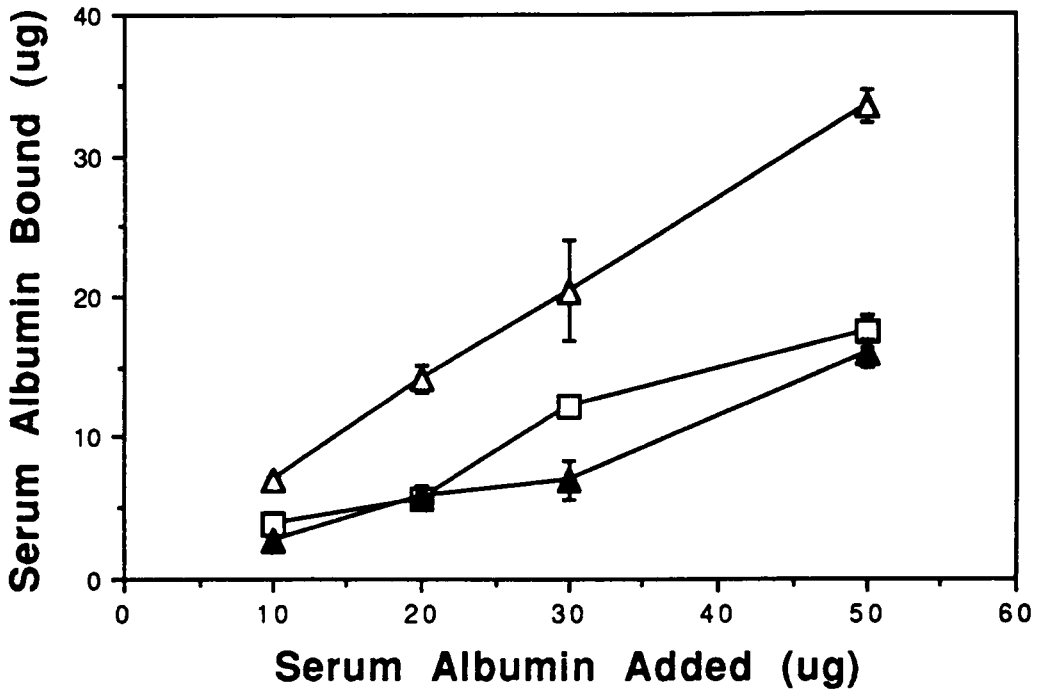

Figure IV-5. Effects of divalent cations on the binding of serum albumin to affinity resin containing DOSG liposomes. In the presence of buffer only (□), buffer containing Ca^{2+} (△) or Mg^{2+} (▲). Data are mean ± s.d. (n=3).

remains unknown. The studies of Kiwada et al. (27,28) using perfused liver showed that liposomes could pass freely through the liver in the absence of blood components and that the liposome uptake by the liver was plasma-dependent. Thus, it has been proposed that these phagocytic cells may possess surface receptors that recognize a blood molecule coating the liposome surface. Such molecule, named "opsonin", is most likely a protein or lipoprotein. Furthermore, Moghimi and Patel (21) have detected liver specific opsonin activities in the rat serum.

Like most liposomes, DOSG liposomes have a short circulation time and high liver accumulation (Fig. IV-1). We attempted to use affinity columns prepared with immobilized DOSG liposomes to purify the putative liver specific opsonins from human serum. We found that serum albumin was the major protein associated with DOSG liposomes (Fig. IV-4). It is well known that serum albumin can bind and transport a variety of molecules including anions, divalent cations such as Ca^{2+} and Mg^{2+}, fatty acids, and drugs (for review, see 15). Serum albumin is also known to induce leakage of liposomes (29,30). It is one of the major protein components associated with liposomes when incubated in serum (31). However the interaction of serum albumin with liposomes has not been investigated. Here, we find that the interaction of serum albumin and DOSG liposomes is mediated by divalent cations, since removal of these divalent cations significantly lowers

the amount of serum albumin associated with DOSG liposomes (Fig. IV-3). The interaction appears to be mediated preferentially by Ca^{2+} and only weakly by Mg^{2+} (Fig. IV-3 and IV-5). This may be related to the fact that serum albumin is known to bind preferentially with Ca^{2+} over Mg^{2+} by approximately three-fold higher amounts (22).

Serum albumin is known to be taken up by the liver. It has been suggested that the cell surface of liver endothelial or Kupffer cells may possess serum albumin binding proteins (for review, see 32). Thus, we propose that DOSG liposomes are mainly taken up by the liver due to its association with serum albumin via an interaction with divalent cations, particularly Ca^{2+}. This may also explain why the incorporation of G_{M1} or PEG-PE could not prolong the circulation time of DOSG liposomes (Fig. IV-2) as the activity of G_{M1} and PEG-PE could not overcome the large abundance of albumin in serum, approximately 40-50 mg/ml (21).

These studies suggest that serum albumin could be a newly identified liver specific opsonin. If this is the case, serum albumin should increase the uptake of liposomes, such as DOSG liposomes, by macrophages. To test the hypothesis, we have studied the uptake of liposomes by cultured macrophages in the presence of serum albumin. The results are reported in the part V.

