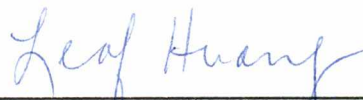


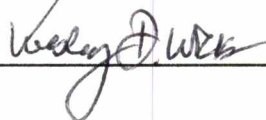
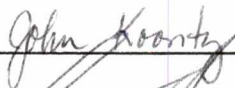
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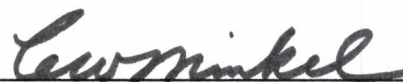


Leaf Huang, Major Professor

We have read this dissertation
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Accepted for the Council:



Vice Provost
and Dean of The Graduate School

pH-SENSITIVE LIPOSOMES FROM TEST TUBE TO ANIMAL MODEL

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Dexi Liu

May 1990

DEDICATION

This dissertation is dedicated to my parents, Mr. Xianzhi Liu (刘先知) and Mrs. Cuilin Zhang (张翠玲); my sisters, Shuyun (淑云) and Aiyun (爱云); my brother Qiangqiang (强强); and my friends, Minghui Zhao (赵明辉) and Zhide Zhang (张志德).

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My warm and sincere thanks go to my wife Guisheng Zhang for her love, support, sacrifice, suggestions and encouragement throughout the years. A special thanks to my two year old son for his invisible contributions to my work.

ABSTRACT

The therapeutic potential of liposomes as drug-delivery vehicles depends on the efficient delivery of liposome-entrapped compounds to cells. A number of studies have demonstrated in culture cells that pH-sensitive liposomes composed of dioleoylphosphatidylethanolamine and oleic acid (DOPE/OA) show a high efficiency of delivery. However, these liposomes have not been successfully used in animals due to the poor stability of liposomes in serum and the rapid uptake by the reticuloendothelial system (RES) once administered into animals. The studies described in this dissertation were intended to explore the mechanisms of instability of liposomes in serum and the RES uptake and to engineer a new type of liposome which is serum-stable, pH-sensitive and exhibits prolonged circulation in animals.

The stability of liposomes in blood was studied in a simplified in vitro system, i.e., in human plasma at 37°C. It was demonstrated that the stability of liposomes composed of dioleoylphosphatidylethanolamine and oleic acid is size dependent. Small liposomes ($d \leq 200$ nm) are stable in plasma. Large ones are not stable, but the stability of large liposomes in plasma can be increased by including cholesterol in the liposome membranes. More detailed experiments with purified serum proteins show that the small liposomes are actually stabilized by lipoproteins. Apo A1 in the high density lipoproteins (HDL) shows a very strong stabilization effect. The stabilized liposomes are no longer pH-sensitive because of the insertion of proteins and/or lipids into the bilayer of liposomes and removal of OA from liposomes by serum

albumin. The biodistribution of DOPE/OA based liposomes shows that ganglioside GM₁, GT_{1b} as well as high phase transition temperature lipids such as distearoylphosphatidylcholine (DSPC), once incorporated into the DOPE/OA liposome membranes, can increase the circulation time in blood due to a significant decrease of RES uptake of the liposomes. The DOPE based liposomes containing a double chain pH sensor, dipalmitoylsuccinylglycerol (DPSG), which tends to be resistant to removal from liposomes and hence maintains the pH-sensitivity of the liposomes, and GM₁, which meant to increase the circulation time of liposomes, show a good compromise between pH-sensitivity (40-50% release of entrapped contents at pH 4.0) and a relatively prolonged residence time in the circulation.

The stability of DOPE based liposomes in plasma is important when compared with that of PC based liposomes. It seems that the small liposomes composed of DOPE/OA interact with the serum proteins in different mechanisms from those of the PC based liposomes. The results suggest that PE based liposomes may have a better chance to serve as an efficient drug carrier than the PC based liposomes in vivo as far as the pH-sensitivity and high efficiency of delivery are concerned.

PREFACE

Perhaps one of the most important reasons that liposomal research has attracted so much attention worldwide is its potential as a drug carrier. It has been shown during the last thirty years since the discovery of liposomes, that drugs can be efficiently delivered into cells in simplified conditions, mainly in cultured cells in vitro. The delivery efficiency depends not only on liposome compositions but also on the physical properties of the target cells. It is believed in general that liposomes containing antibody on their surfaces (named immunoliposomes) show better delivery efficiency than the ones without. But the therapeutic potential in animal has not been realized largely due to problems that do not exist in cultured cells.

The first project I started for my graduate study was to see if we could apply liposome technology to AIDS therapy, i.e. using immunoliposomes as a drug carrier to deliver drugs to HIV-infected cells, hopefully in vitro and later in vivo. The project was initiated with developing a monoclonal antibody against HIV. After one full year of work with the collaboration of Dr. Premkumar E. Reddy at the Winstar Institute, Philadelphia, a monoclonal antibody against a peptide epitope of HIV GP₁₂₀ membrane protein was developed and named DX-4. The characterization study showed that the antibody was IgG₁ and showed a very high affinity to the peptide. But the tests with several clinical isolates of HIV in two different laboratories showed little or no binding to the virus. It was concluded that the peptide sequence we

used had been mutated or changed among the major clinical isolates and the project was then dropped.

The results recorded here are from the second project, which was to elucidate the behavior of liposomes, which show a high delivery efficiency on cultured cells, in an animal model. It is considered a further step toward clinical application of liposomes as a drug delivery system. The goal of this project is to study the interaction of pH-sensitive liposomes with serum proteins and their biodistributions in an animal model; and to engineer a new type of liposome which would overcome the problems that do not exist in an in vitro system, and to keep the properties of liposomes which are essential for efficient drug delivery.

This dissertation is organized in a format of a scientific paper for each chapter and it can be generally divided into four parts. The first part includes chapter I, which introduces the concept of liposomes as a drug carrier with a particular emphasis on all essential aspects that deserve consideration for liposome mediated drug delivery. The reasons or the rationale that liposomes with a composition of dioleoylphosphatidylethanolamine (DOPE) and oleic acid (OA) were chosen for this project are presented. The second part of the dissertation deals with the behavior of DOPE/OA liposomes in plasma and the possible mechanisms and solutions for the problems which are derived from the liposome's interaction with the plasma components. This part covers chapters II, III, IV and V. The third part of the dissertation describes the relationship between the liposome composition and liposome biodistribution in animal models (Chapter VI). The last part

(Chapter VII) describes an attempt to design a new type of liposome which could be used for drug delivery in vivo.

It may be a pity that I cannot include in this dissertation all of the work that I have accomplished in the last five years, but I have always enjoyed doing projects which have derived from the main project by simply asking why and how these results are observed. The results from this kind of "side project" may not be less important than the ones presented here. But it would require a big effort to put them together as a huge dissertation. Hopefully, the results not recorded here will soon be summarized and submitted to the appropriate scientific journals for publication.

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ABBREVIATIONS

ApoA1:	apolipoprotein A1
BSA:	bovine serum albumin
chol:	cholesterol
DOC:	deoxycholic acid
DOPC:	dioleoylphosphatidylcholine
DOPE:	dioleoylphosphatidylethanolamine
DPH:	1,6-diphenyl 1,3,5-hexatriene
DPSG:	1,2-dipalmitoyl-sn-3-succinyl glycerol
DSPC:	distearoylphosphatidylcholine
EGTA:	ethylenebis (oxyethylenenitrilo) tetraacetic acid
GM ₁ :	monosialoganglioside
GT _{1b} :	trisialoganglioside 1b
³ H-CE:	hexadecyl [³ H]cholestanyl ether
HDL:	high density lipoproteins
Igs:	human immunoglobulins
LDL:	low density lipoproteins
LUV:	large unilamellar vesicles
MLV:	multilamellar large vesicles
OA:	oleic acid
PBS:	phosphate buffered saline
PC:	phosphatidylcholine
PE:	phosphatidylethanolamine
PS:	phosphatidylserine
RES:	reticuloendothelial system.

SUV: small unilamellar vesicles

VLDL: very low density lipoproteins

CHAPTER I

INTRODUCTION

Use of carriers for the transport of drugs to target areas has been recognized as a promising method of improving drug selectivity. Many types of carriers such as macromolecules, viruses and synthetic particles (for a review, see 1) have been proposed and undoubtedly, each of these will serve a particular need. It is, apparent, however, that the utility of most known carriers is limited by the range and quantity of drugs which they can accommodate, also by their ability to prevent exposure of the encapsulated drug from the normal biological environment, and by their access to the areas in need of treatment. In addition, there are difficulties related to the toxicity of the carrier components, and to the preparation of the carrier-drug complex. Liposomes, with the properties of biodegradability, nontoxicity, a high drug loading capacity, and ease of manipulation, have attracted more attention than any other carriers.

In the following section a number of aspects that bear relevance to the liposome as a drug carrier in vivo are outlined.

Liposomes

Liposomes or lipid vesicles are microscopic particles consisting of one or more concentric phospholipid bilayers enclosing an aqueous compartment(s) (Fig.I-1). They are formed spontaneously when a film of phospholipid is hydrated in an aqueous buffer solution (2). Once liposomes are formed they do not dissociate into the individual lipid

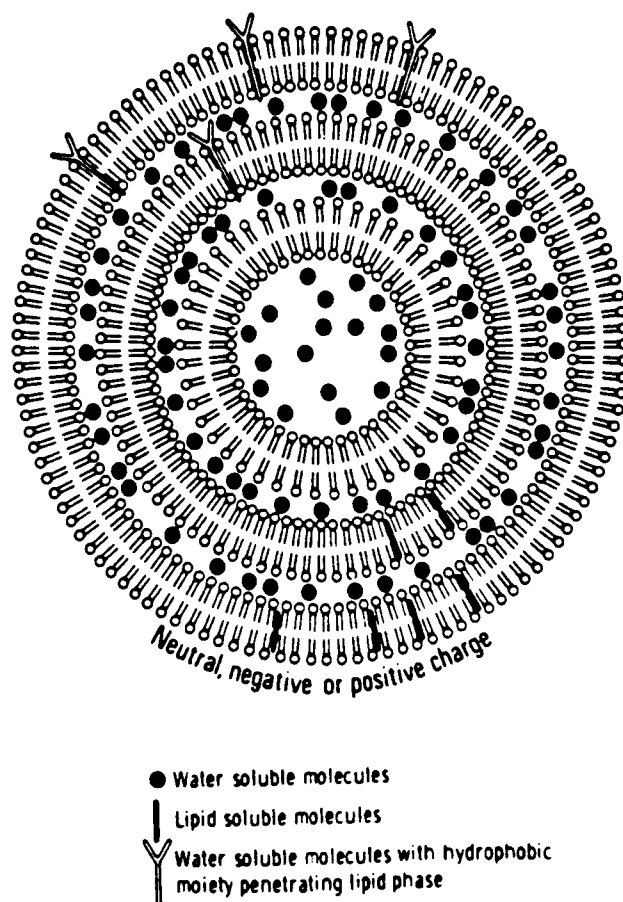


Figure I-1. Schematic presentation of liposome structure (adapted from Toos Daemen, 1987).

components under the normal condition. They are destroyed, however, if an excess of detergent is present.

Not all of the phospholipids form bilayer structures under physiological conditions. A great number of studies on the subject of lipid polymorphism (for a review, see 41), which is schematically shown in Fig I-2, have demonstrated that most of the polar phospholipids including PC, PS, PG and sphingomyelin readily form stable bilayers in an aqueous medium at the physiological pH, ionic strength and temperature. However, single chain lysophospholipids and some other polar lipids such as fatty acids readily aggregate to form micelles in physiological fluids. There is a third class of lipids, including the unsaturated PE, which readily aggregate to form the inverted micelles, or the so-called inverted hexagonal (H_{II}) phase. The headgroup of PE is poorly hydrated comparing to the headgroup of PC. Intermolecular H-bonding between the headgroups of PE also contributes to the low hydration level of the PE molecules. Unsaturated acyl chains, containing mostly cis double bonds, further expand the volume which the hydrocarbon chains occupy. The combined effects of the headgroup and acyl chains provide an unique physical property to the unsaturated PE, such as dioleoyl PE (DOPE), to assume the H_{II} phase conformation. No stable bilayer liposomes can be prepared with pure DOPE at the physiological conditions. However, it is well known that lipids which stay in hexagonal phase can be stabilized by lipids which form a micellar phase to form stable liposome bilayers.

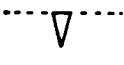
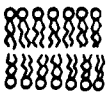
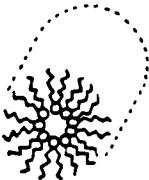
Lipid	Phase	Molecular Shape
Lysophospholipids Detergents	 Micellar	 Inverted Cone
Phosphatidylcholine Sphingomyelin Phosphatidylserine Phosphatidylglycerol	 Bilayer	 Cylindrical
Phosphatidylethanol- amine (unsaturated) Cardiolipin - Ca^{2+} Phosphatidic acid - Ca^{2+}	 Hexagonal (H_{II})	 Cone

Figure I-2. Polymorphic phases and corresponding dynamic molecular shapes of component lipids (adapted from ref. 41).

Depending on the preparative methods applied, liposomes can be obtained with single (unilamellar vesicles) or multiple bilayers (multilamellar vesicles) while the size of the liposomes can be varied by sonication, dialysis, or extrusion through polycarbonate filters (6). Unilamellar liposomes with a diameter of greater than 100 nm are called large unilamellar vesicles (LUV) and those with a diameter of less than 100 nm are named small unilamellar vesicles (SUV).

Drugs can be entrapped within the aqueous space of the liposomes by hydrating the phospholipids in a buffer containing water-soluble drugs, or within the phospholipid bilayer by mixing lipophilic drugs with the phospholipids before the film formation. It is also possible to incorporate glycolipids in the phospholipid bilayer, or to couple immunoglobulins to liposomes that can recognize the cell-surface antigens (for reviews, see 7).

Fate of Liposomes in vivo

When liposomes are introduced into any compartment of the body two things may happen: (1) liposome encapsulated drug may be released with or without the disintegration of the lipid bilayers under the influence of the components in the body fluid, and/or (2) liposomes can be transported via the circulation to the various tissues where some or all of the dose may be taken up by the cells. No matter if liposomes are used as a carrier for the sustained release of drug or as a carrier for the target specific drug delivery, the stability of liposomes in the body fluids with respect to the encapsulated drug is of central

importance. Therefore, the factors which regulate liposome stability in vivo will be discussed first.

Stability of Liposomes in Blood

Although different compartments of the body have different molecular compositions, the factors in blood which regulate the liposome stability are the focus of the study, because many destabilization factors encountered by liposomes upon intravenous (i.v.) administration are also found in other body fluids when the liposomes are introduced by alternative routes (8). This is due to the fact that liposomes administered via different routes eventually reach the circulation.

Most of the work on the stability studies of liposomes are done by incubation with the human plasma in vitro with a reporter marker entrapped inside the liposome. Fluorescence markers such as calcein (9) and carboxyfluorescein (10) are more commonly used than radioactive markers.

A simple experimental procedure is used to distinguish between the markers entrapped inside the liposomes (self-quenched) and those which are free in the aqueous environment, e.g. buffer, plasma or serum. The percentage of total marker molecules which are liposome entrapped in a given condition can be easily measured. Systematic studies with phosphatidylcholine (PC) based liposomes have demonstrated that liposomes which retain the aqueous marker most effectively regardless of the size or the type of entrapped materials are those which: 1) contain, as the major lipid component, phospholipids of high transition

temperatures, 2) are enriched with cholesterol (chol), and 3) contain, phospholipids which exhibit intermolecular hydrogen bonding, such as sphingomyelin. The breakdown of liposomes composed of only egg PC or DMPC in the absence of chol seems to be caused by the fact that liposomal PC readily incorporates into the plasma high density lipoproteins (HDL) and the liposome entrapped solutes are released (11,12,13). Other phospholipid components such as sphingomyelin, distearoylphosphatidylcholine (DSPC) are transferred to the HDL to a much lesser extent. Chol inhibits such transfer process.

How does the HDL recognize the transferable liposomal phospholipids? As first proposed by Tall and Small (19), and later by others (18), liposomes composed of certain phospholipids are susceptible to adsorption by ApoA1 which dissociates from the HDL and forms a complex with the liposomal phospholipids. The complex may then become associated with the HDL. ApoA1 binding to the liposomes may depend on the phospholipid composition of the liposomes.

Deposition of Liposomes in vivo

While the detailed mechanisms of liposome interactions with the blood components are unknown, extensive work has been completed to look at the fate of liposomes by following the distribution of the radiolabeled marker in animals such as rats, mice, rabbits, and in humans (for review see 19).