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PART V

SERUM ALBUMIN INCREASES THE UPTAKE BY CULTURED MACROPHAGES OF LIPOSOMES COMPOSED OF 1,2-DIOLEOYL-3-SUCCINYLGLYCEROL

Abstract

We have reported that i.v. injected liposomes composed of 1,2-dioleoyl-3-succinylglycerol (DOSG) are almost exclusively taken up by the liver. Such high affinity of uptake might be caused by the preferential association of serum albumin with DOSG liposomes via an interaction with divalent cations. Here we report that serum albumin increases the uptake of DOSG liposomes by differentiated THP-1 macrophage cells. Thus serum albumin was at least partially responsible for the opsonization of DOSG liposomes. The opsonization process requires the presence of Ca^{2+} and Mg^{2+}. There appears to be no preference over which divalent cation is present in the medium. However serum albumin did not increase the uptake of liposomes composed of egg phosphatidylcholine/cholesterol (ePC/Chol) or dioleoylphosphatidylserine (DOPS) even in the presence of Ca^{2+} and Mg^{2+}. Thus serum albumin appears to be an opsonin only for DOSG liposomes but not for other types of liposomes.

Introduction

Liposome encapsulation of drugs reduces the toxic side effects of anti-cancer (1), anti-fungal (2), anti-bacterial (3) and anti-viral drugs (4). Unfortunately, i.v. injected liposomes are rapidly cleared from the blood circulation by the RES, limiting their use as a systemic drug delivery system. Many plasma proteins become associated with liposomes

when liposomes are injected into animals (5). Some of these proteins may act as opsonins, which enhance the uptake of liposomes by the RES, particularly the liver Kupffer cells and the splenic macrophages (6). Recently it was reported that the circulation time of liposomes could be prolonged by the inclusion of G_{M1} (7), PEG-PE (8) and hydrogenated plant phosphatidylinositol in the liposome composition (9). These amphiphiles are believed to reduce the RES uptake of liposomes by the following two mechanisms: (a) increasing the hydrophilicity of the liposome surface thus reducing nonspecific interactions of liposomes with RES cells, and (b) sterically preventing the coating of opsonins to liposomes thus reducing specific interaction of liposomes with RES cells (8,10). Even with the discovery of the molecules which prolong the circulation time of liposomes, it is still necessary to understand the properties and mechanisms of opsonins in order to optimally design liposomes as a drug carrier system.

The identity of opsonins is not clear. Moghimi and Patel (6) had detected opsonin activities in rat serum. These opsonins appear to be organ-specific as the properties of liver and spleen opsonins are different (11). We have found that serum albumin, via an interaction with divalent cations, might also play a role in opsonization, particularly with DOSG liposomes (part IV).

To verify this hypothesis, we studied the interactions of DOSG liposomes with THP-1 cells, a human monocytic leukemia

cell line (12,13), in the presence of serum albumin and divalent cations, such as Ca^{2+} and Mg^{2+}. We find that serum albumin increases the uptake of DOSG liposomes in the presence of Ca^{2+} and Mg^{2+}. These data suggest that serum albumin is indeed responsible for the opsonization of DOSG liposomes.

Materials and Methods

Materials:

DOSG was custom synthesized by Avanti Polar Lipids. The purity of DOSG was confirmed by thin-layer chromatography using hexane: ethylether: methanol: acetic acid (70/30/5/1, v/v) as a developing solvent system. All other phospholipids were also obtained from Avanti. Cholesterol, human serum albumin, normal human serum, Tris and EDTA were from Sigma Chemical Co. Diaminodipropylamine and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide were purchased from Pierce and PMA was from Calbiochem. Tissue culture medium, fetal calf serum, penicillin and streptomycin were obtianed from Gibco. [^{3}H]CE was synthesized by Amersham and purified as described (14).

Liposome preparation:

Solvent-free lipids containing [^{3}H]CE (11 μCi/μmol) were vacuum dessicated for at least 30 min. The lipids were hydrated overnight with TBS, pH 7.5, at room temperature. Liposomes were then extruded through polycarbonate filters

(Nucleopore Co.) to obtain size uniformity. Liposome size was estimated by a Coulter N4SD sub-micron particle analyzer (Hialeah, Fl), as in the range of 180 - 225 nm in diameter.

Cell culture:

THP-1 cells were obtained from Dr. Gabriel Lopez-Berestein. The cells were grown in RPMI-1640 medium supplemented with 10% fetal calf serum, streptomycin (100 μg/ml) and penicillin (100 units/ml) in Falcon 200-ml flasks in a 95% air / 5% CO_2 humidified incubator at 37°C. They were sub-cultured once a week at an initial density of 2-4 X 10^5 cells/ml.

Incubation of liposomes with cells:

Sixty thousand cells/well were seeded in Corning 24-well plates in 1 ml medium. PMA (100 ng/ml) was added to the cells to induce macrophage differentiation. After 3 days of culture, liposomes were added to the washed cells at a final concentration of 50 nmol in 0.2 ml of medium, and incubated for 20 h in RPMI-1640 medium or 8 h in Hank's solution at 37°C in a 95% air / 5% CO_2 humidified incubator. After the desired incubation time, the cells were washed three times with TBS before harvesting in 0.4 ml NaOH (0.1 N). The amount of liposomes associated with cells was determined by ^3H radioactivity.

Preparation of spin columns:

The preparation of DOSG affinity resin was described in the part IV. The affinity resin was packed into 1-ml tuberculin syringes plugged with glass wool. They were then centrifuged (Damien Series bench top centrifuge; 2,000 rpm, 5 min, room temperature) in 13 X 100 mm glass culture tubes. A series of fills and centrifuges were done until the bed volume approximated 0.7 ml. The column was washed with TBS (2 ml) and centrifuged to assure that the gel was uniformly packed and that excess buffer was removed.

Preparation of albumin-poor serum:

Two hundred μl of normal human serum was loaded to the spin columns and centrifuged (2,600 rpm, 30 min, 4°C). The unbound serum components were collected in glass culture tubes. The bound serum albumin was eluted off the column by TBS containing 10 mM EDTA. The concentration of proteins was determined by Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, CA). When all the proteins had been eluted by EDTA, the columns were washed with 5 ml of TBS, followed by 2.5 ml of TBS containing 3 mM Ca^{2+}. The columns could then be re-used for another cycle of serum albumin depletion. This procedure was repeated eight times on the same two hundred μl original serum to obtain the albumin-poor serum. The yield was approximately 160 μl.

SDS-PAGE:

Ten percent SDS-PAGE under reducing conditions was done according to the method of Laemmli (15).

Results

Effects of increasing serum and liposomes concentration on liposomal uptake:

THP-1 cells, a human monocytic leukemia cell line (12,13), was chosen as the model macrophages to study liposome uptake. THP-1 cells had been used to study lipoprotein and scavenger receptors (16,17). However they had not been extensively used for liposome uptake studies. Thus, we have first examined the effects of increasing serum and liposome concentration on the uptake of liposomes by the differentiated THP-1 cells.

A. Increasing serum concentration

The non-exchangeable and non-metabolizable lipid marker, [^{3}H]CE (18), was incorporated into liposomes to study liposome uptake by differentiated THP-1 cells. Zero to 40 percent human serum was added to the media during the incubation of liposomes with THP-1 cells. As shown in Fig. V-1, liposome uptake decreased as the concentration of serum in the incubation increased. This was probably because the non-specific uptake of liposomes by macrophages was reduced by increasing serum proteins in the liposome incubation. This effect of reducing non-specific liposome uptake could be seen

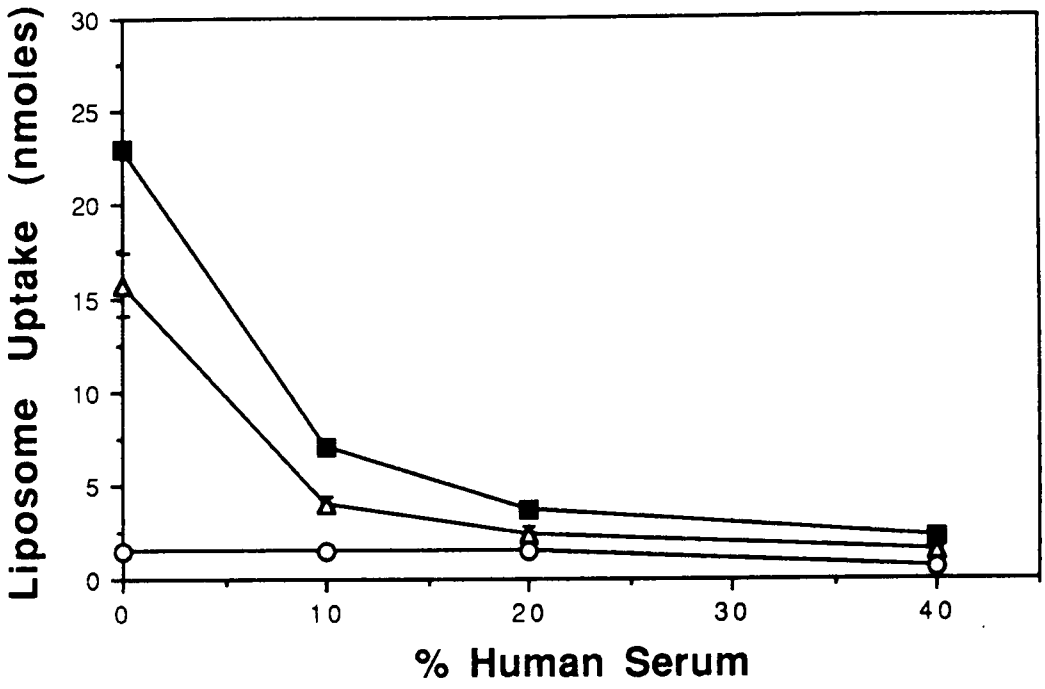

Figure V-1. Effects of human serum concentration on liposome uptake by differentiated THP-1 cells. ePC/Chol (2/1, m/m) liposomes (○), DOPS liposomes (△) and DOSG liposomes (■). Fifty nmol of liposomes were incubated with THP-1 cells for 20 h at 37°C. Data are mean ± s.d. (n=3).

even when 10 % serum concentration was used.

It was also noted that the uptake of ePC/Chol liposomes was lower than that of DOPS and DOSG liposomes. This was expected since liposome uptake is higher when opsonin attracting lipids such as PS are included in the liposome composition (10).