Most liposomes introduced i.v. are eventually cleared from the blood by the phagocytic cells of the reticuloendothelial system (RES) which usually results in the localization of the liposomes in liver and

spleen (for reviews, see 8, 9 and 10). The structural characteristics of the endothelial barrier in the liver are schematically presented in Fig. I-3. Removal of liposomes from the circulation by the phagocytic cells of the RES could be modified to a certain extent by the liposome size, the injection dose, and by the liposome surface characteristics which include charge, hydrophobicity and the presence of the targeting ligands. Smaller vesicles are cleared more slowly than their larger counterparts. For example, at a 4 mg/kg lipid dose, the circulation half-life ($t_{1/2}$) of small vesicles (~30 nm) is greater than 50 min, whereas vesicles with a diameter of about 600 nm exhibit a $t_{1/2}$ of approximately 5 min (20). Liposomes, like other particles introduced into the circulation, are cleared in a dose-dependent manner; higher doses usually lead to the longer circulation times. The $t_{1/2}$ for PC/chol (1:1) liposomes with a diameter of 100 nm increases from 20 min to 3 hours upon increasing the lipid dose from 0.4 to 40 mg/kg (20). These effects are presumably related to the saturation of the RES at higher doses (21). Multiple dose studies usually involve the concept of RES blockage where the effect of predose of small liposomes on the clearance of large liposomes, and vice versa, indicates that both types of liposome are cleared by the same mechanism, i.e., by the same tissue site. However, the interpretation of such data is far from straightforward, largely because the liposomes of different sizes exhibit very different clearance rates (22), as well as the possibility that they may be taken up by different cell types at different tissue sites (23,24). Other factors such as the charges on the vesicles are also important for the circulation time; vesicles containing

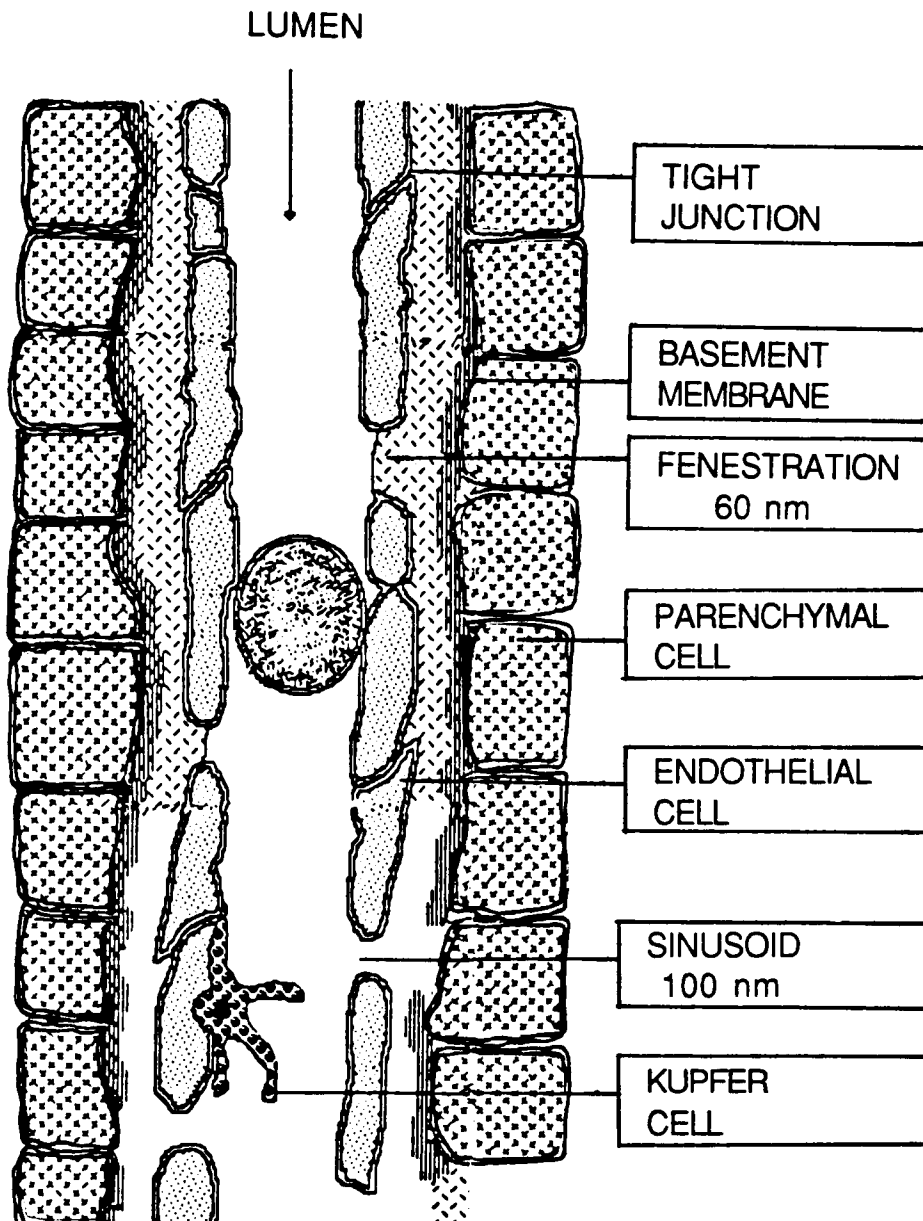


Figure I-3. The endothelial barrier in liver.

negatively charged lipids are cleared more rapidly than those containing neutral or positively charged lipids (25).

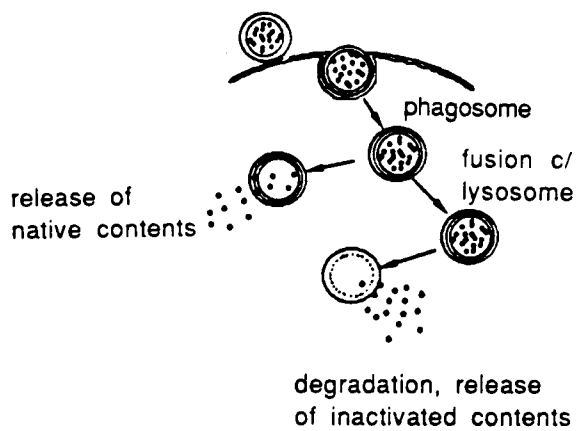
By modifying the liposome membrane characteristics, Allen, et al. (26) and Gabizon and Papahadjopoulos, (27) have demonstrated that ganglioside GM₁ exhibits the activity of decreasing the uptake of liposomes by the RES. Other negatively charged lipids with a bulky headgroup, e.g. sulfatides and phosphatidylinositol, also show a similar although small effect in prolonging the circulation half-life of liposomes. Liposomes composed of saturated PC generally stay longer in the blood than those composed of unsaturated PC (27).

The mechanism(s) whereby liposomes are recognized and removed from the circulation by phagocytic or other cells is a largely unexplored area. Three possibilities exist. Firstly, these cells possess surface receptors which recognize the specific liposomal lipids and endocytose the liposomes of the appropriate lipid composition. Secondly, the receptor might recognize a blood molecule coating the liposomes. Such molecule, named "opsonin", is most likely a protein or lipoprotein. Thirdly, the combination of the two processes may exist.

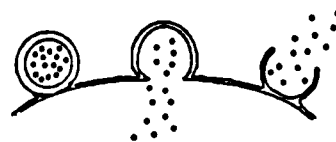
Liposome-cell Interactions

From the point of view of in vivo drug delivery, it is relevant to describe the way(s) by which liposomes deliver their contents into the target cells (for review, see 28). Four basic mechanisms of liposome-cell interaction are schematically represented in Fig.I-4. These mechanisms need not be mutually exclusive. The first pathway for

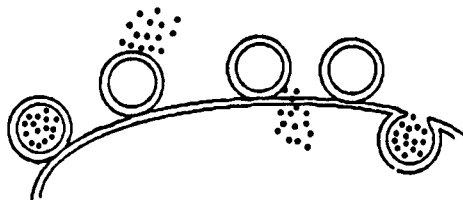
A) ENDOCYTOSIS OF LIPOSOMES



B) FUSION



C) SURFACE ADSORPTION OF LIPOSOMES



D) LIPID EXCHANGE

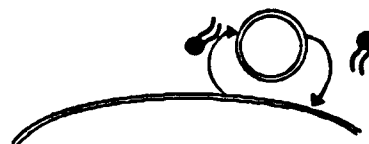


Figure I-4. Various mechanisms of liposome-cell interaction (modified from Pagano and Weinstein, 1978).

liposomes to enter the cells is through an endocytosis process (Fig.I-4A). When liposomes penetrate the cell via endocytosis, they first appear in the early endosomes where they encounter the acidic pH environment. Liposomes are then transferred to the more acidic late endosomes and finally enter the even more acidic lysosomes. The early and late endosomes are collectively called "phagosomes", particular in the macrophages. If phagosome fuses with a lysosome it can be expected that the liposomal contents will be exposed to the lysosomal acid hydrolysis and will be degraded/inactivated. Drugs which are resistant to the hostile intralysosomal environment may then act intralysosomally. Alternatively, drugs may be released to the cytoplasm from a prelysosomal/endosomal compartment, either by liposome-endosome membrane fusion or by the destabilization of both the liposome and endosome membranes.

In the case of fusion with the plasma membrane (Fig.I-4B), the liposomal contents are released directly into the cytoplasm. The contents may also be released to the extracellular space. The fusion mechanism is a very attractive route for delivering entrapped compounds to cells because some viral fusion proteins can be reconstituted into the liposome membranes thus endowing the liposomes with fusogenic activity (29).

The third type of interaction is the stable surface adsorption of the liposome which does not lead to either endocytosis or fusion (Fig. I-4C). The entrapped drug of adsorbed liposomes may still be released at the cell surface, resulting in a transiently high concentration of

the drug at the adsorption site (30). The drugs may then enter the cell by pinocytosis or by a transport mechanism.

Finally, exchange of lipid molecules (Fig.I-4D) may take place between the liposome and the cell membrane. Lipid exchange occurs without the internalization of the aqueous contents of the liposomes. However, if the encapsulated drug is hydrophobic it may enter the cell during the exchange process or it may diffuse into the cell as free drug molecules.

Recent research suggests that the major pathway of liposome uptake by cells is endocytosis via the coated pits which is followed by intracellular processing of the liposomes (31,32). Therefore, liposomes and their contents are eventually delivered to the lysosomes and degraded. Several groups have taken advantage of this observation to design new types of liposome which exploit pH changes in the endocytic pathway. As a result, pH-sensitive liposomes primarily composed of PC were first described by Yatvin and his colleagues (33). And later, three other groups including this laboratory have developed the pH-sensitive liposomes which contain PE as the major lipid component (34,35,36).

pH-Sensitive Liposomes

Currently used pH-sensitive liposomes consist mainly of an unsaturated PE such as dioleoyl PE (DOPE). DOPE, at the physiological conditions, aggregates to form the H_{II} phase. But it can be stabilized into the bilayer phase by the addition of a second lipid component such as oleic acid (34,36) or other weakly acidic amphiphiles. When the

medium is acidified, protonation of the second lipid component leads to a rapid phase transition which drives the DOPE from a bilayer to a non-bilayer phase.

In cell culture, it has been demonstrated that the pH-sensitive liposomes composed of DOPE/OA (8:2) show an efficient cytoplasmic delivery of the entrapped fluorescent dyes (37). On the other hand, pH-insensitive liposomes containing PC are not effective. Cytotoxic drugs (37), toxin (38) and DNA (39) have also been efficiently delivered to the target cells. In all cases, PE based, pH-sensitive liposomes are superior in delivery efficiency to the PC based, pH-insensitive liposomes.

The exact mechanism of the high delivery efficiency of pH-sensitive liposomes has not been elucidated. A generally accepted hypothesis is that pH sensitive liposomes release their contents to the endosome interior when the pH is dropped to a certain range (usually from 7.4 to 6 or lower) followed by a fusion process between liposome and endosome membranes such that the fused endosomes become leaky and release their contents into the cytoplasm.

However, the experimental results, collected at the beginning of the project described in this dissertation, indicate that pH sensitive liposomes are quite unstable in the plasma or serum (40), thus hampering their use in drug delivery in vivo.

The goals of this project were to study the interactions of pH-sensitive liposomes with the plasma components, to investigate the behavior of the pH-sensitive liposomes in vivo, and to design stable

and long circulating, pH-sensitive liposomes for the in vivo applications of drug delivery.

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CHAPTER II

THE ROLE OF CHOLESTEROL IN THE STABILITY OF pH-SENSITIVE, LARGE UNILAMELLAR LIPOSOMES PREPARED BY DETERGENT-DIALYSIS METHOD

Abstract

Large unilamellar liposomes prepared by an octylglucoside-dialysis method were examined for stability at 37°C in the presence or absence of human plasma, using the release of the entrapped calcein as a fluorescence marker. The liposomes were acid-sensitive as they were composed of dioleoylphosphatidylethanolamine, oleic acid and cholesterol. The stability of the liposomes in the absence of plasma was significantly enhanced with increasing cholesterol content. However, the maximal calcein release at pH 5 decreased linearly with increasing cholesterol content of the liposome, indicating that cholesterol had reduced the acid-sensitivity of the liposomes. In the presence of human plasma, calcein release exhibited a biphasic behavior with a fast (plasma-sensitive) and a slow (plasma-resistant) component. Inclusion of cholesterol in the liposomes resulted in an increased proportion of the plasma-resistant component. Liposomes pretreated with human plasma, after removal of excess plasma and the released calcein by gel filtration, showed a remarkable stability both in the presence and absence of human plasma. The acid sensitivity of the

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plasma-treated liposomes with 40% cholesterol was the same as that of the untreated ones. These results are discussed in terms of the mechanism by which these liposomes deliver their contents to the cytoplasm of the cells via the endocytic pathway, a known biological activity of the type of liposome described here.

Introduction

Liposomes have been widely used as a delivery vehicle for drugs, enzymes, antibodies and DNA (for recent reviews, see 1). One of the primary findings of the liposomal drug delivery is that the liposomes are largely endocytosed by the cells and the final cellular destination is the lysosomal compartment (2,3). Many drugs, especially the macromolecular drugs, are hydrolysed in the lysosome, and/or cannot effectively escape from the lysosome, thus resulting in poor biological activities of the delivered drug (3).

In recent years, pH-sensitive liposomes have been developed by several investigators (4-7) for improved cytoplasmic delivery of the liposomal drugs. These liposomes are primarily composed of phosphatidylethanolamine, a lipid with a high tendency of forming hexagonal phase (8). The bilayer phase of PE is stabilized with a weakly acidic amphiphile which, when protonated at an acidic pH, triggers the destabilization of the PE liposomes. The cytoplasmic delivery activity of these liposomes is considerably enhanced as compared to the conventional pH-insensitive liposomes. Fluorescent dyes (5,9,10), anticancer drugs (11), toxins (12), and DNA (13,14) are

efficiently delivered to the cells cultured in vitro. However, the in vivo delivery activity of these liposomes is severely limited by the instability of the liposome in the presence of plasma or serum. It has been shown by us that the large unilamellar liposomes prepared by the reverse-phase evaporation method composed of DOPE and OA (8:2 molar ratio) rapidly aggregate and become leaky when exposed to serum (15). It was concluded that this type of pH-sensitive liposomes is not suitable for delivery of water-soluble drugs in vivo.

Nevertheless, an attempt was made to deliver intact plasmid DNA to the target cells in mouse (13). The pH-sensitive liposomes used in the study were prepared by a detergent-dialysis method, modified from the original procedure of Philippot et al. (16). In addition, chol was included in the lipid composition to increase the trapping efficiency of DNA (16). The liposome entrapped (or associated) DNA was injected into nude mice bearing the target lymphoma cells. Successful delivery and expression of the marker gene were detected in the target cells (13). The results of these experiments strongly suggest that at least a portion of the liposome is stable under the in vivo conditions. It has thus prompted us to study the stability, both in the presence and absence of plasma, of the pH-sensitive liposomes prepared by the detergent-dialysis method. Furthermore, we have investigated the role of chol in liposome stability.

Materials and Methods

Materials

DOPE was purchased from Avanti Polar Lipids. OA, chol, calcein and octylglucoside were purchased from Sigma Chemical Co. SM-2 beads were purchased from Bio-Rad. Freshly collected human plasma was obtained from Fort Sanders Regional Medical Center (Knoxville, TN). [^{14}C]oleic acid (57 mCi/mmol) was purchased from New England Nuclear. [^{14}C]octylglucoside (314 mCi/mmol), a kind gift from Dr. Carol Cunningham was originally obtained from New England Nuclear.

Liposome Preparation

Large unilamellar vesicles were prepared according to Philippot et al (16) with modifications. Ten micromoles of lipid were dried with a gentle stream of N_2 gas. The dry lipid was kept under vacuum for a minimum of 30 min and suspended in 900 μl of PBS buffer containing 50 mM calcein and 0.55 mM EGTA which was isotonic to human plasma. [^3H]cholestanyl ether was included in the lipid mixture to monitor the lipids (17). The lipid suspension was sonicated with a bath sonicator (Laboratory Supplies, Hicksville, NY) and the pH was adjusted to pH 8.0. One hundred μl of octylglucoside (100 μmol) was added. The mixture was vortexed and dialyzed at room temperature against 100 ml of the buffer used above containing 1g of washed SM-2 beads for 48 hours with slow stirring. The liposomes were separated from free calcein by using Bio-Gel A-1.5m column chromatography equilibrated with PBS (160 mM NaCl, 3.2 mM KCl, 1.8 mM KH_2PO_4 , 0.12 mM Na_2HPO_4 , 1.2 mM EGTA pH 8.0) which was isotonic to the calcein containing buffer. The

liposomes had an average diameter of 500 ± 200 nm as measured by dynamic laser light scattering using a Coulter NS4D instrument.

Dissociation of Oleic Acid and Octylglucoside During Liposome

Preparation

Trace amounts of [^{14}C]OA or [^{14}C]octylglucoside were included in different preparations of liposomes. Twenty microliters of lipid mixture from the dialysis bag were collected at different times and counted for ^{14}C radioactivity in a scintillation counter.

Pretreatment of Liposomes with Plasma

Five hundred microliters of liposomes were mixed with the same volume of plasma and incubated for one hour at room temperature before passing through a Bio-Gel A-1.5m column.

pH-Sensitivity of Liposomes

Fluorescence intensity was measured by Perkin Elmer LS5 spectrofluorometer with $\lambda_{\text{ex}} = 490$ nm and $\lambda_{\text{em}} = 520$ nm. Liposomes (10 μl) were added to 1.99 ml PBS at the desired pH and incubated at room temperature for 5 min. The pH of the mixture was adjusted back to 8.0 after incubation by adding appropriate amount of NaOH solution. Deoxycholate (final concentration, 0.25%) was then added to completely release calcein from the liposomes. The percent leakage of liposomes was calculated from the dequenching of calcein fluorescence according to the following equation:

$$\% \text{ Release} = \frac{(F_t/F_t') F_f - F_o}{F_t - F_o} \times 100$$

where F_0 is the fluorescence intensity of liposomes in PBS at pH 8.0 before the incubation; F_f is the fluorescence intensity of the liposome after incubation; F_t is total fluorescence intensity of liposomes incubated at pH 8.0 after deoxycholate addition; F_t' is the total fluorescence intensity of liposomes incubated at different pH. F_t/F_t' was used to normalize the amount of liposomes used in the measurement.

Stability of Liposomes in Plasma and Isotonic PBS at 37°C

One hundred microliters of liposomes containing 60 nmol of lipid was added to 900 μ l of buffer (pH 7.4) or plasma prewarmed to 37°C and maintained at 37°C in a water bath. Ten microliters of the mixture was used for the fluorescence measurement at different incubation time points. The percent leakage of liposomal calcein was calculated as above except F_0 is the fluorescence intensity in PBS at time zero and F_t is the total fluorescence intensity of liposome in PBS at time zero. The F_f and F_t' are the fluorescence intensity at a given time point before and after deoxycholate-induced lysis, respectively.

The reproducibility of the leakage measurement was ascertained by measuring three or more different samples and each data point was obtained as the average of duplicate measurements of each sample. The standard deviation was less than 10% of the mean. Repeat measurements on the same sample were very similar to each other.

Results

Dissociation of OA and Octylglucoside From Liposomes During Dialysis

It was important to ascertain that the stability of the liposomes prepared by the detergent-dialysis method was not affected by the residual detergent, octylglucoside, left in the liposome preparation. As one can see in Fig. II-1B, there was an undetectable amount of [^{14}C]octylglucoside left in the liposome preparation after 48 hours dialysis against a buffer containing SM2 beads. In a parallel experiment, [^{14}C]OA was used to see if any OA was lost during dialysis. Total retention of OA was observed under the same dialysis condition. Inclusion of chol in the lipid composition did not change the conclusions, as liposomes with or without chol gave essentially the same results (Fig. II-1A).