B. Increasing liposome concentration

We also studied the effects of increasing liposome concentration on liposome uptake. The liposome incubation was supplemented with 10 % human serum. Zero to 200 nmol (or 0 - 1 mM) liposomes were incubated with the macrophages. Liposome uptake increased as the concentration of liposomes increased (Fig. V-2). Increase in uptake could be seen more significantly with DOPS and DOSG liposomes than ePC/Chol liposomes. Under the experimental conditions, the uptake of DOPS and DOSG liposomes by differentiated THP-1 macrophage cells did not reach saturation even when 200 nmol liposomes were added. The higher levels of uptake of DOPS and DOSG liposomes were probably due to the presence of these opsonin attracting lipids, which show high affinity towards RES cells (19,20).

These data showed that THP-1 cells could be used for liposome studies. Similar to the isolated bone marrow macrophages (10), a correlation between liposome uptake and liposome composition was observed with THP-1 cells. For instance, when opsonin attracting lipids were included in the

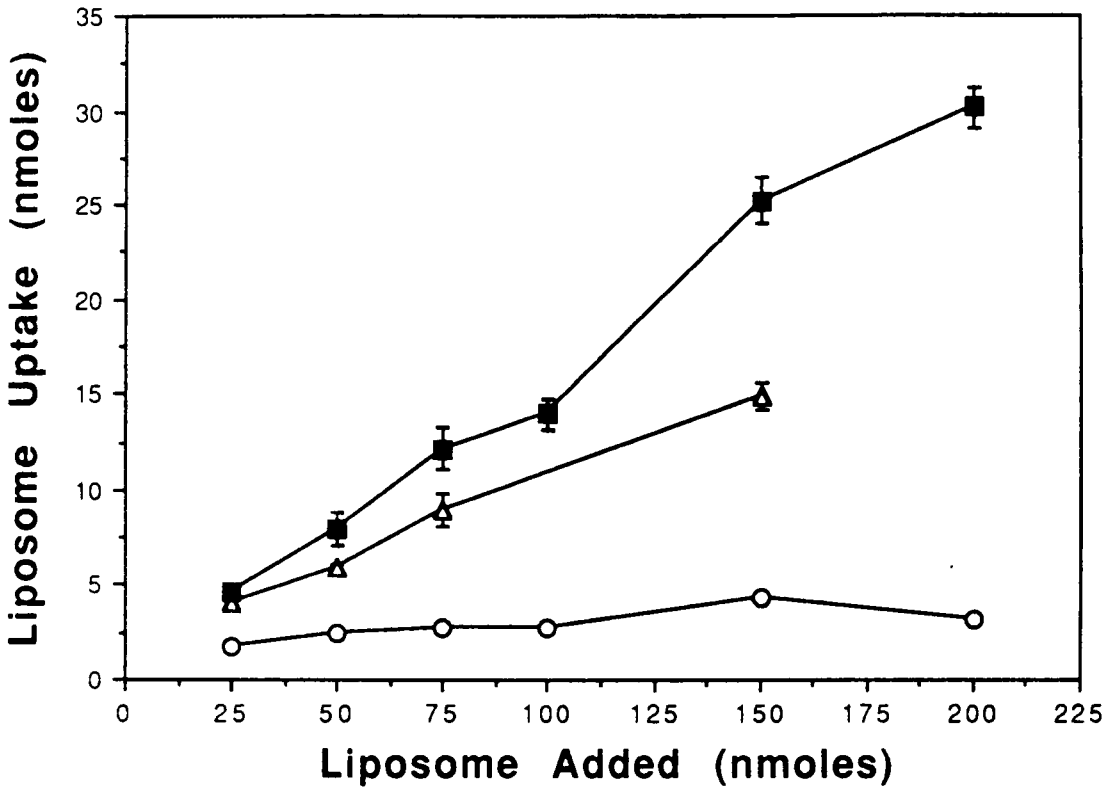

Figure V-2. Effects of liposome concentration on liposome uptake by differentiated THP-1 cells. ePC/Chol liposomes (○), DOPS liposomes (△) and DOSG liposomes (■). Ten percent human serum was used. Liposome incubation was for 20 h at 37°C. Data are mean ± s.d. (n=3).

liposome composition, higher uptake of liposomes was observed. When G_{M1} or PEG-PE was incorporated in the liposome membranes, a reduced liposome uptake was found (data not shown).

Effects of serum albumin and divalent cations on liposome uptake:

We proceeded to test the effects of serum albumin and divalent cations, such as Ca^{2+} and Mg^{2+}, on the uptake of liposomes by differentiated THP-1 cells. During the incubation of macrophages with liposomes, we had included serum albumin and various concentrations of Ca^{2+} and Mg^{2+}. We found that the uptake of ePC/Chol and DOPS liposomes was not affected either in the presence of Ca^{2+} or Mg^{2+} (Fig. V-3). However the uptake of DOSG liposomes increased in the presence of both Ca^{2+} and Mg^{2+}. The uptake of DOSG liposomes in the presence of either divalent cation was similar. Half-maximal uptake of DOSG liposomes was seen when 0.3 mM of either divalent cation was used.