Effect of Chol on the Acid Sensitivity of pH-Sensitive Liposomes

In order to systematically investigate the role of chol in the stability of the pH-sensitive liposomes, liposomes containing 0-50% chol were prepared. In all cases, the molar ratio of DOPE to OA was kept at 2. For example, liposomes containing no chol had a composition of DOPE:OA = 6.66:3.33, and those liposomes containing 40% chol had a composition of DOPE:OA:chol = 4:2:4. The acid sensitivity of the liposomes was investigated by measuring calcein release after an acid treatment. The results shown in Fig. II-2 clearly indicate that although the pH value at which half-maximal calcein release took place remained at about pH 7, the maximal calcein release which took place at pH 4-5 decreased linearly with increasing chol content of the

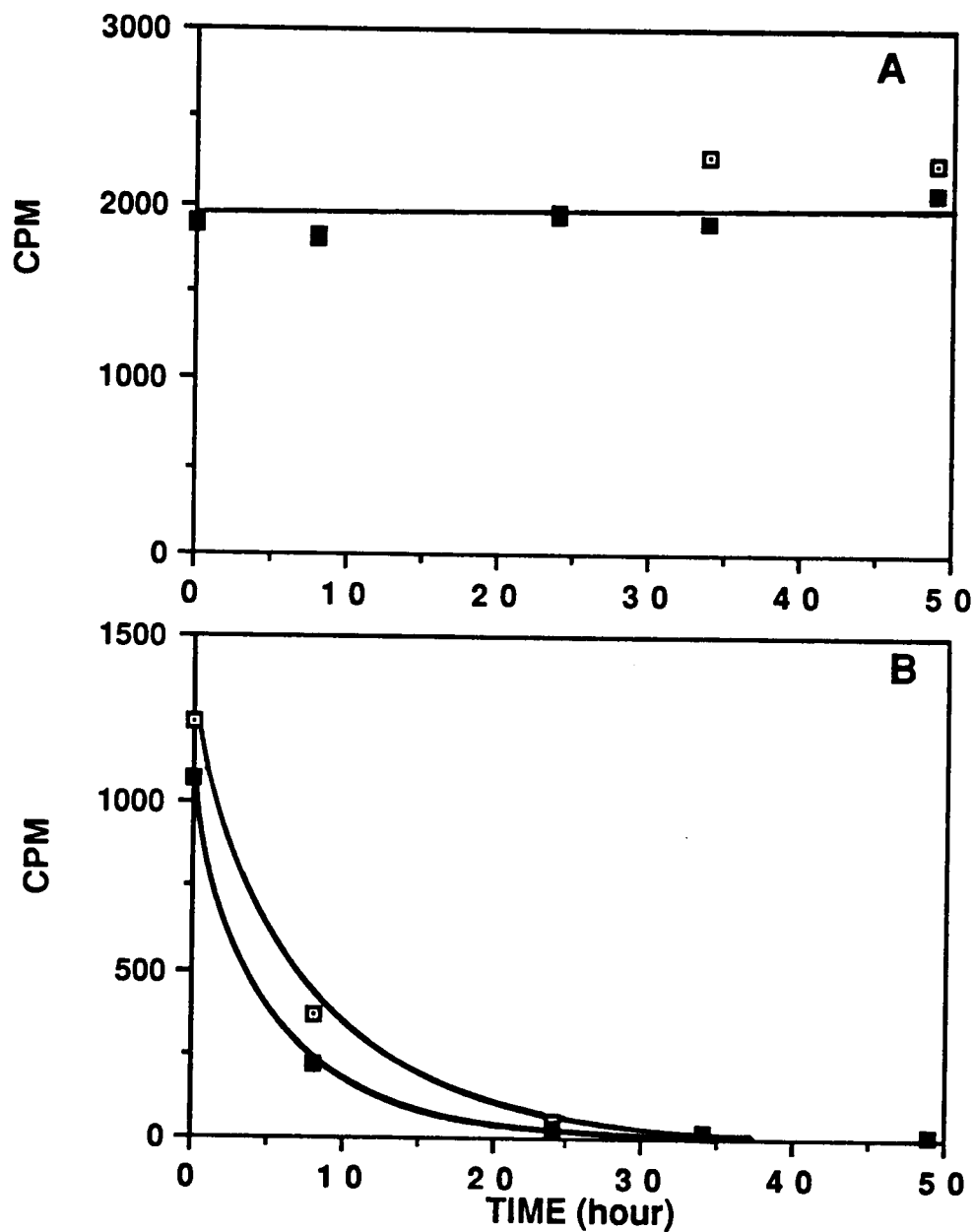


Figure II-1. Dissociation of OA and octylglucoside from liposomes during dialysis. A trace amount of [^{14}C] OA or [^{14}C] octylglucoside was included in separate liposome preparation either without or with 40% chol. 20 μl of lipid mixture from the dialysis bag was collected at different time and counted for ^{14}C -radioactivity. A, dissociation of OA; B, dissociation of octylglucoside; ($\square$) liposomes without chol; ($\blacksquare$) liposomes with 40% chol.

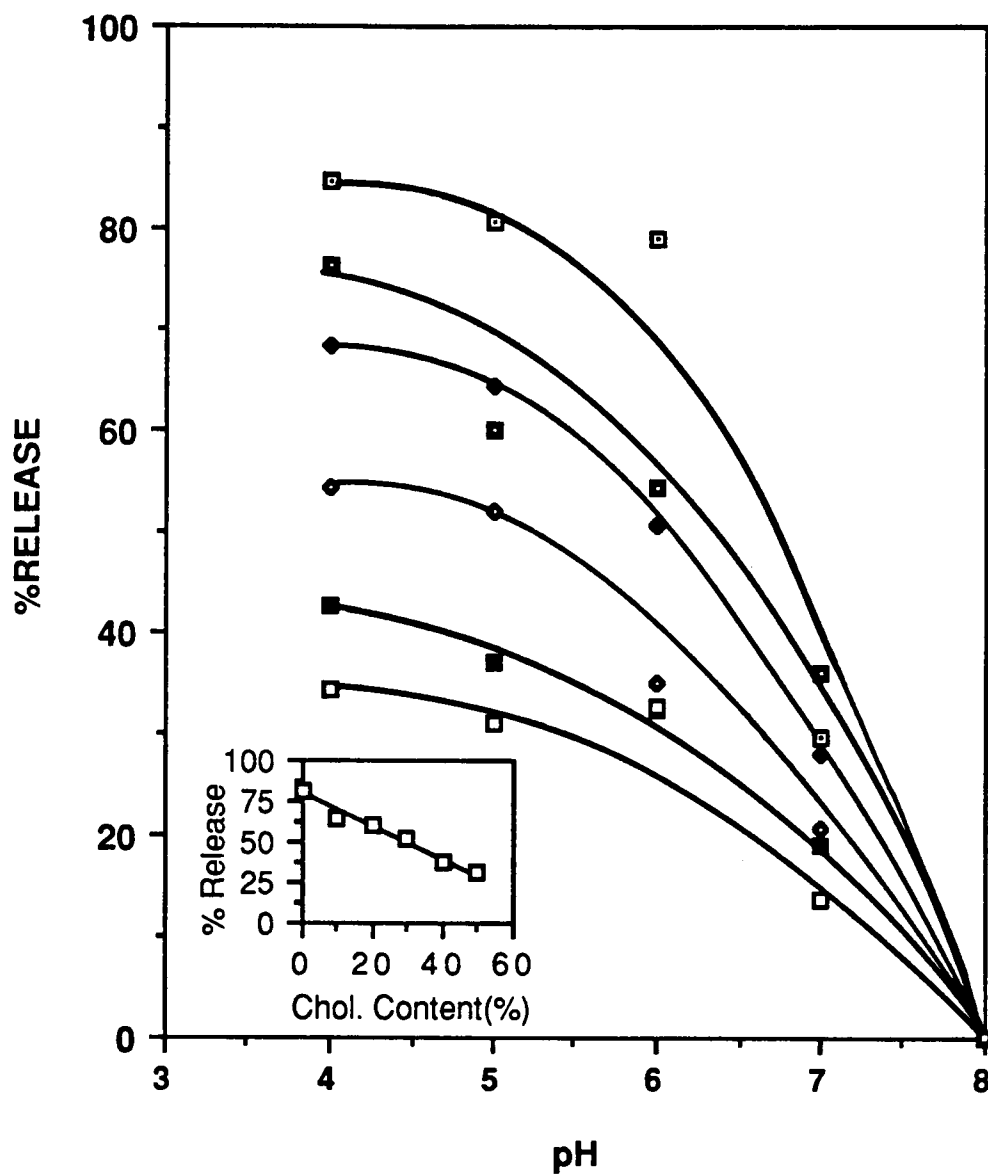


Figure II-2. Effect of cholesterol on the acid sensitivity of pH-sensitive liposomes. % calcein release from liposomes is plotted against the pH of the incubation buffer. (□) without chol; (■) with 10% chol; (◆) with 20% chol; (◇) with 30% chol; (■) with 40% chol; (□) with 50% chol. Insert, the percent calcein release at pH 5 vs. the chol content.

liposomes (Fig. II-2 insert). Therefore, chol in the liposome membrane caused the liposomes to become less acid-sensitive. For example, at 40% chol content, only about 40% calcein could be released at pH 4. This number was approximately half of those of liposomes containing no chol.

Effect of Chol on the Stability of pH-Sensitive Liposomes

Chol also affected the release kinetics of calcein from liposomes incubated in PBS at 37°C (Fig. II-3). The release of calcein at 37°C followed first order kinetics (plots not shown) with the rate constant decreasing with elevated chol content of the liposomes. Thus, inclusion of chol in the liposome membrane significantly enhanced the stability of the pH-sensitive liposomes in a protein-free, phosphate buffered saline solution.

It is also important to investigate the stability of the liposomes in the presence of plasma. Liposomes were diluted 10-fold with human plasma and the release of calcein was measured at different time of incubation at 37°C. As it can be seen in Fig. II-4, the kinetics of release was biphasic. The fast phase (plasma-sensitive phase) was so fast that it was difficult to measure with our method. The slow phase was very slow; essentially no significant calcein leakage was detected up to 8 hour incubation (Fig. II-4). Interestingly, the component of the slow, or plasma-resistant, phase was dependent on the chol content of the liposome. As the chol content in the liposome increased, the resistant phase component became larger (Fig. II-4, insert). For example, liposomes with 40% chol only released about 60% calcein after

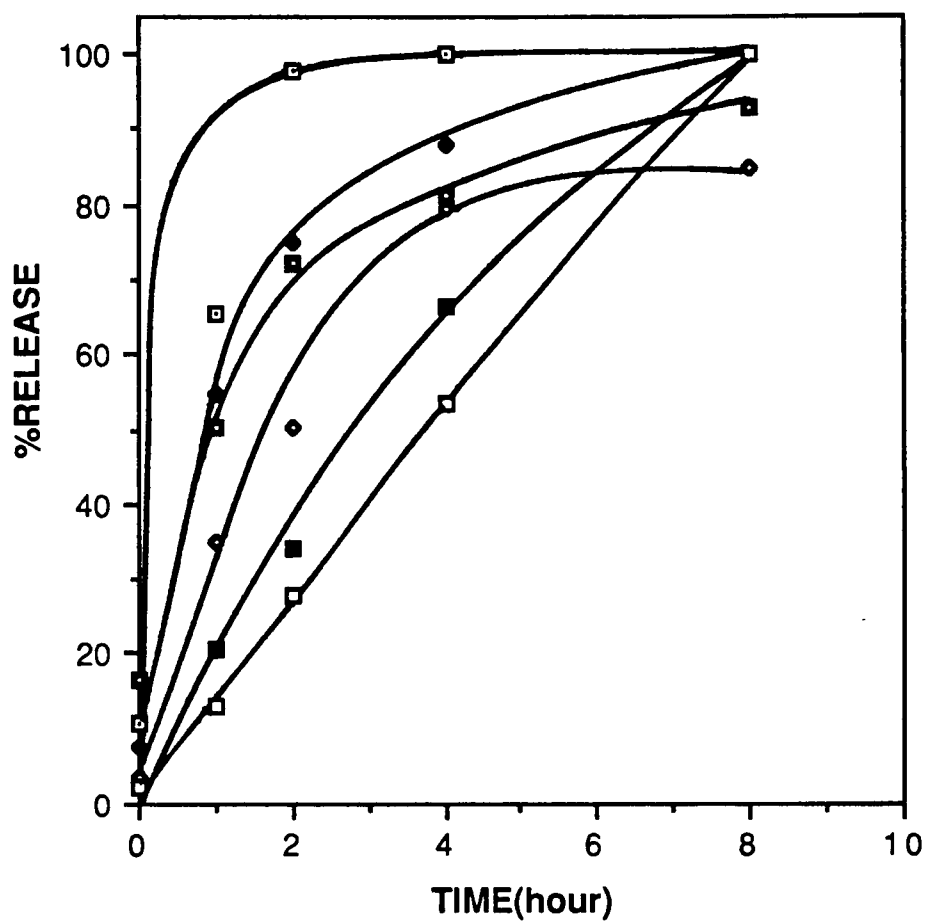


Figure II-3. Effect of cholesterol on the stability of liposomes in PBS (pH 7.4) at 37°C. % calcein release from liposomes is plotted against the time of incubation. (□) without chol; (■) with 10% chol; (◆) with 20% chol; (◇) with 30% chol; (■) with 40% chol; (□) with 50% chol.

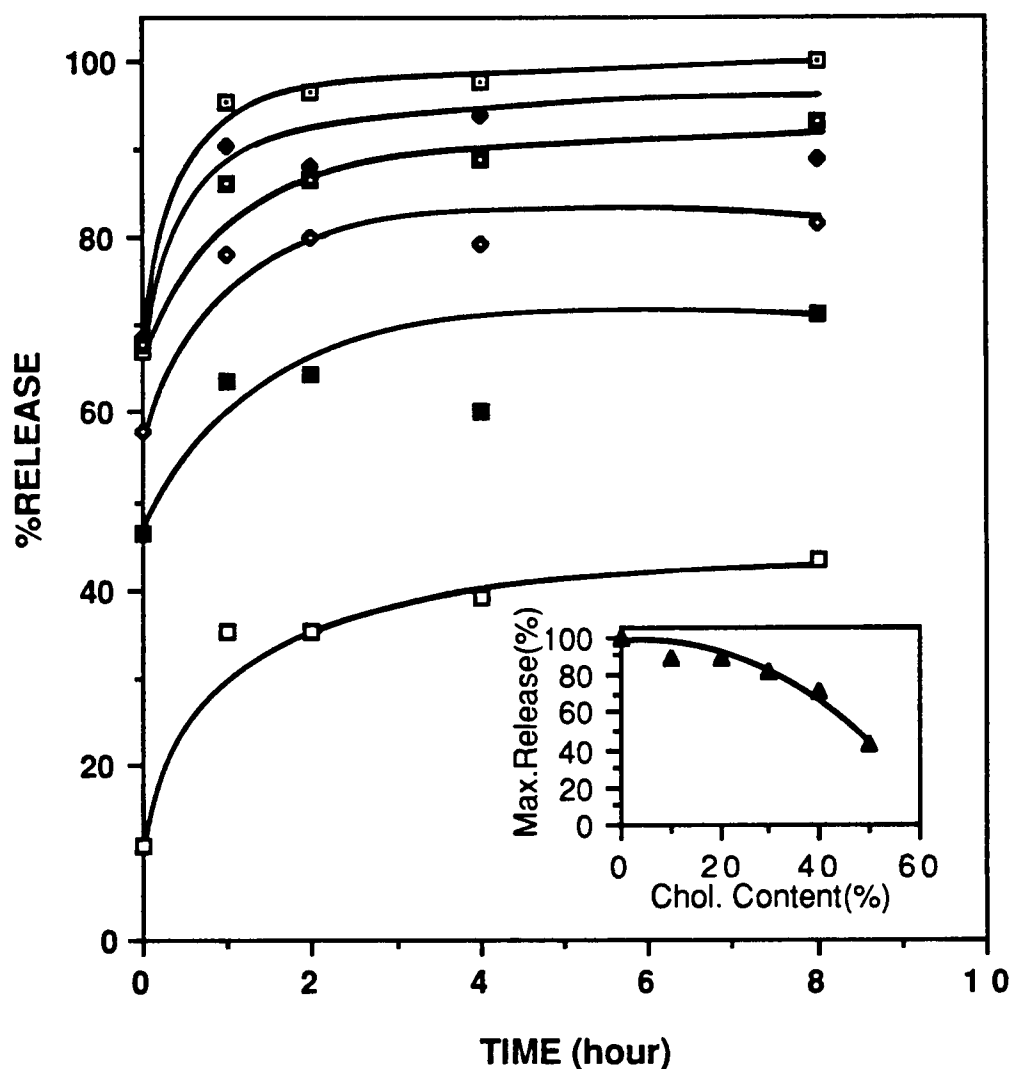


Figure II-4. Effect of cholesterol on the stability of pH-sensitive liposomes in human plasma at 37°C. % calcein release from liposomes is plotted against the time of incubation. (□) without chol; (■) with 10% chol; (◆) with 20% chol; (◇) with 30% chol; (■) with 40% chol (□) with 50% chol. Insert, maximal calcein release vs. chol content of the liposome.

8 hours of incubation; whereas the liposomes without chol released 100% in the same time period. The kinetics of the calcein release from liposomes was essentially the same when the plasma concentration was reduced from 90% in the routine experiments to 50 and 25% in some experiments. Below 25% of plasma, calcein release was slower and less complete (data not shown).

Acid Sensitivity of Plasma Treated Liposomes

It was interesting to see if the plasma-resistant component of the chol-containing liposomes is sensitive to acid. Liposomes containing 40% chol were exposed to 50% plasma for 1 hour at room temperature, causing the release of calcein from the plasma-sensitive component. The liposomes were then chromatographed on a BioGel A1.5m column to remove free calcein and most, if not all, of the plasma proteins. The resulting liposomes were then tested for the acid induced release of calcein. As shown in Fig. II-5, the acid sensitivity of the plasma-treated liposomes containing 40% chol was indistinguishable from one without the plasma treatment. Thus, the acid sensitivity of the plasma-resistant component was the same as that of the original liposome population.

Stability of Plasma Treated Liposomes

One explanation of the above data was that the plasma-liposome interaction was reversible such that the plasma-treated liposomes reverted back to untreated state after the removal of plasma proteins. This possibility was investigated by measuring the calcein release at

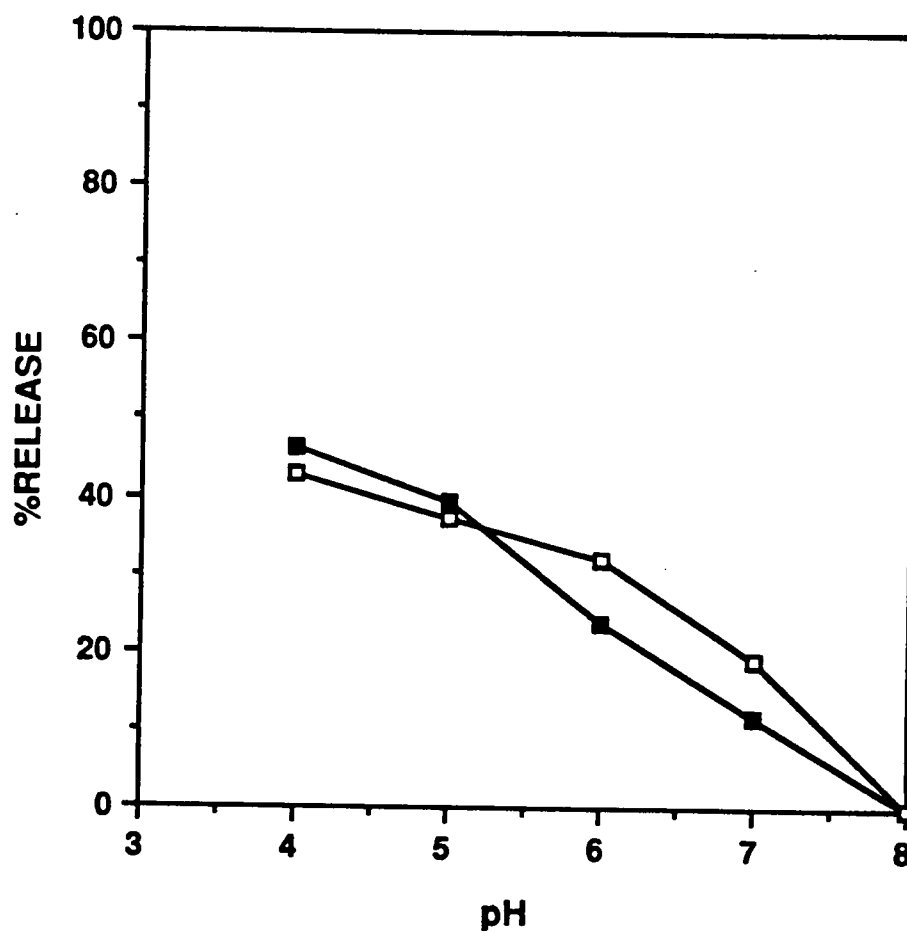


Figure II-5. Acid sensitivity of plasma-treated and untreated liposomes. % calcein release of liposome containing 40% chol was measured at different pH. ($\square$) plasma untreated; ($\blacksquare$) plasma-treated.

37°C in the presence and absence of human plasma for the plasma-treated liposomes (Fig. II-6). The plasma-treated liposomes showed striking stability both in the presence or absence of plasma; only 20-30% of calcein release was observed after 8 hours of incubation. This is to be compared with the untreated liposomes which were much more unstable either in PBS or in 90% plasma (Fig. II-6). Thus, the plasma treatment of the liposomes was not reversible; the plasma-resistant component of the liposome stayed resistant after the excess plasma was removed.

Dissociation of OA From Liposomes in Plasma

To examine whether OA in the liposome were transferred to the plasma component(s), liposomes containing trace amount of [^{14}C]OA were pretreated with plasma, then chromatographed on Bio-Gel A-1.5 M gel filtration column to separate liposome (void volume) and the plasma component(s) (included volume). The results presented in Table II-1 clearly show that most (60% or greater) of the liposomal OA had transferred to the plasma component(s). Furthermore, liposomes containing 40% chol showed more loss of OA than those containing no chol.

Discussion

In the absence of chol, the stability of the pH-sensitive liposomes composed of DOPE and OA prepared by the detergent-dialysis method is very similar to that of the liposomes of the same composition prepared by the reverse phase evaporation as previously reported (15). These liposomes, although relatively acid-sensitive, are unstable at

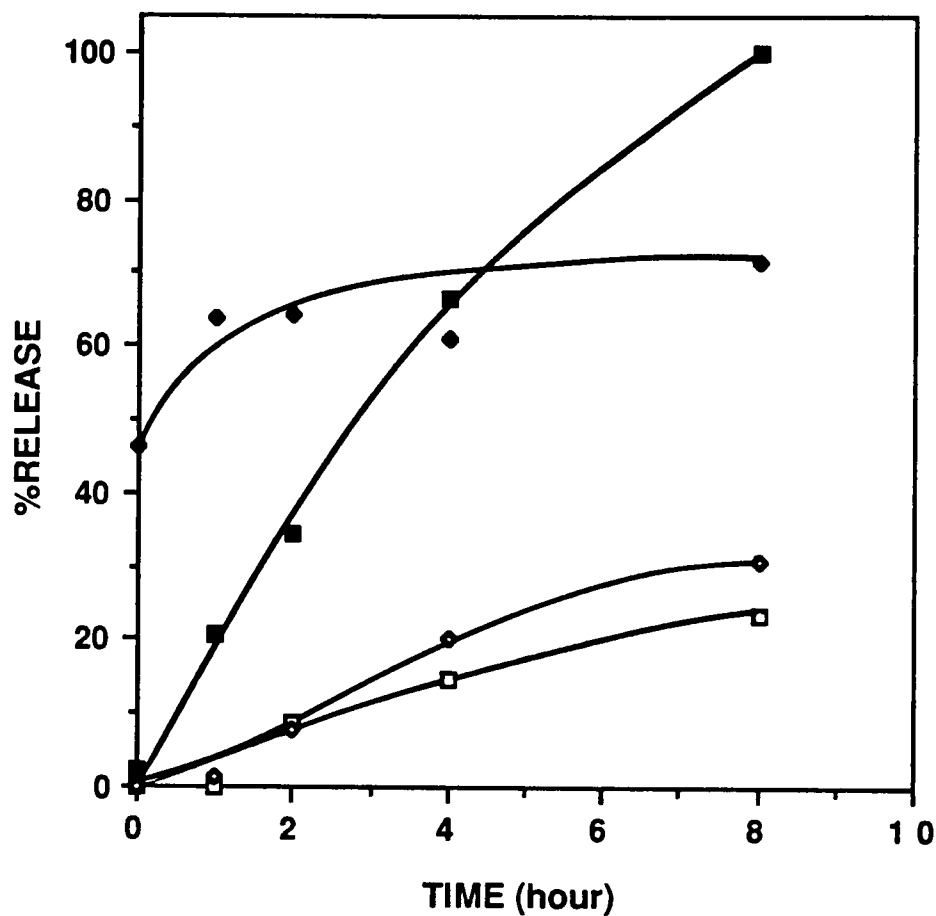


Figure II-6. Comparison of the stability of plasma-treated and untreated liposomes containing 40% cholesterol in plasma and PBS at 37°C. % calcein release from liposomes is plotted against the time of incubation. (■) untreated liposomes in PBS; (◆) untreated liposome in plasma; (□) plasma-treated liposome in PBS; (◇) plasma-treated liposome in plasma.

Table II-1. Transfer of OA from liposomes to plasma component(s)¹.

Chol content in liposome ² (%)	Plasma treatment ³	% [¹⁴ C]OA eluted in	
		void volume	included volume
0	no	100	0
	yes	41	59
40	no	100	0
	yes	22	78

1. Liposomes containing [¹⁴C]OA, with or without plasma treatment, were chromatographed on Bio-Gel A0.5M to separate liposome (void volume) from the plasma component(s) (included volume). Data are average of the two experiments.
2. Lipid composition was DOPE:OA (2:1) with 0 or 40% chol.
3. Plasma treatment was the same as described in Materials and Methods.

37°C, especially in the presence of plasma. However, the presence of chol significantly enhances the stability of the liposomes. For example, the first-order rate constant of the calcein release decreased from $1.8\% \text{ min}^{-1}$ to $0.2\% \text{ min}^{-1}$ when the liposome's chol content increased from 0 to 50%. Such a remarkable effect of chol must be related to the effect of chol on the phase transition behavior of PE as previously reported by Epand and Bottega (18). Chol lowers the bilayer to hexagonal phase transition temperature of dielaidoyl PE up to the mole fraction of about 0.1. At mole fractions above about 0.3, the effect of chol is to stabilize the bilayer phase. In the current study, the chol content ranged between 10-50%. Thus, only the bilayer stabilization effect of chol was seen.

Epand and Bottega have attributed the bilayer stabilization effect of chol to the unfitness of chol structure (e.g. single hydrocarbon chain) into the inverted hexagonal packing of PE. Furthermore, chol is known to form a weak complex with phospholipids in the bilayer phase via hydrogen bonds with the carbonyl oxygen of the neighbouring phospholipids (19). While the conformation of the phospholipid in the hexagonal phase packing is not known, chol might not be able to form hydrogen bonds with the neighbouring PE, thus raising the free energy level of the hexagonal phase.

pH-sensitive liposomes without chol rapidly released calcein when exposed to human plasma. This behavior has previously been observed with liposomes prepared by reverse phase evaporation method with the same lipid composition (15). Inclusion of chol in the lipid composition dramatically changed the stability of the liposomes in the

plasma-containing medium. Apparently, there were two components in the chol-containing liposomes: a component which was rapidly lysed by the action of the plasma and a component which was essentially resistant to plasma (Fig. II-4). The proportion of the two components depended on the chol content; liposomes with higher chol content showed a larger plasma-resistant component and vice versa (Fig. II-4 insert). This result can be understood by assuming that there are two opposing processes occurring when the liposome encounters plasma: a lytic reaction and a stabilization reaction. The stabilization reaction is apparently missing or the rate of the reaction is negligibly slow when the liposome does not contain cholesterol, because only rapid lysis of the liposome was observed in this case. The rate of stabilization must increase with increasing cholesterol content in the liposomes, since increasing amount of the stable liposome was found at the steady state. The lytic reaction is a fast process such that the initial rate of the reaction could not be measured with our method (Fig. II-4). This process may be directly or indirectly related to the transfer of OA from liposome to some plasma component(s) (Table II-1), most likely albumin, leaving the liposomes with a low level of OA which is insufficient to stabilize the bilayer phase of the liposomes. Insertion into the bilayer of apolipoproteins such as those in the HDL is known to cause liposome lysis (for a review, see 20). Small unilamellar liposomes with a high radius of curvature interact more extensively with lipoproteins or apolipoproteins than do the large multilamellar liposomes of the same composition. Bilayers at phase transition temperature are most sensitive to the insertion of the

apolipoproteins. Both the extraction of OA and interaction with the lipoproteins may be operating for the lytic reaction of the liposome. The molecular nature of the proposed stabilization reaction is not clear at the present time. DOPE is a lipid molecule with a weakly hydrated head group (21) and with bulky acyl chains, which are the reasons for its high tendency to form the inverted hexagonal phase at the physiological conditions (22). Bilayer phase of lipid molecules such as DOPE can be stabilized by adding amphiphiles of the opposite molecular characteristics, i.e. molecules with strongly hydrated head group and relatively non-bulky acyl chain (22,23). A number of plasma components, both proteins and lipids, can be the candidates for the stabilizer, e.g. lysophospholipids, gangliosides, and apolipoproteins. Identification of the stabilization reaction and the stabilizer will have to await further investigations.

An alternative explanation differing from the above kinetic considerations is also available. The liposome population could simply be heterogeneous in the lipid composition. For example, the chol distribution among the liposomes could be uneven, such that the chol-rich liposomes are resistant to lysis by plasma and the chol-poor liposomes are sensitive to lysis (24). Increasing the average chol content in the liposomes would increase the chol-rich proportion in the liposome population, resulting in more resistant liposomes. Heterogeneity of the liposomes prepared by the detergent-dialysis method has been reported before. Liposomes containing reconstituted HLA antigen (25) and palmitoyl antibody (26) show heterogeneous

distribution of the protein among the liposomes. The heterogeneity might be originated from the uneven distribution of various lipids in the mixed micelles from which the liposomes are derived (27). Heterogeneous distribution of chol is, however, difficult to demonstrate experimentally, because no significant difference in liposome size, charge or density is expected. Although the resistant liposomes can be isolated from the excess plasma proteins after the plasma treatment, they are unsuitable for the lipid composition analysis. This is because the plasma-sensitive liposomes, although losing all the entrapped calcein during the incubation with plasma, co-purify with the plasma-resistant liposomes under the chromatographic conditions used in the present study (data not shown).

While the hypothesis of the liposome heterogeneity remains speculative, it provides a theoretical basis for the observed acid sensitivity of the chol-containing liposomes. Data in Fig. II-2 show that there was a subpopulation in the chol-containing liposomes which was acid stable. This subpopulation increased with increasing average chol content of the liposome. It has been shown that the inclusion of chol in the pH-sensitive liposomes reduces the sensitivity of the liposome to acid treatment (4). The PE bilayer stabilization activity of chol discussed above may become important when the OA is protonated at acidic pH. Thus, liposomes enriched with chol were still stable even when the OA was protonated. Liposomes poor in chol would rapidly lyse at the same pH because of the lack of the stabilizing chol.

These observations are important in view of our previous work using the detergent-dialysis liposomes composed of DOPE:OA:chol (4:2:4)

to deliver plasmid DNA to the target lymphoma cells in mouse (13). According to the data presented here, a portion of the liposomes might be resistant to and/or stabilized with plasma component(s), and delivered the DNA to the target cells. The delivery mechanism most likely involved an acid-induced destabilization mechanism, because the plasma-treated liposomes are still partially acid-sensitive (Fig. II-5). Thus, the pH-sensitive, cholesterol-containing liposomes prepared by the detergent-dialysis method show a good potential as a target-specific, cytoplasmic delivery vehicle for water-soluble drugs, such as DNA.

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CHAPTER III

SIZE DEPENDENT STABILIZATION OF pH-SENSITIVE LIPOSOMES COMPOSED OF
DIOLEOYLPHOSPHATIDYLETHANOLAMINE AND OLEIC ACID BY HUMAN PLASMAAbstract

Small unilamellar liposomes, composed of dioleoylphosphatidyl-ethanolamine (DOPE) and oleic acid (OA), prepared by sonication, were incubated in the presence of human plasma at 37°C. The release of entrapped calcein after 8 hours incubation was about 15% in plasma, compared with about 70% in phosphate-buffered saline under the same conditions. In contrast, dioleoylphosphatidylcholine (DOPC):OA liposomes under the same conditions release about 70% in plasma and only 10% in PBS. Total release of calcein from the DOPE:OA liposomes was observed in a PBS solution containing bovine serum albumin, and the release was completely blocked by preincubation of the liposomes with plasma. These results indicate that the unstable DOPE:OA liposomes are stabilized by incubation with plasma. The stabilization process was very fast, being completed within one minute. Only relatively small liposomes ($d \leq 200$ nm) were completely stabilized by plasma; larger liposomes were progressively less stabilizable. SDS-polyacrylamide gel electrophoresis of liposomes which had been incubated with plasma and then washed indicated that several proteins were tightly associated

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with liposomes. Using liposomes containing [^{14}C]OA, it was found that about 70% of the original OA was extracted after 1 hour incubation with human plasma at 37°C. Thin layer chromatographic analysis of the plasma-treated liposomes showed the presence of the plasma lipids in the liposomes. These results suggest that liposomes composed of DOPE:OA are stabilized by protein and/or lipid components from human plasma and that the composition of the liposomes is altered. The mechanism of stabilization is discussed in terms of the surface pressure of small vesicles with a high degree of curvature. The results presented here are relevant to the use of PE-containing liposomes for drug delivery in vivo.

Introduction

Liposomes have been extensively tested in experimental animals and in humans as carriers for drugs, enzymes, biological response modifiers and nucleic acids (for a recent review, see 1). Of primary concern is the stability of the liposomes during storage and in the blood. The former often limits the clinical use of the liposome and the latter determines the carrier potential of the liposome. It is well known that liposomes composed of PC as the major matrix lipid are generally stable in a simple buffer upon storage (2) and this type of liposome is widely used by many investigators. However, unsaturated PC-based liposomes rapidly release the entrapped contents upon exposure to serum or plasma, unless cholesterol is included as one of the major lipid components (3). Plasma components such as albumin, lipoproteins

and fibronectin readily interact with the liposomal bilayer (4). Lipoproteins such as HDL are particularly important in determining liposome stability in the blood (5). Apolipoproteins are transferred to liposome membranes (6,7), and perhaps serve as opsonins for the uptake of liposomes by macrophages (8). Different sets of serum proteins are found to interact with liposomes depending on the cholesterol content of the liposomes (9).

Liposomes composed predominantly of PE, and particularly an unsaturated PE such as DOPE, have become increasingly important for the liposomal drug delivery in recent years (10-16). The equilibrium phase of DOPE at physiological temperature and pH is the hexagonal H_{II} phase (17). However, the bilayer phase, in the form of liposomes, can be prepared by mixing DOPE with at least one other amphiphile (lipid or protein) (10,11,13,18,19). These liposomes are generally less stable than PC-based liposomes upon storage due to their tendency to revert to the H_{II} phase. Indeed, special conditions such as acidic pH, or binding to target cells, often trigger a rapid destabilization of the liposomes, making the liposomes suitable for intracellular drug delivery (14,15,16,20). Thus, DOPE liposomes stabilized with weakly acidic amphiphiles such as fatty acids (11,14,15,16), acyl amino acids (13,21), and other double-chain lipids (22), are useful for efficient cytoplasmic delivery of drugs. DOPE liposomes stabilized with acylated antibody, known as target-sensitive immunoliposomes (19), are useful for target-cell surface drug delivery (20) and in vitro diagnosis of virus (23). At least one such liposome preparation, i.e. the large unilamellar liposomes composed of DOPE:OA (8:2), is not stable in

serum; rapid liposome aggregation and content leakage take place within minutes after exposure to serum (24). Inclusion of cholesterol in such liposomes improves the stability (25). Thus, the behavior of large unilamellar liposomes composed primarily of DOPE is similar to that of liposomes composed primarily of PC.

We report here the observation that the small unilamellar liposomes of the same lipid composition, i.e. DOPE and OA, though quite unstable in buffer, become rapidly stabilized upon exposure to plasma or serum. We have isolated the stabilized liposomes and characterized their composition. Furthermore, we have shown that the plasma stabilization of liposomes depends on the liposome size.

Materials and Methods

Materials

DOPE and DOPC were purchased from Avanti Polar Lipids. OA, cholesterol, BSA, Ficoll, and calcein were obtained from Sigma Chemical Co. Freshly collected human plasma was obtained from Fort Sanders Regional Medical Center, Knoxville, TN. [^{14}C] oleic acid (57 mCi/mmol) was purchased from New England Nuclear. TLC plates were from Beckman.

Preparation of Small Unilamellar Liposomes

Ten μmole lipids containing either DOPE or DOPC (6.67 μmole) and OA (3.33 μmole) were dried in a stream of N_2 gas. The dry lipids were vacuum desiccated for a minimum of 30 min, and hydrated for 5 hours at room temperature in 1 ml of 50 mM calcein. The osmolarity of this solution was adjusted to 320 mOsm/kg with 10X of PBS, pH 8.0. Trace

amount of a non-exchangeable lipid marker hexadecyl [³H] cholestanyl ether was included to monitor the lipids. The lipid suspension was sonicated with a bath sonicator (Laboratory Supplies, Hicksville, NY) and the pH was adjusted and maintained at 8.0 during sonication. The liposome suspension was left for 2 hours at room temperature to facilitate the annealing process. Free calcein was then separated from liposomes by using BioGel A-0.5M column chromatography using PBS which was isotonic to the calcein containing buffer as the eluent. The size of liposome was measured by dynamic laser light scattering using a Coulter N4SD instrument. Liposomes with a diameter of 95 ± 10 nm were used for most of the experiments.