It was also observed that the uptake of liposomes was higher than what was observed previously, as in Fig. V-1 and V-2. The higher liposome uptake was probably due to non-physiological uptake of liposomes by macrophages as only serum albumin and not other serum proteins were present in the incubation medium. Thus, we decided to test the effects of serum albumin and divalent cations on liposomal uptake in the presence of other serum proteins as well.

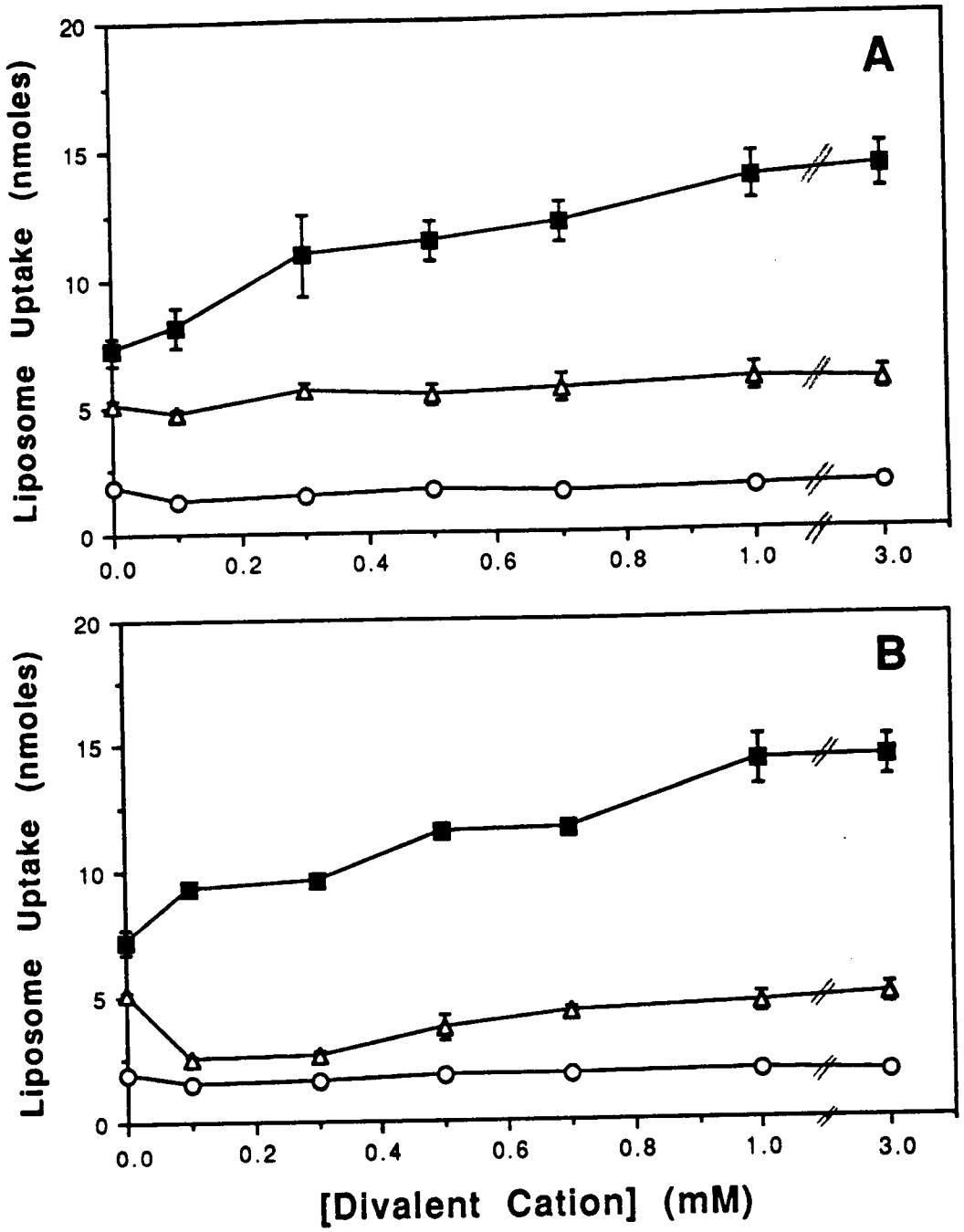

Figure V-3. Effects of serum albumin and divalent cations on the liposome uptake by differentiated THP-1 cells. ePC/Chol liposomes (○), DOPS liposomes (△) and DOSG liposomes (■). (A) and (B) represent Ca^{2+} and Mg^{2+}, respectively. Fifty nmol of liposomes were incubated with cells for 8 h at 37°C. Data are mean ± s.d. (n=3).

Depletion of serum albumin and divalent cations from human serum:

Before exploring the effects of serum albumin and divalent cations on liposome uptake, it was necessary to reduce or deplete the amount of serum albumin in the serum. If not, the amount of serum albumin added would be too small when compared with the amount of serum albumin that already existed in the serum. Thus we decided to prepare albumin-poor serum. We reported in part IV that serum albumin had a high affinity towards DOSG liposomes. DOSG-affinity columns were used to deplete albumin from normal human serum. As shown in Fig. V-4, the concentration of serum albumin in the albumin-poor serum (Lane 2) was much lower than that in the whole serum (Lane 1). The concentration of serum albumin was estimated to be mg/ml. But the concentration of the other serum proteins remained approximately the same. This result showed that serum albumin was the major protein removed by the affinity-column. However, the concentrations of Ca^{2+} and Mg^{2+} in the albumin-poor serum were also reduced to 0.1 and 0.2 mM, respectively, as measured with atomic absorption spectrometry.