Liposome Stability Assay

Fifty microliters of the liposome suspension containing about 50-150 nmole lipids were added to 450 μ l prewarmed (37°C) human plasma or PBS (pH 7.4). Ten microliters of the mixture were added into a cuvette containing 1.99 ml PBS for fluorescence measurement. The fluorescence intensity was measured with excitation wavelength of 490 nm and emission wavelength of 520 nm. The total fluorescence intensity was obtained by adding 50 μ l of 5% deoxycholate to lyse the liposomes. Release of the entrapped calcein from the liposomes was calculated with the following equation:

$$\% \text{ Release} = \frac{(F_t/F) \cdot F_x - F_o}{F_t - F_o} \quad (\text{equation 1})$$

where F_0 is the fluorescence intensity of the liposomes in PBS at room temperature at time zero. F_t is the total fluorescence intensity of liposome in PBS after addition of DOC. F_x is the fluorescence intensity of the liposome incubated either in plasma or PBS at 37°C at time X. F is the total fluorescence intensity of the liposomes in plasma after addition of DOC. F_t/F is a correction term which is used to minimize the pipeting and other systematic errors in the measurement. The value of F_t/F ranged from 0.9 to 1.1.

Treatment of Liposomes With Plasma

Liposome suspensions containing 1 μ mole lipids (in 100 μ l) were incubated with plasma of various dilutions for 1 hour at 37 °C. Excess plasma proteins were separated from liposomes by either floating the liposomes in a discontinuous Ficoll gradient as described below or by chromatography on a Bio-Gel A-0.5M column.

pH Sensitivity Assay

Ten microliters of liposomes containing about 3 nmole lipid were incubated in a cuvette containing 1.99 ml of PBS at desired pH for 5 min. before the pH was returned to 8.0 by adding appropriate amounts of NaOH. Equation 1 was used to calculate % calcein release, except that of F_0 was the fluorescence intensity at pH 8.0 and F_x was the fluorescence intensity at pH 8.0 after incubation at different pH. F_t and F were the total fluorescence intensity after lysis of liposomes with DOC.

Preparation of Liposomes of Various Diameters

Large unilamellar vesicles were prepared by a detergent dialysis method. Ten μ mole of lipid was hydrated in 900 μ l buffer containing 50 mM of calcein and sonicated as described above. One hundred microliters of octylglucoside (100 μ mole) was added after sonication and pH adjustment. The mixture was vortexed and dialysed at room temperature against 100 ml of the same buffer containing 1g washed SM-2 beads for 48 hours with slow stirring. The liposomes were extruded through polycarbonate filters of defined pore size (manufactured by Nuclepore Corp.) to obtain unilamellar liposomes of defined size. Free calcein was then removed by gel filtration on BioGel A-0.5 M column as above. The size of the liposomes was measured by dynamic laser light scattering as above.

BSA Induced Leakage of DOPE:OA Liposomes

Same amount of liposome as in the stability assay was incubated with 450 μ l BSA with 22, 44 or 88 mg/ml for 15 minutes at 37 °C. Ten microliters of the mixture were added to a cuvette containing 1.99 ml of PBS. The release was calculated according to equation 1.

Size Change of DOPE:OA Liposomes After Sonication

Liposomes (20 mM) were mixed either with equal volume of PBS or human plasma after sonication and the size was measured by either dynamic laser light scattering as described above or by negative-stain electron microscopy using 1% uranium acetate as a stain as described previously (26).

Association of Plasma Components with Liposomes

Liposomes containing 5 μ mole lipids (in 0.5 ml) were incubated with 0.5 ml of human plasma at 37°C for 1 hour. The mixture was then mixed with a Ficoll solution (in PBS, pH 8.0) to obtain a final Ficoll concentration of 20% and placed into a 15 ml COREX centrifuge tube. Six milliliters of 15% Ficoll were overlayed, followed by 0.5 ml PBS. The mixture was centrifuged in a Sorvall HB4 swinging bucket rotor for 1 hour at 5,000 rpm at room temperature. The liposomes which floated to the PBS/15% Ficoll interface were collected. The resuspended liposomes were floated one more time with an identical procedure before being used for protein determination by the Lowry assay (27) and for the SDS PAGE analysis using a 7.5% polyacrylamide under denaturing conditions (28). The lipid was extracted from floated liposomes by using standard methods (29) and the amount of lipid was determined by a chromate assay (30) using DOPE:OA (2:1) as standard. The recovery of lipid was determined by using [3 H] cholestanyl ether as a trace marker. The lipid extract was also analysed by thin layer chromatography on a silica gel plate using chloroform:acetone:methanol:acetic acid:H₂O (6:8:2:2:1) as a developing solvent.

The dissociation of oleic acid from liposomes was determined by using a trace amount of [14 C]OA with the same flotation procedure as described above. After one flotation, the gradient was fractionated from the bottom and 14 C-cpm of the fractions was determined. The 14 C-cpm at bottom fractions was considered as the dissociated OA. The dissociation of OA was confirmed by gel filtration on a Bio-Gel A-1.5M

column (1.4 X 60 cm) and by sucrose gradient centrifugation. The [^3H] cholestanyl ether was used as a lipid marker in these experiments.

Results

Stability of the Small Sonicated Liposomes in Buffer and Plasma

Stability of liposomes was measured by the release of a water soluble, self-quenching fluorescence dye, calcein, from the liposomes. When liposomes composed of DOPC:OA (2:1) or DOPC:OA:Chol (2:1:2) were incubated in PBS at 37°C, little calcein was released up to 8 hrs. (Fig. III-1). However, when the incubation was carried out in 90% human plasma, cholesterol-free liposomes rapidly released calcein (approximately 70% after 2 hrs). The cholesterol-rich liposomes, on the other hand, were quite stable in the plasma, showing little calcein release even after 8 hrs. Therefore, the PC-based liposomes are stable in buffer and unstable in plasma unless cholesterol is present in the liposome membrane.

The stability of the corresponding PE-based liposomes was also studied (Fig. III-2). Small unilamellar liposomes composed of DOPE:OA (2:1) or DOPE:OA:chol (2:1:2) were not stable in PBS; continuous calcein release was observed up to 70-80% in 8 hrs. Inclusion of cholesterol in the liposomes did not stabilize the liposome. Interestingly, calcein release from either type of liposome was not observed when the liposomes were incubated in 90% plasma. Inclusion of cholesterol made little difference in terms of the stability after plasma treatment. Thus, the behavior of the PE-based liposome is just opposite to that of the PC-based liposomes. These PE-based liposomes

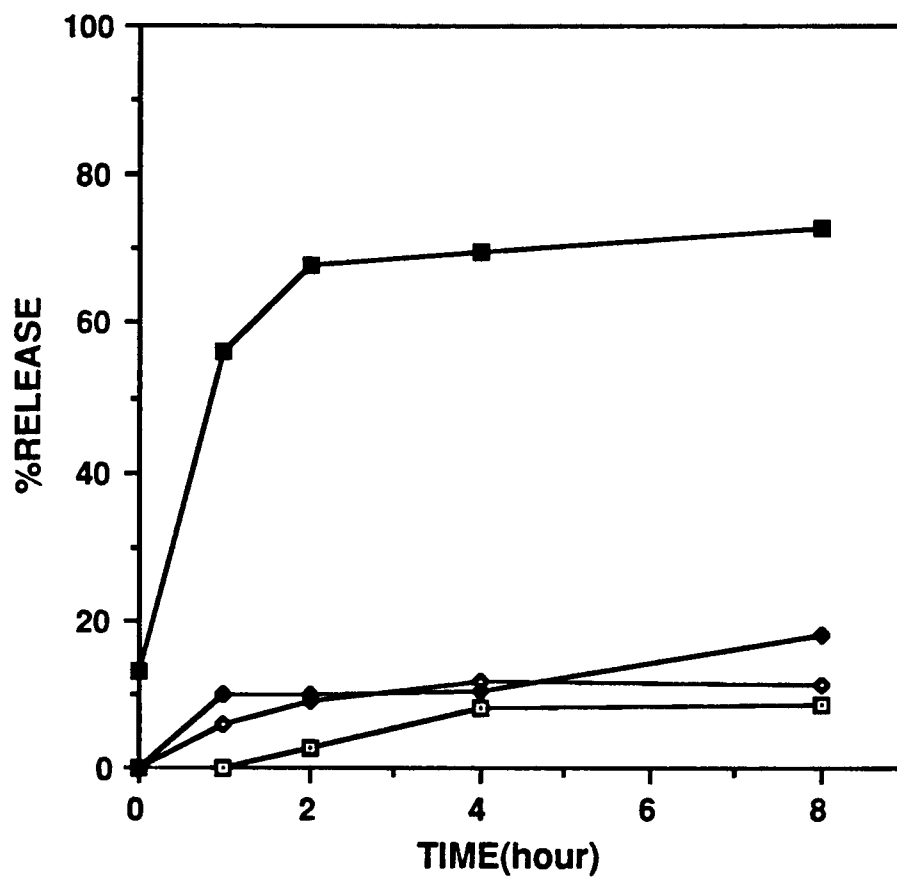


Figure III-1. Stability of PC-based liposome in 90% plasma or PBS at 37°C. (□) DOPC:OA (2:1) in PBS; (◇) DOPC:OA:chol (2:1:2) in PBS; (■) DOPC:OA (2:1) in plasma; (◆) DOPC:OA:chol (2:1:2) in plasma.

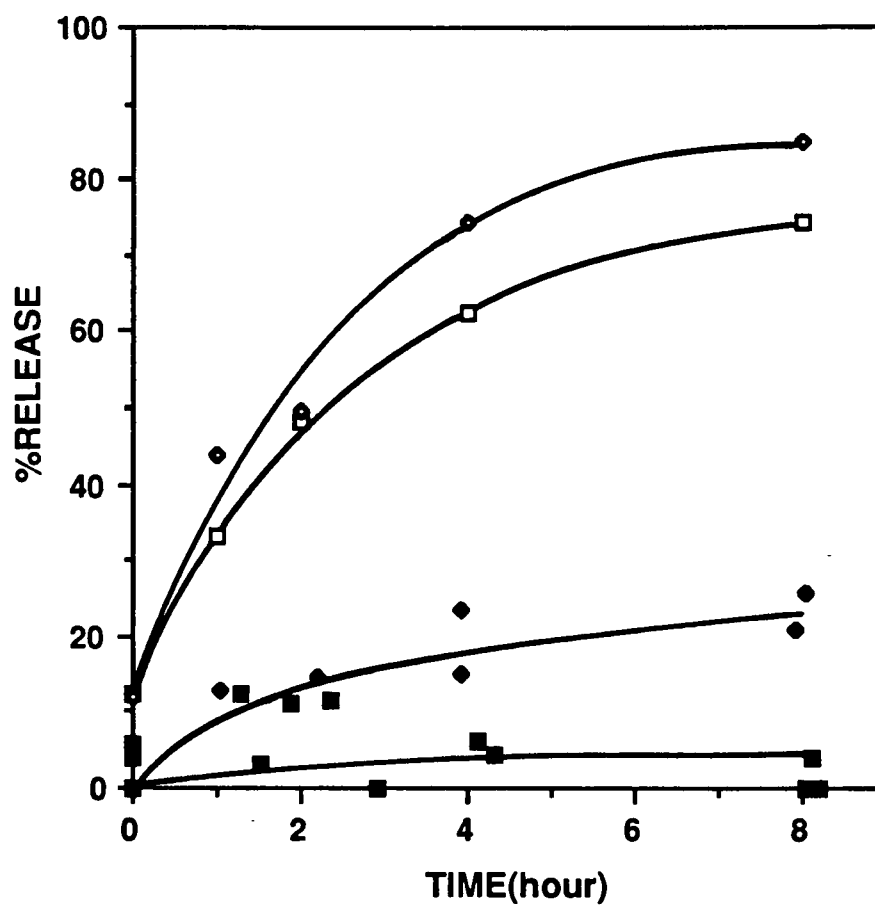


Figure III-2. Stability of DOPE-based liposomes in 90% plasma or PBS at 37°C. (□) DOPE:OA (2:1) in PBS; (◇) DOPE:OA:Chol (2:1:2) in PBS; (■) DOPE:OA (2:1) in plasma; (◆) DOPE:OA:chol (2:1:2) in plasma.

are unstable in buffer, but quite stable in plasma. Furthermore, cholesterol improves the stability of PC-based liposomes in plasma, but shows little effect on the PE-based liposomes.

In order to examine if the incubation with plasma has stabilized the PE-based liposomes, liposomes were incubated with 50% plasma at 37°C for 1 hour, separated from the plasma components by chromatography on BioGel A-0.5m column (see Experimental Procedures), and retested for calcein release in PBS or in 90% plasma at 37°C. As can be seen in Fig. III-3, the plasma-treated liposomes now became quite stable in PBS; only about 20% calcein release was observed after 8 hrs. Subsequent incubation in 90% plasma did not cause any significant calcein release. Again, cholesterol-free and cholesterol-rich liposomes behaved identically. Thus, preincubation of the PE-based liposomes in plasma had rendered the liposome stable. In fact, the stabilized liposomes could be stored at 4°C for a few months without leakage of calcein (data not shown).

Eight different batches of human plasma taken from different individuals gave the same stabilization effect. Fresh or frozen plasma also showed the same activity. The activity was also seen in serum, whether dialysed against PBS or not, indicating that the activity resides in some macromolecular component(s) common in the serum and the plasma.

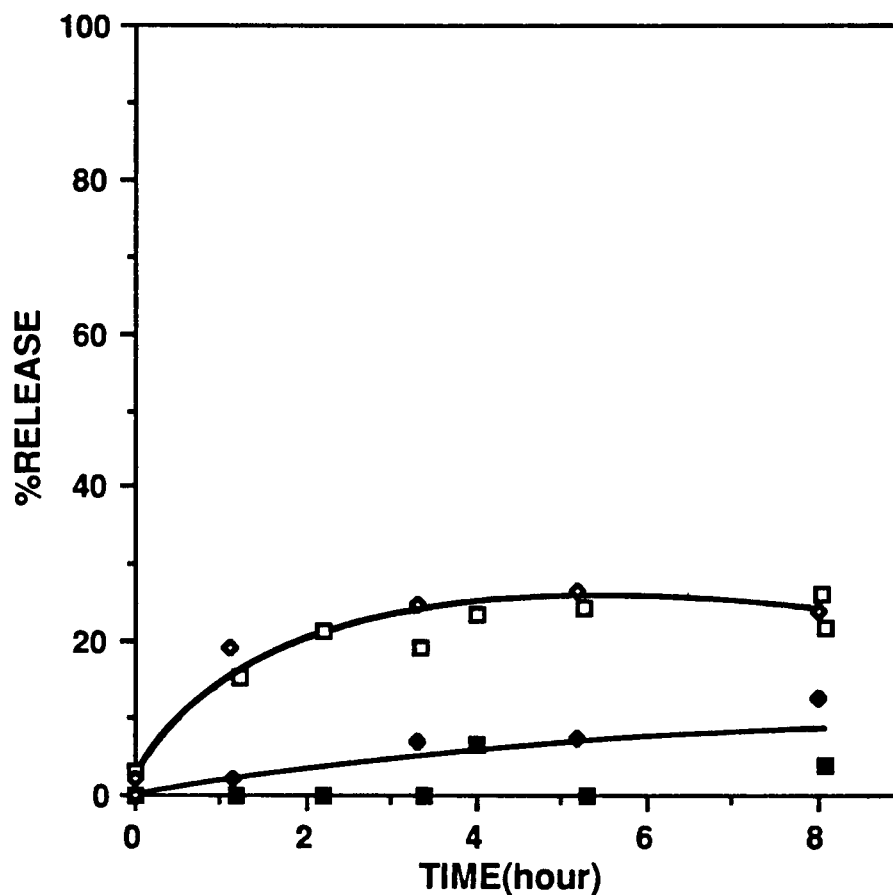


Figure III-3. Stability of plasma treated PE-based liposomes in 90% plasma or PBS at 37°C. (□) DOPE:OA (2:1) in PBS; (◇) DOPE:OA:chol (2:1:2) in PBS; (■) DOPE:OA (2:1) in plasma; (◆) DOPE:OA:chol (2:1:2) in plasma.

Plasma Blocks the Aggregation and Fusion of the DOPE:OA Liposomes

When the DOPE:OA (2:1) liposomes were freshly sonicated, they appeared to be small unilamellar vesicles as revealed by negative-stain electron microscopy (Fig. III-4A). The average diameter of the liposomes was 91 ± 25 nm ($n=232$). Upon incubation at 37°C in PBS, the liposomes began to fuse (Fig. III-4B) with an increase in the average diameter to 128 ± 36 nm ($n=216$) after 24 hours of incubation. The progress of liposome aggregation and fusion was also followed by dynamic laser light scattering. The average particle diameter grew from approximately 88 nm immediately after sonication to 128 nm during 24 hours of incubation in PBS at room temperature. Thus, the destabilization of the small sonicated liposomes was accompanied by liposome fusion. However, this process could be completely blocked by adding human plasma to the liposome suspension. Fig. III-4C shows the morphology of the plasma-treated, freshly prepared liposomes, reisolated by gel filtration. Note that the liposomes were still unilamellar and non-aggregated. The average diameter was 79 ± 25 nm ($n=148$), very close to that of the freshly sonicated liposomes without plasma treatment. The average diameter of the liposomes ($d = 78 \pm 26$ nm, $n=197$) did not change after 24 hour incubation in plasma at 37°C (Fig. III-4D). The inhibitory effect of plasma on the aggregation and fusion of the liposomes could also be revealed by the light scattering measurements. If plasma was added immediately after the sonication, the average particle diameter did not change with time.