Effect of serum albumin and divalent cations on liposome uptake in albumin-poor serum:

We had incubated differentiated THP-1 cells with liposomes in the presence of 10 % albumin-poor serum. Serum albumin in the presence or absence of divalent cations was

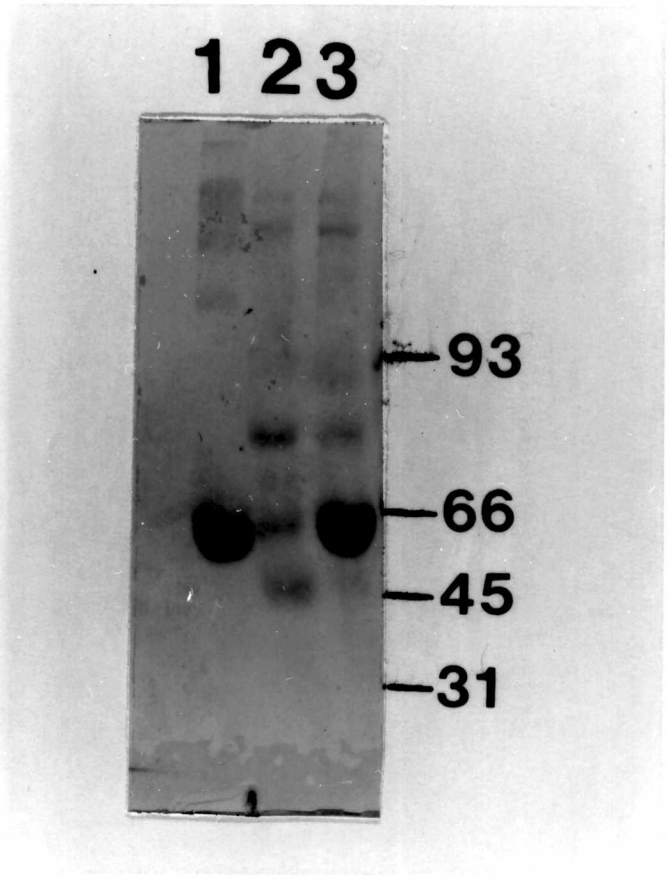

Figure V-4. SDS-PAGE analysis of albumin-poor serum. Lane 1, human serum albumin standard; 2, albumin-poor serum; and 3, whole serum. Approximately the same amount (7 μg) of total protein in each sample was loaded.

also added to study their effects on the liposome uptake. In the absence of divalent cations, the uptake of all three different types of liposomes did not increase with the addition of 0.88 mg serum albumin (Fig. V-5). However, when 1 mM divalent cations was added to the incubation media, the uptake of DOSG, but not DOPS or ePC/Chol, liposomes reproducibly increased by about 45%. The uptake of DOSG liposomes was similar when either Ca^{2+} or Mg^{2+} was used.

These data show that albumin is indeed an opsonin since it could increase the uptake of DOSG liposomes in the presence of Ca^{2+} and Mg^{2+}. However serum albumin is only responsible for the opsonization of DOSG liposomes, as the uptake of ePC/Chol and DOPS liposomes are not affected.

Discussion

We previously reported that DOSG liposomes show a high level of liver accumulation and that serum albumin, via an interaction with divalent cations, has a high affinity to bind with DOSG liposomes (part IV). Since serum albumin is known to be taken up by the liver (21), we propose that DOSG liposomes are taken up by the liver cells due to, at least in part, their association with serum albumin. To test this hypothesis, we have incubated differentiated THP-1 macrophages with liposomes and studied the effects of serum albumin and divalent cations on liposome uptake.