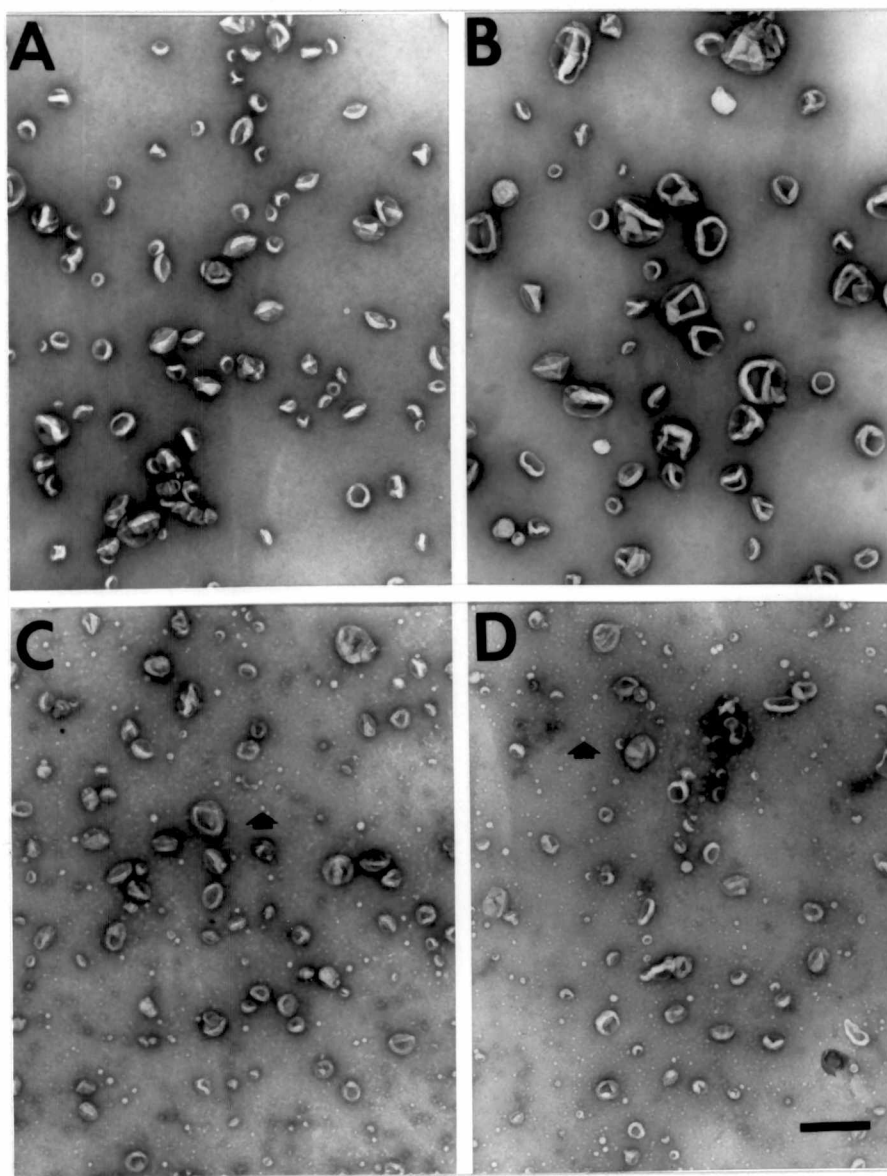


Figure III-4. Electron micrographs of DOPE:OA (2:1) liposomes. The samples were negatively stained with 1% uranyl acetate. Bar is 0.2 μm . The arrow shows lipidic particle.

- A. freshly sonicated liposomes mixed with equal volume of PBS.
- B. liposomes incubated in PBS for 24 hours at 37°C.
- C. freshly sonicated liposomes mixed with equal volume of plasma.
- D. liposomes incubated in 50% plasma for 24 hours at 37°C.

Effect of Plasma Treatment on Albumin-Induced Liposome Lysis

Liposomes composed of unsaturated PE and OA are not stable in albumin solutions (22). This is probably due to the extraction of OA from liposome by albumin. We have investigated if the plasma stabilized liposomes could be lysed by albumin (Fig. III-5). Liposomes not treated with plasma were very sensitive to BSA; complete lysis was observed at BSA concentration of 22 mg/ml or higher. However, if liposomes were pretreated with plasma, liposome lysis was completely inhibited. Complete protection of liposomes by plasma was observed at a plasma concentration of 25% or above; 10% plasma only gave partial protection. These results imply that the stabilization of liposome by plasma is a stoichiometric reaction, i.e. the stabilization factor(s) in the plasma can be titrated.

Proteins Associated with the Plasma-stabilized Liposomes

As part of the chemical characterization of the plasma-stabilized liposomes, liposomes were twice floated on a discontinuous Ficoll gradient after the incubation with plasma and the proteins associated with the liposomes were analysed by SDS-PAGE under reducing conditions (Fig. III-6). There were 7 major polypeptides associated with DOPE:OA liposomes, 4 of them having Mr 237, 81, 73 and 66 kd. The Mr of the last three could not be accurately determined with the gel conditions used. Similar polypeptide patterns were found for other liposome compositions, i.e. DOPE:OA:chol (2:1:2); egg PC; egg PC:Chol (6:4); DOPC:OA:chol (2:1:2). Thus, these major bands, although associated with the liposomes, are not the likely candidates for the

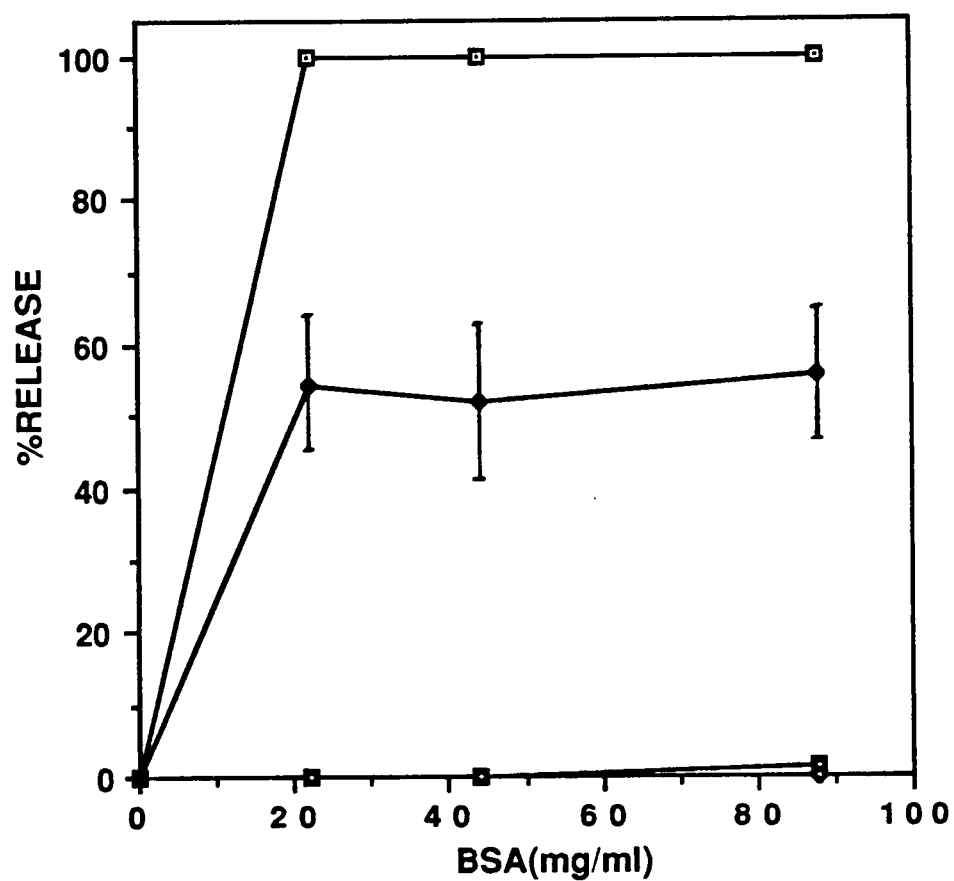


Figure III-5. BSA induced leakage of DOPE:OA (2:1) liposome.
(□) untreated; (◆) 10% plasma treated; (□) 25% plasma treated; (◇) 50% plasma treated.

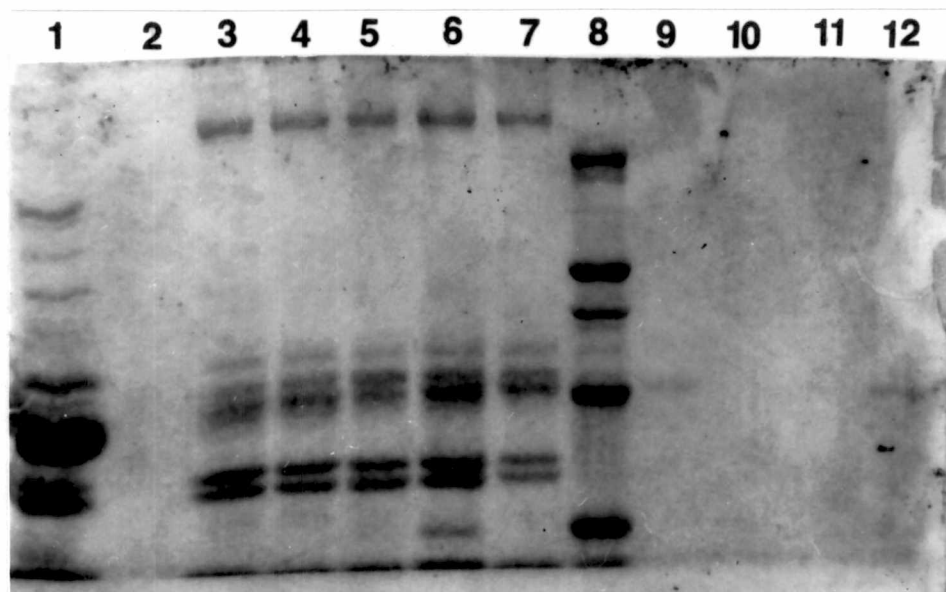


Figure III-6. Plasma proteins associated with plasma treated liposomes. Liposomes were incubated with either PBS or plasma for 1 hour at 37°C. The liposomes were then floated in a discontinuous Ficoll gradient (see text for detail) and analysed on a 7.5% polyacrylamide gel in the presence of SDS and mercaptoethanol. 1) Whole human plasma, 2) Blank, 3) Egg PC liposomes treated with plasma, 4) Egg PC:chol (6:4) liposome treated with plasma, 5) DOPC:OA:chol (2:1:2) liposomes treated with plasma, 6) DOPE:OA (2:1) liposomes treated with plasma, 7) DOPE:OA:chol (2:1:2) liposomes treated with plasma, 8) molecular weight standards (myosin, β -galactosidase, phosphorylase B, BSA, ovalbumin), 9) whole human plasma floated in the absence of liposome, 10) egg PC liposome treated with PBS, 11) DOPC:OA:chol (2:1:2) liposomes treated with PBS, 12) DOPE:OA:chol (2:1:2) liposomes treated with PBS.

plasma factor responsible for the stabilization of the DOPE:OA liposomes. One possible exception is a polypeptide with an electrophoretic mobility close to that of the ovalbumin used in the molecular weight standards, i.e. Mr approximately equals 42.7 kd. This band was uniquely associated with the DOPE:OA liposomes. However, since it was absent in the proteins associated with the DOPE:OA:chol liposomes which were also stabilizable by plasma, the role of this polypeptide in the stabilization of the PE-based liposome could not be ascertained. Furthermore, all of the above named large polypeptides associated with the DOPE:OA liposomes could be removed with a trypsin digestion, yet the liposomes still retained calcein (data not shown). Therefore, it is not likely that these polypeptides are responsible for the liposome stabilization. These results, however, do not rule out the possibility that some polypeptide(s) which is at low concentration in plasma but has strong stabilization activity is the liposome stabilizer. It is possible that the polypeptide(s) which serves as a stabilizer for DOPE/OA liposomes may not be identified on the gel because of the large amount of proteins nonspecifically associated with liposomes.

Lipids Associated with the Plasma Stabilized Liposomes

We first checked if OA had been extracted away from the DOPE:OA liposomes by using [^{14}C]OA as a marker. After incubation with the plasma, liposomes were floated on a Ficoll gradient and the distribution of [^{14}C]OA in the gradient was determined. Approximately $28 \pm 5.0\%$ ($n=5$) of the total [^{14}C]OA was found in fractions at top portion of the gradient. The top fractions contained most of the liposomes as

judged by the turbidity and quenched calcein fluorescence of the liposomes. These results indicate that approximately 72% of the original OA was extracted from the liposomes during the incubation with the plasma. The inclusion of cholesterol did not block the OA extraction.

The lipids of the plasma-treated liposomes, original composition DOPE:OA (2:1), were extracted and separated by thin-layer chromatography on Silica gel. Fig. III-7 shows the chromatogram. Apparently, the lipid composition of the plasma-treated liposomes was more complex than the original composition. The relative abundance of the lipids in the treated liposomes, as measured by the intensity of the iodine vapor stain and by charring the TLC plate after spraying with sulfuric acid, was in the order of $PE > PC \geq chol > lysoPC > fatty\ acids$. Although the lipids were not quantitated, the protein/lipid ratio of the plasma-treated liposome was determined to be 0.51 ± 0.02 (w/w) ($n=6$). Thus, the plasma-treated liposomes contained about 1/3 protein and 2/3 lipid by weight. Thus, they were "proteoliposomes".

pH-Sensitivity of the Plasma-Treated Liposomes

The DOPE:OA (2:1) liposomes rapidly destabilize in a weakly acidic environment ($pH < 7$) (11,12). Since the composition of the liposome had changed significantly upon treatment with plasma, one would expect that the pH-sensitivity of the liposome would also be modified. This was determined directly and the results are shown in Fig. III-8. Quite obviously, the plasma-treated liposomes, whether originally cholesterol-free or -rich, had become insensitive to acid. Only 10-20%

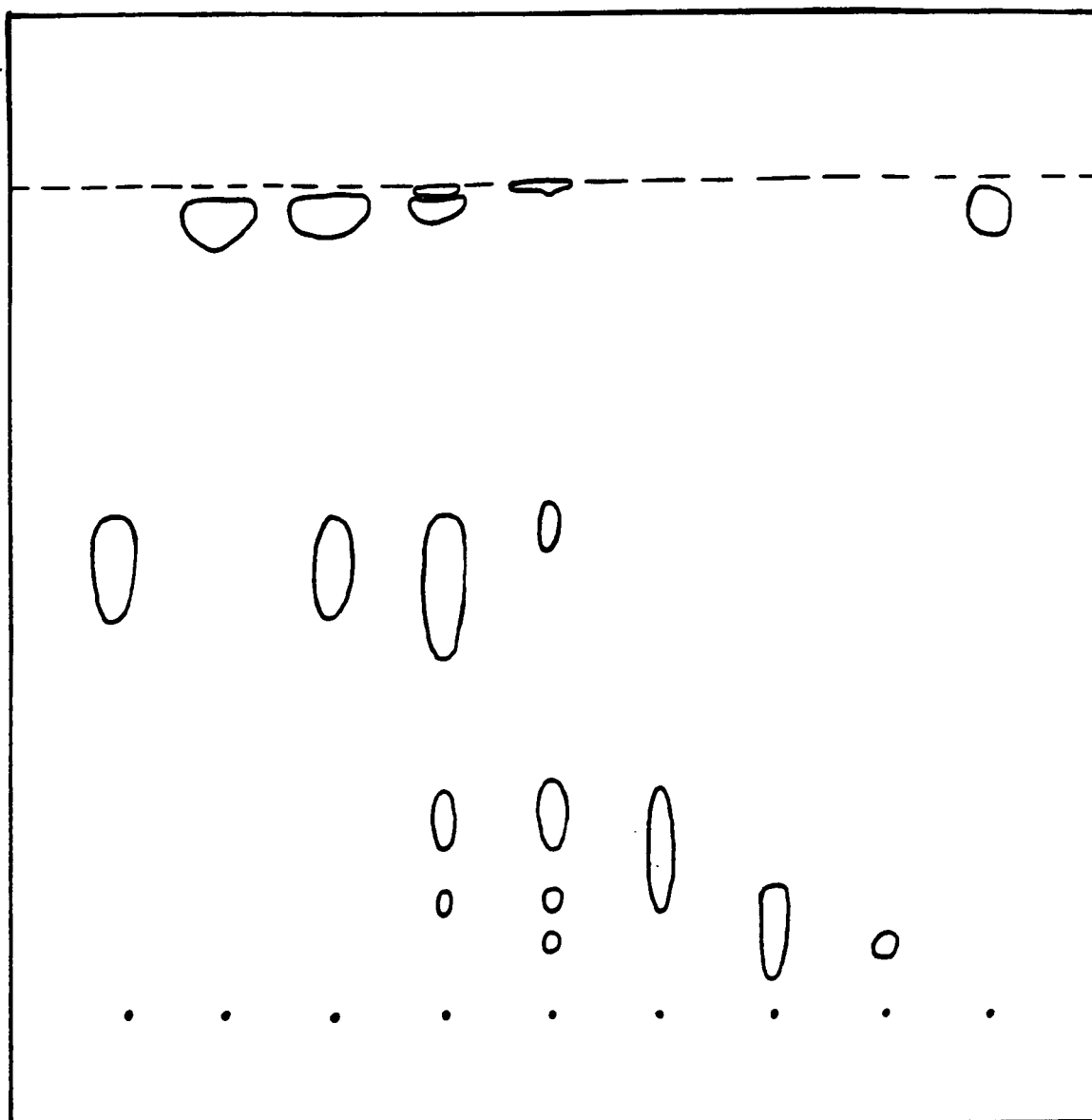


Figure III-7. Thin layer chromatography of lipids extracted from DOPE:OA liposomes treated with plasma. Solvent contains CHCl_3 :acetone:methanol:acetic acid: H_2O (6:8:2:2:1). From left to right: DOPE; OA; liposome treated with PBS; liposomes treated with plasma; lipids extracted from plasma; egg PC; lysoPC; sphingomyoline; cholesterol. One hundred μg of lipids per lane was used.

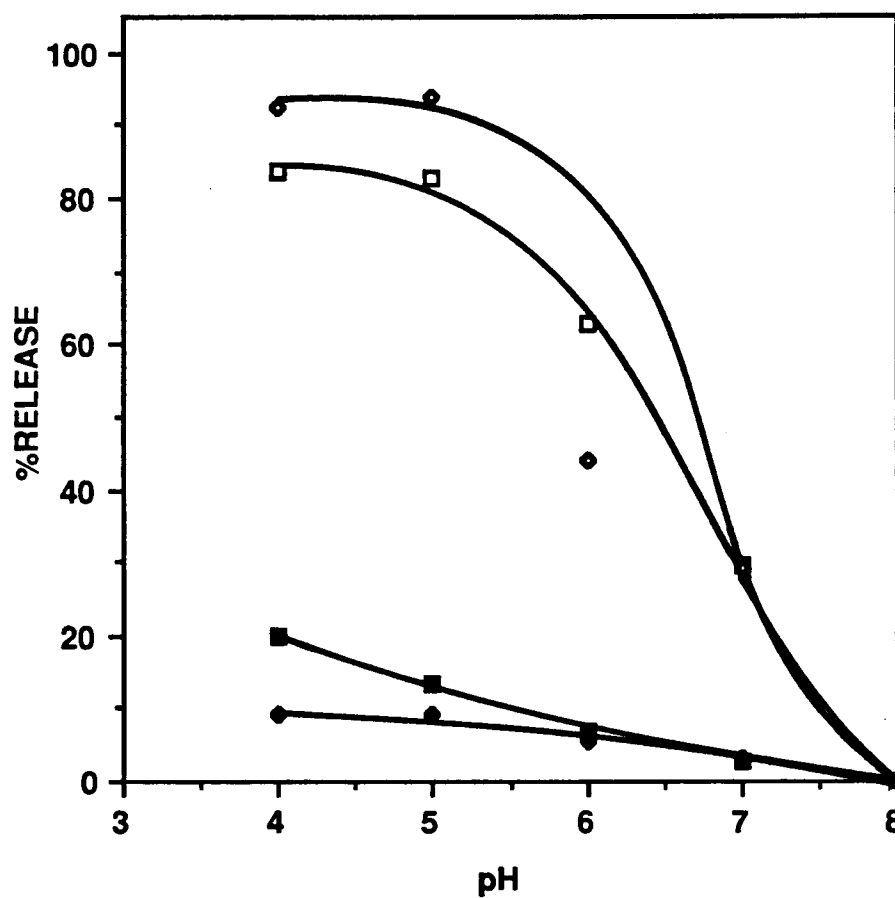


Figure III-8. Acid induced leakage of PE-based liposomes. (□) DOPE:OA (2:1) and (◇) DOPE:OA:chol (2:1:2) treated with PBS; (■) DOPE:OA (2:1); and (◆) DOPE:OA :chol (2:1:2) treated with 50% plasma.

calcein release was observed at pH 4, as compared with 80-95% release at the same pH for the liposomes without the plasma treatment.