A difficult problem encountered in this study was the

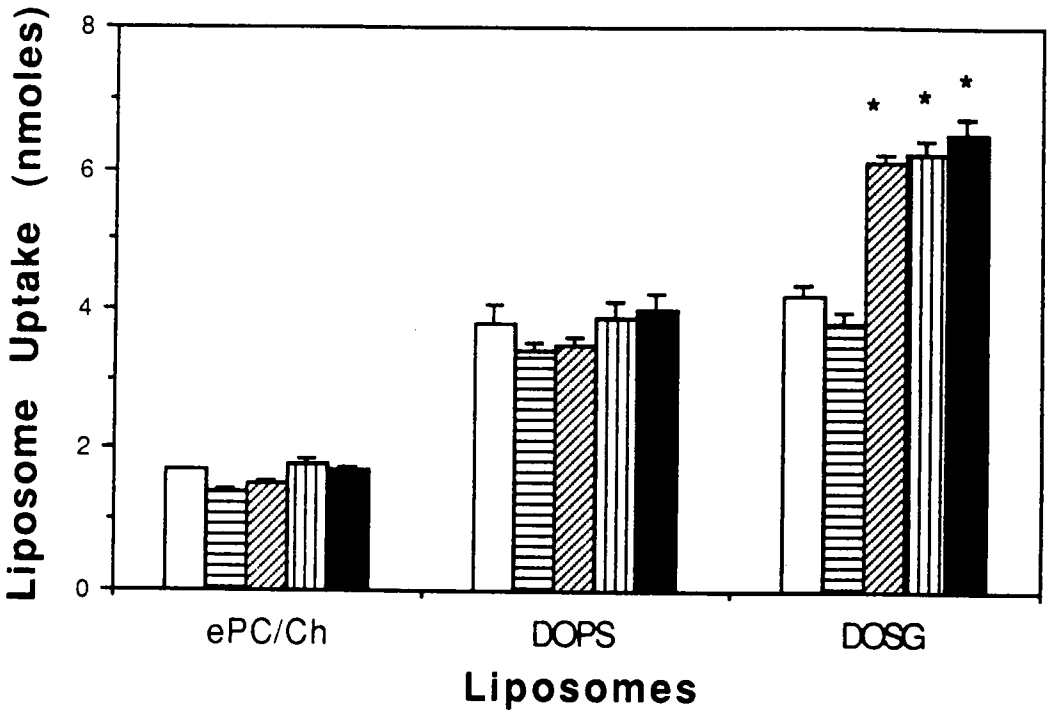

Figure V-5. Effects of serum albumin and divalent cations on liposome uptake. Albumin- poor serum only (□); albumin-poor serum and serum albumin (□); albumin-poor serum, serum albumin and 1 mM Ca^{2+} (□); albumin-poor serum, serum albumin and 1 mM Mg^{2+} (□); and whole serum (■). Data indicated with * are significantly different from the one with albumin-poor serum only (□) for DOSG liposomes, $p < 0.01$ (t test). Fifty nmol of liposomes were incubated with cells for 8 h at 37°C. Ten % albumin-poor serum and 44 mg/ml serum albumin were added. Data are mean ± s.d. (n=3).

high level uptake of liposomes by THP-1 cells in a protein-free medium, especially for the negatively charged liposomes composed of PS or DOSG (Fig. V-1). Such uptake by macrophage-like cells in a non-physiological condition has been reported recently and has been attributed to an interaction with the scavenger receptor (22,23). Inclusion of serum significantly reduces such uptake (Fig. V-1). Since the purpose of this study is to mimic the physiological conditions, we have included at least 10% human serum in the incubation medium to suppress such uptake (Fig. V-2). Furthermore, an albumin-poor serum was prepared in which other major serum proteins were preserved (Fig. V-4). When this serum was included in the incubation medium, the uptake of DOSG liposomes by the differentiated THP-1 cells was low (Fig. V-5), indicating that the putative scavenger receptor mediated uptake was suppressed under this condition.

It should be noted that the procedure to remove serum albumin from human serum by using the DOSG affinity columns also removes divalent cations, since the DOSG liposomes possess a high affinity to both Ca^{2+} and Mg^{2+}. This is the reason why when serum albumin alone was added to the albumin-poor serum, it did not enhance DOSG liposome uptake. Only after either Ca^{2+} or Mg^{2+} at physiological concentrations was added, the uptake of liposomes had resumed to the original level. These results strongly indicate that serum albumin is an opsonin for the DOSG liposomes and that the opsonin

activity depends on the presence of divalent cation, either Ca^{2+} or Mg^{2+}.

We previously showed that DOSG liposomes bind to Mg^{2+} more strongly than Ca^{2+} (part IV). In part IV, it is shown that serum albumin binding to DOSG liposomes is promoted more effectively by Ca^{2+} than by Mg^{2+}. Here we show that DOSG liposome uptake by the differentiated THP-1 cells is enhanced by serum albumin in the presence of either Ca^{2+} or Mg^{2+}. The divalent cation dependence in the cellular uptake of liposomes is likely a complex process. Since little is known about the albumin "receptor" of the liver cells (24-26), it is difficult to speculate on the reason why there is no preference of Ca^{2+} over Mg^{2+} in the serum albumin promoted liposome uptake by the differentiated THP-1 cells.

It is important to note that serum albumin is likely not the only opsonin for the DOSG liposomes. The albumin-poor serum probably contained other opsonins because the liposome uptake in the presence of the albumin-poor serum was about 65% of what was in the presence of the whole serum (Fig. V-5). Complement components such as C3 (27), fibronectin (28) and IgG (29) have been recognized as liposome opsonins which were not studied in our experiments. Serum albumin is also not an opsonin, at least not an important one, for the PS liposomes, as no enhancement of PS liposome uptake was observed when serum albumin and divalent cations were added (Fig. V-5). It has been shown that complement C3 is an opsonin for liposomes

containing PS (27). Serum albumin has also no effect on the neutral liposomes composed of ePC/Chol (Fig. V-5).

In summary, the present study has provided direct support for the hypothesis that serum albumin is an opsonin for the DOSG liposomes (part IV). The opsonin activity is dependent on the presence of divalent cations, Ca^{2+} or Mg^{2+}. To our knowledge, this is the first demonstration that serum albumin has an opsonin activity. Further studies are required to elucidate the mechanism by which serum albumin is recognized by the macrophages.

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PART VI

SUMMARY AND FUTURE PERSPECTIVES

Summary

pH-sensitive liposomes are superior than pH-insensitive liposomes in their drug delivery capabilities (1-3). Conventional pH-sensitive liposomes are composed of DOPE stabilized by a second lipid component that has a titratable headgroup. We have used DOSG and DPSG to prepare a novel single-component pH-sensitive liposome that contains no unsaturated PE. Both DOSG and DPSG vesicles are fusogenic at acidic pH. To our knowledge, DPSG vesicles represent the first described "gel-state" pH-sensitive liposome which are stable at temperatures below the critical chain-melting temperature. DOSG immunoliposomes have comparable cytoplasmic delivery efficiencies similar to the conventional pH-sensitive immunoliposomes composed of DOPE/DOSG and DOPE/DPSG (8/2, m/m). The acid induced destabilization mechanism of DASG bilayers is mainly extensive phase separation followed by formation of non-bilayer phases. This is different from the destabilization mechanism of conventional pH-sensitive liposomes containing unsaturated PE as the latter mainly involves the formation of hexagonal and related non-bilayer phases.

DASG vesicles can be destabilized by divalent cations. The destabilization mechanism is the formation of hexagonal phase. These data imply that divalent cations can stabilize the hexagonal phase of DASG more effectively than protons. This is different from the effect of Ca^{2+} on DOPE/OA liposomes

since lipidic particles and extensive lamellar sheets are observed as the dominant structures (4). Both divalent cations act synergistically with protons in destabilizing DASG liposomes. The destabilization mechanisms of Ca^{2+}/H^{+} and Mg^{2+}/H^{+} are different as different phase structures are observed.

It has been reported that "gel-state" liposomes are more stable in plasma than "fluid-state" liposomes (5). However, we find that the "gel-state" DPSG vesicles are very unstable in plasma while the "fluid-state" DOSG liposomes are partially stable in plasma.

It is interesting to find that DOSG liposomes behave similarly to PS-containing liposomes; they are rapidly removed from the circulation and taken up by the RES and they act as opsonin-attracting lipids. Using affinity columns composed of immobilized DOSG vesicles, serum albumin was found to be the major protein binding to these liposomes. The binding requires the presence of divalent cations but not other serum factors.

The most significant finding of this study is that serum albumin serves as an opsonin for DOSG liposomes; the uptake of DOSG liposomes by differentiated THP-1 macrophages is enhanced in the presence of serum albumin and divalent cations.

We propose that after i.v. injection, DOSG liposomes become associated with serum albumin via an interaction with divalent cations. This association causes DOSG liposomes to accumulate in the liver since serum albumin binds to the liver

cells.

Future perspectives

Liposomes have great potential as drug delivery vehicles. Recently, clinical evaluations of certain liposomal drugs have been undertaken (6). Some of these drugs include adriamycin, daunorubicin, PLAT 23 and vincristine as anti-cancer drugs; amphotericin B as anti-fungal drug; albuterol as bronchodilator; Gentamicin as anti-bacterial agent.

Liposomes injected i.v. are taken up by the RES (for review, see 7). This has made targeting of liposomes to cells other than RES very difficult. The activities of G_{M1} (8), PEG-PE (9) and hydrogenated plant phosphatidylinositol (5) in prolonging the circulation half-life of liposomes and reducing the RES uptake of liposomes are very exciting findings. G_{M1}-containing liposomes (5) and PEG-PE containing liposomes (9) are able to accumulate more significantly in solid tumors than conventional liposomes.

To optimize liposomes as drug delivery vehicles *in vivo*, controlled content release is also very important. For cells that are able to take up liposomes via the endocytic pathway, pH-sensitive liposomes have been developed (10-12). Our lab has even developed pH-sensitive liposomes that are partially stable in plasma, have long circulation half-life and remain acid-sensitive after plasma incubation (13). These long-circulating pH-sensitive immunoliposomes, however, cannot bind

to target cells effectively (14). The binding affinity of antibody in the liposomes to its target cells may have been reduced due to steric hindrance. One possible solution is to conjugate antibody to the liposome with an extended spacer arm. However, this may affect the circulation half-life of liposomes. Another approach is to develop other pH-sensitive liposome compositions and/or other amphiphiles that can confer extended liposome circulation times. To develop such systems, it is necessary to understand the mechanisms of the uptake of liposomes by the RES cells. This could be accomplished by identifying putative opsonins and testing their effect on liposome uptake similar to what has been described in this dissertation. Investigations into the identities of putative dysopsonins may also be helpful. Dysopsonins are serum factors proposed to reduce or inhibit the uptake of liposomes by RES (15). Thus, by knowing the structures of the opsonins and dysopsonins, and their mechanistic role in promoting or inhibiting the liposome uptake by RES, we may be able to develop more efficient drug delivery systems.

For target cells with little or no endocytic activity, pH-sensitive immunoliposomes may not be applicable for efficient drug delivery. Target-sensitive immunoliposomes (16-18) have been developed for such conditions. These liposomes are designed to destabilize upon binding to the target cell, and to release the entrapped contents in high concentrations at the cell surface. The therapeutic potential of these

liposomes is being examined. Particular concerns about these liposomes are their ability to avoid RES uptake, stability in circulation until target binding, and cellular uptake of released drug before diffusion away from the target site.

Given to considerations discussed here, the greatest limitation in using liposomes as drug delivery vehicles *in vivo* is their targetability and their uptake by RES. When we have a better understanding of the mechanisms of the uptake of liposomes by RES, we should be able to design liposomes that can serve as more effective drug carriers.

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