Effect of Liposome Size on the Plasma Stabilization of Liposomes

We have previously reported that large unilamellar liposomes composed of DOPE:OA (2:1) are not stable in human plasma unless cholesterol is included in the lipid composition (25). This report, seemingly conflicting with the results described here, could be explained on the basis of the liposome size. The liposomes used in previous studies were prepared by a detergent-dialysis protocol. The present liposomes were prepared by sonication, resulting in much smaller liposomes. In order to test this hypothesis, a systematic study on the effect of liposome size on the plasma stabilization of liposomes was undertaken. Liposomes were prepared with both methods and the average particle size was determined by laser light scattering. To obtain intermediate-sized liposomes, liposomes prepared by detergent-dialysis method were extruded through polycarbonate filters of defined pore size and the average particle diameter of the extruded liposomes was also determined by laser light scattering. These liposomes were then gel-filtered to remove the free, untrapped calcein, and tested for dye release after 4 hour incubation at 37°C in PBS or in 90% human plasma. The result is shown in Fig. III-9. The stability of the liposomes in PBS was not very dependent on the liposome size; larger liposomes released only slightly more calcein than the smaller ones. However, the dye release from liposomes incubated in the plasma was a sensitive function of the liposome size.

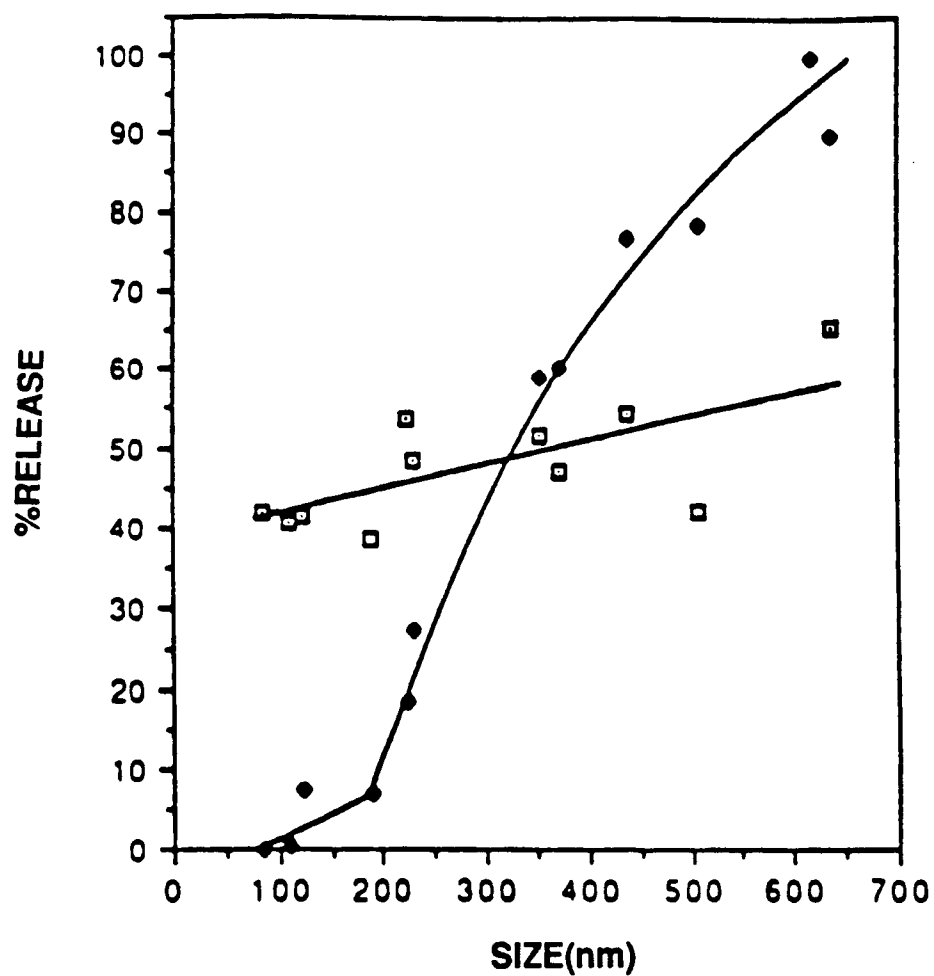


Figure III-9. Size dependent stability of liposomes in plasma or PBS at 37°C. Percent calcein release of DOPE:OA liposomes (50-100 nmole lipid) with various diameters after 4 hour incubation in 90% plasma (◆), or in PBS (□).

Small liposomes ($d \leq 200$ nm) released less than 10% calcein in 4 hours, whereas larger liposomes ($d > 600$ nm) released almost all of the entrapped dye during the same period of incubation. Consistent with previous studies (25), large liposomes were more stable in PBS than in plasma. Small liposomes showed the opposite behavior, i.e. they were more stable in plasma than in PBS. Thus, liposome size determines the extent of liposome stabilization; only small liposomes can be stabilized by the plasma factor(s).

Discussion

The results presented here clearly indicate that small liposomes composed of DOPE and OA, but not those composed of DOPC and OA, are stabilized by some factor(s) in the human plasma. This conclusion is supported by several experimental results. First, only DOPE-based liposomes are stable in the plasma or serum, but not the DOPC-based liposomes unless they contain cholesterol (Fig. III-1 and III-2). DOPE-based liposomes can be isolated and appear to be stable upon subsequent incubation with PBS or albumin (Fig. III-3 and III-5), or upon prolonged storage. Finally, the spontaneous aggregation and fusion of the DOPE:OA liposomes are completely blocked by incubation with plasma (Fig. III-4). These results suggest that some factor(s) in the human plasma has been transferred to the liposome membrane, giving rise to the stabilization effect.

An important observation is the effect of liposome size on the plasma stabilization. Only small liposomes with diameters equal to or smaller than approximately 200 nm are stabilized; liposomes with

greater diameters are less effectively stabilized (Fig. III-9). This result is quite understandable in view of the fact that hydrophobic molecules insert themselves into the membranes of the small liposomes with small radius of curvature more effectively than those of the larger liposomes with large radius of curvature (31). Insertion of an amphipathic protein such as the cytochrome b_5 also prefers small liposomes over larger ones by a factor of at least 20 (32). This is due to the fact that high curvature of the smaller liposome gives rise to a lower surface pressure than the larger liposome (33,34). The surface pressure of a 20 nm PC liposome is 15 dynes/cm, which progressively increases to 46 dynes/cm for a 200 nm liposome (33). Notice that the surface pressure cited here refers to that of the outer monolayer of the unilamellar liposome. Lipids located in the inner monolayer of a small liposome are likely packed much tighter, thus giving rise to a higher surface pressure. The transmembrane gradient of the surface pressure diminishes with increasing diameter of the liposome. This is the reason why small unilamellar liposomes, under appropriate conditions, spontaneously aggregate and fuse with each other to become larger liposomes (35).

The transmembrane gradient of the surface pressure for the small liposomes composed of DOPE:OA is likely to be greater than that of the same size liposomes composed of PC or PC:OA. This is because the head groups of PE hydrates poorly as compared to the headgroup of PC (17). Furthermore, the bulkiness of the unsaturated acyl chains of DOPE prevents a close van der Waal interaction of the hydrocarbon chains.

The combined effect of the head group and acyl chains has been described by a "molecular shape" theory; assigning the shape of an unsaturated PE being that of an inverted cone, i.e. a small head and a bulky tail (17). Amphiphiles of the inverted cone shape favor the packing of the hexagonal H_{II} phase, unless complemented by other amphiphiles of the opposite shape, i.e. molecules which contain a head group with relatively good hydration and saturated acyl chains. Thus, DOPE is better suited to locate in the inner monolayer of a small liposome; because it closely resembles the packing of the hexagonal H_{II} phase, giving rise to a relatively high surface pressure at the inner monolayer. On the other hand, DOPE on the outer monolayer of a small liposome does not pack well at all, giving rise to a relatively low surface pressure. In fact, the distribution of OA is likely asymmetric; more OA should be found in the outer monolayer for the sake of compensation with DOPE than in the inner monolayer. Such a predicted transmembrane gradient in surface pressure could be the driving force for the spontaneous aggregation and fusion of the small DOPE:OA liposomes at 37°C (Fig. III-4), leading to the instability of the liposomes in PBS (Fig. III-1). Removal of OA from the small DOPE:OA liposomes would result in a rapid destabilization of the liposome. Indeed, this was observed when the liposomes were incubated with BSA (Fig. III-5).

Removal of the majority of the OA from the DOPE:OA liposome was also observed when the liposomes were incubated with human plasma, but the liposomes were not destabilized. Instead, stabilization of the liposomes had occurred. We proposed that some plasma factor(s), lipid

and/or protein, must be rapidly inserted into the outer monolayer of the small liposome, thereby increasing the surface pressure of the outer monolayer and stabilizing the entire liposome structure.

Insertion of the plasma factor(s) probably does not, or to a much lesser extent, involve the inner monolayer of the liposome, because the surface pressure of the inner monolayer should be already high. The surface pressure of the outer monolayer of a larger liposome is considerably higher than that of a small liposome (33). Insertion of stabilizing factor(s) from plasma into the large liposomes is less likely. In the event of insertion, it probably inserts all the way to the inner monolayer and leads to liposome leakage, because the surface pressure of the inner monolayer of a larger liposome is about the same as that of the outer monolayer. Furthermore, the model also predicts that small liposomes composed of mainly PC are not stabilized by the same mechanism. Again, the outer monolayer of PC-based liposome exhibits higher surface pressure than the PE-base liposome and is thus less accomodative to the stabilization factor(s) from the plasma. Destabilization of PC-based liposome is also due to the fact that PC is transferred from liposomes to HDL particles (5,36,37).

While the evidence for the action of the stabilization factor(s) is strong, the molecular identity of the factor(s) is not clear. A number of possibilities exist upon examining the composition of the stabilized liposomes. Among the lipids found in the stabilized liposomes, lysoPC is probably the one which has the highest complementarity activity for the bilayer packing of DOPE. This is because

lysoPC contains a phosphorylcholine head group which hydrates well and a single, nonbulky fatty acyl chain which interacts well with other acyl chains, i.e. it is an amphiphile with the shape of that of a cone (17). Mixtures of lysoPC and unsaturated PE form stable bilayer phase (38). However, lysoPC is also a lipid which binds strongly with albumin (39). The fact that the stabilized liposomes stay stable upon subsequent incubation with the fresh plasma (Fig. III-3) indicates that the stabilization component(s) in the stabilized liposome is not removed by the fresh plasma. This is unlikely the case if lysoPC were the stabilization factor. It is possible that the stabilization is brought about by a minor lipid which escaped from our detection method. Gangliosides would be a good candidate. Among the major polypeptide bands shown on the SDS-PAGE (Fig. III-6), none of them is a likely candidate for the stabilization factor. This is because many of them also associate with the DOPC:OA liposomes which are not stabilized. But this can not rule out the possibility that one or more of the smaller blood proteins could serve as the stabilization factor because of the sensitivity of the method. This is particularly interesting because the apolipoproteins from HDL are known to incorporate into liposome membrane (6,7), and all of the HDL's apolipoproteins have Mr smaller than 30kd. Apolipoprotein A₁, in particular, readily inserts into the liposome membrane (6,7,40). Identification of the stabilizer(s) is currently underway in our laboratory.

While the stabilization factor(s) remains unknown, the plasma stabilized small unilamellar liposomes represent a new class of liposomes which show a superb stability both for the long-term storage

and during the incubation with blood. It will be interesting to see if such small liposomes containing a high level of protein stay in the circulation for a relatively long period of time after i.v. injection. It is known that small liposomes have a longer circulation half-life than the larger ones (41). The drug carrier potential of the stabilized liposome also awaits further exploration.

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CHAPTER IV

CHARACTERIZATION OF PLASMA-STABILIZED LIPOSOMES COMPOSED OF DIOLEOYL-
PHOSPHATIDYLETHANOLAMINE AND OLEIC ACIDAbstract

We have previously reported that small unilamellar liposomes ($d \leq 200$ nm) composed of dioleoylphosphatidylethanolamine and oleic acid can be stabilized by incubating with normal human plasma (Liu and Huang, Biochemistry 1989, 28, 7700-7707). The stabilized liposomes were very stable even under relatively harsh conditions such as extreme pH, high salt and trypsin treatment. Fluorescence depolarization of diphenylhexatriene showed that the stabilized liposome had a high microviscosity in the lipid core, which did not decrease even after the majority of proteins were removed by trypsin. These data suggest that plasma proteins inserted into the lipid bilayer are probably responsible for the stabilization activity. After i.v. injection into mouse, stabilized liposomes showed a relatively low affinity to liver and spleen as compared to a conventional liposome composition.

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Introduction

Liposomes composed of unsaturated phosphatidylcholine (PC) are not stable in serum or plasma, unless high concentrations of cholesterol are included in the membrane (for review see 1). Blood proteins such as the high density lipoprotein interact with the liposome membrane and cause a rapid liposome leakage (1). We have recently reported that small ($d \leq 200$ nm), but not large, unilamellar liposomes composed of dioleoylphosphatidylethanolamine and oleic acid show unusual stability characteristics (2). These liposomes are not stable at 37°C in a simple buffered solution, but become stabilized when incubated with normal human plasma or serum. This behavior is just opposite to what is commonly found for the cholesterol-free, PC liposomes. We have also reported that plasma proteins and lipids are found in the stabilized liposomes and that oleic acid is extracted from liposomes by some plasma components (probably albumin). We now report a few additional observations about the plasma-stabilized liposomes which include the stability of the stabilized liposomes under extreme conditions, the microviscosity of the lipid core, and the in vivo biodistribution of the stabilized liposomes.

Materials and Methods

Materials

Dioleoyl PE, egg PC, PS were purchased from Avanti Polar Lipids, Inc. Oleic acid, cholesterol, inulin, calcein and trypsin were obtained from Sigma Chemical Co. Freshly collected human plasma was

obtained from Fort Sanders Regional Medical Center, Knoxville, TN. Tyraminyl inulin was synthesized according to Sommerman et al. (3) followed by standard radioiodination with ^{125}I performed by the chloramine T methods.

Liposome Preparation

Small unilamellar vesicles containing either calcein (50 mM in PBS which was isotonic to plasma) or [^{125}I]tyraminyl inulin were prepared by sonication according to Liu and Huang (2). A trace amount of hexadecyl [^3H]cholestanyl ether was included in the lipid to monitor the lipid.

Treatment of Liposomes Under Different Conditions

Calcein containing liposomes (20 mM) were incubated with an equal volume of human plasma for 1 hour at 37°C followed by gel chromatography using a Bio-Gel A 1.5m column to separate free plasma components from liposomes. To test the stability of liposomes, 200 μl of liposomes containing 0.4 μmole of lipids were mixed with either PBS, KCl (1M in PBS) or trypsin containing buffer in a final volume of 400 μl . The ones mixed with PBS were adjusted to a desired pH by NaOH or HCl and left at room temperature for 1 hour. The fluorescence intensity was measured with a LS-5 fluorescence spectrophotometer. Release of the entrapped calcein from the liposomes was calculated according to Liu and Huang (2).

Liposomes for protein association measurement were prepared in a simple PBS buffer and mixed with a Ficoll solution to a final Ficoll concentration of 20% after incubation with plasma. The mixture was

placed in a 15 ml COREX centrifuge tube. Six milliliters of 15% Ficoll were overlayed, followed by 0.5 ml PBS. The mixture was centrifuged in a Sorvall HB4 swing bucket rotor for 1 hour at 5000 rpm at room temperature. The liposomes floated to the PBS/15% Ficoll interface were collected and treated as described above. The liposomes were floated one more time after treatments. Protein content was determined by Lowry assay.

Membrane Fluidity Measurement

Fluorescence depolarization of DPH in liposomes was measured by a method similar to that described by Shinitzky and Barenholz (4) by including 0.2% (mole/mole) of DPH in lipid composition. The anisotropy was calculated using the following equation:

$$r = \frac{I_{\parallel} - I_{\perp} \times G}{I_{\parallel} + 2 I_{\perp} \times G}$$

where $I_{\parallel}$ is the fluorescence intensity parallel to and $I_{\perp}$ is the intensity perpendicular to the plane of polarization of the excitation beam. G is the correction factor obtained using horizontally polarized light ($G = I_{\perp} / I_{\parallel}$). Fluorescence was measured with excitation at 360 nm and emission at 430 nm.

Biodistribution of Liposomes

One hundred fifty microliters [^{125}I]tyraminyl inulin containing liposome (1 μmole lipid) was administered intravenously by tail vein

injection into Balb/c mice (6-8 weeks old, male). After 5 hours, the mice were anaesthetized with diethyl ether and bled by eye puncture. Blood collected was weighed for each mouse. The mice were then killed by cervical dislocation and all organs were collected. The individual organ was counted for ^{125}I -counts in a gamma counter. The total radioactivity in the blood was determined by assuming the total volume of blood was 7.3% of the total body weight (5).

Results

Effect of Different Treatments on Stability and Protein Association of Plasma Stabilized Liposomes

We have previously shown that small ($d \leq 200$ nm) sonicated liposomes composed of DOPE and OA can be stabilized by incubation with normal plasma or serum (2). These liposomes contain a large amount of serum protein (~ 0.5 mg protein/mg lipid). In order to further test the stability of the plasma-stabilized liposomes, we have incubated them in relatively harsh conditions and examined the loss of protein from liposome and the release of entrapped calcein (Table IV-1). Incubation in alkaline (pH 10) or high salt (0.5M KCl) solutions for 1 hour at room temperature did not cause any loss of protein from liposomes. Only a small fraction ($< 30\%$) of the entrapped calcein was released during incubation. Incubation of liposome in an acidic (pH 3) solution caused about 30% loss of protein, but less than 20% release of calcein. These results indicate that the stabilized liposomes are quite stable even in relatively harsh conditions. This is to be compared with the

Table IV-1. Stability of plasma-stabilized liposomes.

Treatment ^a	Protein Content ^b (%)	Calcein Release ^c (%)
pH 7.4	100 (n=9)	0
pH 3.0	73.8 $\pm$ 2.8 (n=9)	17.9 $\pm$ 1.3
pH 10.0	99.9 $\pm$ 6.7 (n=12)	21.0 $\pm$ 5.9
KCl	98.3 $\pm$ 11.2 (n=9)	27.3 $\pm$ 3.5
Trypsin	33.0 $\pm$ 6.0 (n=12)	7.9 $\pm$ 3.5

^aPlasma-stabilized liposomes (2.5 μ mole in 0.5 ml PBS) were incubated with either equal volume of PBS with a final pH of 7.4, 3.0, 10.0 or with PBS containing 1 M KCl at room temperature for 1 hour or with PBS containing 1.0 mg/ml trypsin for 1 hour at 37°C.

^bNormalized to the protein content of the plasma stabilized liposomes treated with the pH 7.4 buffer which was approximately 0.5 mg protein/mg lipid.

^cThe pH was adjusted back to 7.4 and calcein release was measured fluorometrically immediately after the treatment (n=4).

original DOPE:OA liposomes which show a complete release of entrapped calcein at pH below 5 (2).

Membrane Fluidity of Liposomes

It seems that the stabilization of liposomes by plasma treatment is related to the association of proteins with liposomes. There are at least two possibilities that can apply for the protein association. The first is that proteins are attached to the surface of liposomes. The other is that part of protein or all proteins insert into the bilayer of liposomes. In the later case, the microviscosity of the membrane is expected to increase significantly. We have examined this possibility by measuring the fluorescence depolarization of a hydrophobic probe, DPH, which is widely used to measure the microviscosity of a lipid membrane (for review, see 9). As can be seen in Table IV-2, a large increase of fluorescence depolarization of DPH was observed in the plasma-stabilized liposomes. The microviscosity of the lipid core, calculated according to Shinitzky (9), had increased from 0.7 to 1.1 poise by the plasma treatment. The removal of most of the liposome-associated proteins by trypsin did not reduce the membrane microviscosity. This result indicates that the liposome stabilization is probably due to the insertion of plasma proteins into the lipid bilayer.

Biodistribution of Liposomes in vivo

The biological property of the stabilized liposomes, i.e. biodistribution in vivo, was tested in mouse as an animal model. Liposomes were treated with mouse serum which gave a stabilization

Table IV-2. Comparison of membrane rigidity of DOPE/OA liposomes upon the interaction with plasma.

Liposome Treatment	Fluorescence Anisotropy ^c	Microviscosity ^f (poise)
PBS	0.084 ± 0.002	0.727 ± 0.020
Plasma ^a	0.111 ± 0.003 ^d	1.066 ± 0.043 ^d
Plasma then trypsin ^b	0.113 ± 0.004 ^e	1.086 ± 0.049 ^e

^aLiposomes containing 0.2 mol % DPH were incubated with PBS or 50% plasma for 1 hr at 37°C, followed by chromatography through a Bio-Gel A1.5m column.

^bPlasma-treated liposomes were further incubated with trypsin (0.5 mg/ml final) for 1 hr at 37°C. Trypsin inhibitor (2.5 mg/ml final) was added to stop the reaction. Liposomes were gel-filtered through a Bio-Gel A1.5m column to remove free proteins.

^cMeasured in 5 determinations.

^d_p < 0.0002 when compared with values for PBS treatment.

^e_p < 0.0001 when compared with values for PBS treatment.

^fCalculated according to Shinitzky and Barenholz (9).

activity identical to that of human plasma. The stabilized liposomes were injected into the tail veins of Balb/c mice and the biodistribution of an entrapped aqueous marker, [^{125}I] tyraminyl inulin. This radioactive marker is rapidly ($t_{1/2}$ = few minutes) excreted from the mouse (3). As can be seen in Table IV-3, DOPE:OA liposomes mainly accumulated in liver (about 31% of injected dose). Blood was the second highest compartment for liposome distribution with approximately 8 percent. The rest had spread in various other organs and tissues. DOPE:OA liposomes stabilized with mouse serum and then injected into mouse showed a similar biodistribution with a higher level of liver uptake. Serum-stabilized liposomes which were trypsin-treated before the injection also showed a similar distribution.

Discussion

Two important features of liposome stabilization by plasma or serum are that 1). only small ($d \leq 200$ nm) liposomes are stabilized, and 2). only PE-, but not PC-, based liposomes are stabilized (2). We have interpreted these observations by the facts that 1). The DOPE molecule has a small and poorly hydratable head group (10) and relatively bulky acyl chains, 2). amphipathic molecules like DOPE are poorly packed in a concave surface such as the outer monolayer of a small liposome of high curvature (11), 3). lipids and proteins having a richly hydrated head group and relatively non-bulky hydrophobic portion can be easily inserted into the outer monolayer of the small liposome enriched with DOPE, bringing about liposome stabilization. Thus, we suspect that the

Table IV-3. Comparison of in vivo distribution of DOPE/OA liposomes treated under different conditions in vitro.^a

Tissue	DOPE:OA (2:1)			PC:chol:PS (10:5:1)
	Untreated (n=5)	Serum Treated (n=5)	Serum Treated and Trypsinized (n=5)	Untreated (n=3)
Blood	8.0±0.9	8.3±1.1	7.1±0.4	2.7±0.7
Spleen	2.9±0.4	3.9±0.4	2.7±0.3	3.6±0.7
Liver	31.4±0.7	43.9±1.2	40.9±1.4	24.8±3.2
Lung	0.4±0.1	0.3±0.1	0.3±0.0	0.1±0.0
Chest	1.8±0.3	2.2±0.3	1.9±0.2	0.5±0.2
Intestine	2.1±0.5	2.2±0.2	2.2±0.3	0.9±0.1
Heart	0.2±0.0	0.2±0.1	0.1±0.0	0.3±0.5
Trunk	1.5±0.5	2.2±0.3	2.6±0.4	1.1±0.4
Head	2.8±0.7	2.5±0.3	2.1±0.2	1.7±0.2
Upper Limb	1.2±0.3	1.2±0.3	0.8±0.2	0.9±0.5
Lower Limb	1.2±0.3	1.3±0.1	0.8±0.2	0.8±0.4
Kidney	1.7±0.3	1.0±0.1	0.9±0.1	0.5±0.0
Skin	2.3±0.3	2.1±0.6	1.7±0.4	6.6±2.5
Tail	1.8±0.6	2.5±1.0	6.2±3.2	0.9±0.1
% Recovered	60.2±2.9	74.8±2.6	70.2±2.8	45.4±4.2
Res/Blood ^b	4.3±0.6	5.8±0.7	6.2±0.5	11.0±3.7 ^c

^aData expressed as % injected dose. Liposomes were labeled with [¹²⁵I]tyraminyl inulin.

^bRES/blood = % doses in liver and spleen/% dose in blood.

^cSignificantly different from the other three, $p < 0.01$ (F test).

apolipoproteins in the plasma or serum are prime candidates of the liposome stabilizer, because they contain amphipathic helices which interact strongly with phospholipids to form discoid structures (8,12). Insertion of the amphipathic helix to the outer monolayer of the small liposomes would promote tighter molecular packing and hence more stable bilayer. These helices would be resistant to trypsin digestion because they are partly "buried" in the membrane. They would also increase the microviscosity of the lipid core of the liposome membrane due to lipid-protein interactions. The insertion into lipid bilayer of several proteins, such as cytochrome b5 (6), mellitin (7), and apolipoproteins (8) has been demonstrated with PC based liposomes.

The uptake of liposomes in liver and spleen is generally regarded as uptake by the reticuloendothelial system (RES) (for recent review see 1). The RES/blood ratio is a sensitive index for the relative affinity of liposome to the RES, because lower level of RES uptake is usually accompanied with a higher level in the blood (13). The RES/blood ratios for the three DOPE:OA liposome preparations are not significantly different from each other. This result is not surprising because the DOPE:OA liposomes would be coated with the same proteins, once injected into the blood of the animal, as the ones stabilized with serum in vitro. SDS-PAGE of the serum-stabilized liposomes treated with trypsin and exposed to serum again, showed nearly identical protein bands on the gel (data not shown). If some of the associated proteins serve as opsonins for RES uptake, as has been suggested by other investigators (13,14), similar degrees of RES uptake of these three liposome preparations are expected. On the contrary, the

biodistribution of liposome of a commonly used lipid composition, i.e. egg PC:cholesterol:PS (10:5:1), showed a significantly higher affinity for RES with a RES/blood ratio significantly greater than those of the other three. These results indicate that the serum-stabilized liposomes originally composed of DOPE:OA show a relatively low affinity to RES and hence stay in blood for a relatively long period of time. Recently, Allen et al (15) and Gabizon and Papahadjopoulos (13) have independently developed liposomes which would stay in circulation for a relatively long period of time. One of the important ingredients of liposome composition is glycolipids such as ganglioside GM₁. The present liposome system does not contain any glycolipids. Nevertheless, the liposomes already show the signs of prolonged circulation time. This is an important result because liposomes with prolonged circulation half-life generally show a relatively high level of tumor accumulation (13). Thus, the drug delivery potential of the DOPE:OA liposomes looks favorable and will be further evaluated in future experiments.

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CHAPTER V

INTERACTIONS OF SERUM PROTEINS WITH SMALL UNILAMELLAR, pH-SENSITIVE LIPOSOMES COMPOSED OF DIOLEOYLPHOSPHATIDYLETHANOLAMINE AND OLEIC ACID: HDL, APOLIPOPROTEIN A1 AND AMPHIPATHIC PEPTIDES STABILIZE LIPOSOMES

Abstract

Small unilamellar liposomes composed of dioleoylphosphatidyl-ethanolamine (DOPE) and oleic acid (OA) are stabilized by incubation with normal human serum or plasma (Liu and Huang, Biochemistry (1989) 28, 7700-7707). The present report describes a systematic study of interactions of purified serum proteins and lipoproteins with these liposomes. Albumin destabilized liposomes by extracting OA from the liposomes; whereas immunoglobulins and lipoproteins (HDL, LDL and VLDL) had no effect. However, HDL and, to some extent, VLDL showed a rapid stabilization activity against the lytic effect of albumin. HDL added together with or shortly after the addition of albumin completely abolished the liposome leakage and aggregation effects induced by albumin. SDS-PAGE analysis of the HDL-stabilized liposomes revealed that apolipoprotein A1 was associated with liposomes. Purified apolipoprotein A1, but not a lipid mixture resembling the lipid composition of HDL, showed comparable liposome stabilization activity

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as HDL. Furthermore, synthetic peptides resembling the amphipathic helices found in apolipoprotein A1 also showed strong liposome stabilization activity. Peptides which were able to form amphipathic helices of a wedge shape were more effective stabilizers than those which could not. These data indicate that HDL plays a major role in human serum or plasma for the liposome stabilization activity. HDL exerts its activity probably by the interactions of the amphipathic helices of apolipoprotein A1 with the hydrophobic voids found on the outer surface of the highly curved, small liposomes.

Introduction

One of the crucial aspects of using liposomes as a drug delivery vehicle is to understand their interactions with plasma or serum components. Previous work has revealed that a number of serum proteins become associated with liposomes (for reviews, see 1,2). For example, albumin, immunoglobulins, fibronectin and other serum proteins are found to coat the surfaces of multilamellar liposomes composed of PC and cholesterol, and additionally containing phosphatidylserine or stearylamine (1). Interaction of HDL with multilamellar liposomes composed of PC causes the lysis of liposomes and transfer of liposomal PC to HDL (3,4,5). Insertion of apolipoprotein(s) into membrane of small unilamellar liposomes at or near the chain-melting temperature causes a rapid leakage of the entrapped contents (6,7). Activated complement components also induce liposome leakage by forming pores in the liposome membrane (8). Some as yet unidentified serum protein(s)

is likely to be opsonin(s), which promotes the uptake of liposomes by macrophages, primarily those in liver and spleen (9).

It is well known that liposomes composed of unsaturated PC are not stable in serum or plasma, unless cholesterol is included in the lipid composition (10). We have previously reported that small unilamellar liposomes (average diameter ≤ 200 nm) composed of DOPE and OA, unlike the same size liposomes composed of DOPC and OA, are not lysed by the serum. Instead, they are stabilized by some serum components (11). The stabilized liposomes contain a number of serum proteins and lipids. Most of the proteins associated with the stabilized liposomes are probably not responsible for stabilization, because liposomes treated with trypsin which removed about 70% of the associated proteins are still stable. The stabilized liposomes show a significantly higher degree of membrane microviscosity, probably due to the insertion of serum protein into the liposome membrane (12). The serum-stabilized liposomes stay in the circulation for a relatively long period as compared to other PC-based liposomes of similar size (12). Thus, the serum-stabilized, PE-containing liposomes are potentially useful for drug delivery.

In order to further elucidate the interactions of this novel liposome with serum proteins, we have initiated a series of experiments using purified serum proteins, lipoproteins, ApoA1, and peptide analogs of ApoA1. Stability of liposomes is studied by measuring the release of an entrapped fluorescent dye, calcein, and by light scattering and electron microscopy. The results of these studies have

been useful to identify the serum components which interact with and stabilize the PE-containing liposomes.

Materials and Methods

Materials

DOPE, egg PC, phosphatidylserine, phosphatidylinositol, phosphatidic acid and sphingomyelin were purchased from Avanti Polar Lipids, Inc. Calcein, BSA, human γ -globulins, cholesterol, cholesteryl ester, glycerides, Ficoll and oleic acid were obtained from Sigma Chemical Co. [^{14}C]oleic acid (57 mCi/mmol) was purchased from New England Nuclear.

Isolation of Lipoproteins and Apolipoproteins

HDL, LDL and VLDL were isolated from freshly collected normolipemic plasma in a potassium bromide density gradient using the ultracentrifugation procedure according to Chung et al. (13). The individual lipoproteins were washed and reisolated employing similar gradient ultra-centrifugation methods with different density gradients. The appropriate fractions from each ultracentrifugation were pooled. ApoA1 was isolated from Apo-HDL by gel filtration and ion-exchange chromatography (14). The purified lipoproteins and apolipoproteins were then dialyzed against PBS (phosphate buffered saline, 178 mM NaCl, 3.5 mM KCl, 2.0 mM KH_2PO_4 , 1.3 mM Na_2HPO_4 and 1.3 mM EGTA). The protein concentration was determined by the Lowry assay method (15).

Synthesis of Peptides

The peptides were synthesized by the solid-phase peptide synthesis procedure as previously described by Anantharamaiah et al. (16). Release of peptides from the solid support was achieved by the modified HF procedure (17). Crude peptides were purified by reversed-phase HPLC. Their purity was confirmed by sequencing and amino acid analysis. The sequence and characteristics of the peptides have been described (16).

Preparation of Liposomes

Lipids containing DOPE and OA with a molar ratio of 2 to 1 were dried using a N₂ stream and vacuum desiccated for a minimum of 30 min., and hydrated for 3-5 hours at room temperature in a calcein (50 mM) containing buffer. The osmolarity of this buffer adjusted with 10X PBS was equal to that of dialyzing buffer in preparation of lipoprotein. A trace amount of hexadecyl [³H]cholestanyl ether was included in order to monitor the lipid. The lipid suspension was sonicated with a bath sonicator for 10 min. at room temperature (Laboratory Supplies, Hicksville, NY) and the pH was adjusted and maintained at pH 8.0 during sonication. The liposome suspension was left for 2 hours at room temperature to facilitate the annealing process before free calcein was separated from liposomes by using a Bio-Gel A0.5M column equilibrated with PBS. The size of liposome used in the experiments was 90-120 nm as determined by quasi elastic light scattering using a Coulter N4SD instrument.

Interaction of Serum Proteins with Liposomes

Liposomes (10 μ l containing 5-15 μ g of lipid) were added to a cuvette containing 1.99 ml of PBS for the fluorescence measurement with constant stirring. The serum protein was normally added at 2 min. The fluorescence intensity was measured with excitation wavelength of 490 nm and emission wavelength of 520 nm. The total fluorescence intensity was obtained by adding 50 μ l deoxycholate (5%) to lyse the liposomes. Release of the entrapped calcein from the liposomes was calculated with the following equation:

$$\% \text{ Release} = \frac{\text{Fx}-\text{Fo}}{\text{Ft}-\text{Fo}}$$

where Fo is the fluorescence intensity of the liposomes at 1 min i.e. before the addition of serum protein. Ft is the total fluorescence intensity of liposomes after addition of deoxycholate. Fx is the fluorescence intensity at different time or the fluorescence intensity before the addition of deoxycholate in the presence of stabilizer. For the stabilization of liposomes by apolipoproteins and lipoproteins against the lysis of BSA, the apoproteins were added to cuvette 1 min. after addition of liposomes and before the addition of BSA.

Removal of OA from liposomes by BSA

Liposomes containing trace amounts of [^{14}C]OA were incubated with different amounts of BSA in the presence or absence of HDL at 37°C for 1 hour before passing through a Bio-Gel A1.5M column. The fractions were counted for ^{14}C and ^3H . The percent removal of OA from liposomes was calculated by dividing the counts of ^{14}C from nonliposomal

fractions with the total ^{14}C counts. Liposomes were eluted in the void volume fractions as monitored by both ^3H counts and calcein entrapment.

Electron Microscopy of Liposomes with Different Treatments

Liposomes treated with either PBS or BSA (in the presence or absence of HDL) for 1 hour at 37°C were passed through a BioGel A1.5M column to remove excess proteins. Liposomes were examined with negative-stain electron microscopy using 1% uranium acetate as a stain (18).

Association of Serum Proteins with Liposomes

Liposomes containing 2.5 mg of lipids were incubated with HDL or Igs with a ratio of 0.2 (protein to lipid ratio) for 1 hour at 37°C . The mixture was then mixed with a Ficoll solution (in PBS, pH 8.0) to obtain a final Ficoll concentration of 20% (w/w) and placed into a 15 ml COREX centrifuge tube. Six milliliters of 15% (w/w) Ficoll were overlayed, followed by 0.5 ml PBS. The mixture was centrifuged in a Sorvall HB4 swinging bucket rotor for 1 hour at 5000 rpm at room temperature. The liposomes floated to the PBS and 15% Ficoll interface were collected. The liposomes were floated one more time with an identical procedure before SDS-PAGE analysis. SDS-PAGE was done using 15% acrylamide gel under the reducing conditions (19).

Results

Effect of Serum Proteins on Liposome Stability

In order to study the interaction between liposomes and serum proteins, we have examined the effects of BSA, HDL, LDL, VLDL and Igs, which were selected according to their abundance in plasma and known functions on liposomes (2). As can be seen in Fig. V-1, none of these proteins except BSA caused any significant release of calcein at concentrations tested. On the other hand, BSA induced a complete calcein release from the liposomes. Thus, the destabilization activity of liposome is rather unique to albumin, probably related to its strong affinity for oleic acid.

Stabilization of Liposomes Against the Lytic Effect of Albumin

Although most of the proteins investigated above exerted no lytic activity on liposomes, some of them showed a striking effect of stabilization against the lysis induced by BSA. Fig. V-2 shows an example with HDL. When BSA was added at 2 min., it caused a rapid and complete release of calcein ($t_{1/2} \sim 40$ sec). If BSA and HDL were both added at 2 min, no rapid liposome lysis was observed. Even if HDL was added 1 min after the addition of BSA, a partial protection of liposome against the BSA-induced lysis was seen. Addition of more BSA (at 6 min) to the protected liposomes no longer produced any calcein release. This experiment clearly indicated the liposome stabilization effect of HDL against the lysis by BSA.

Destabilization of liposomes induced by BSA was accompanied by a rapid increase in turbidity as measured by 90° light scattering (data

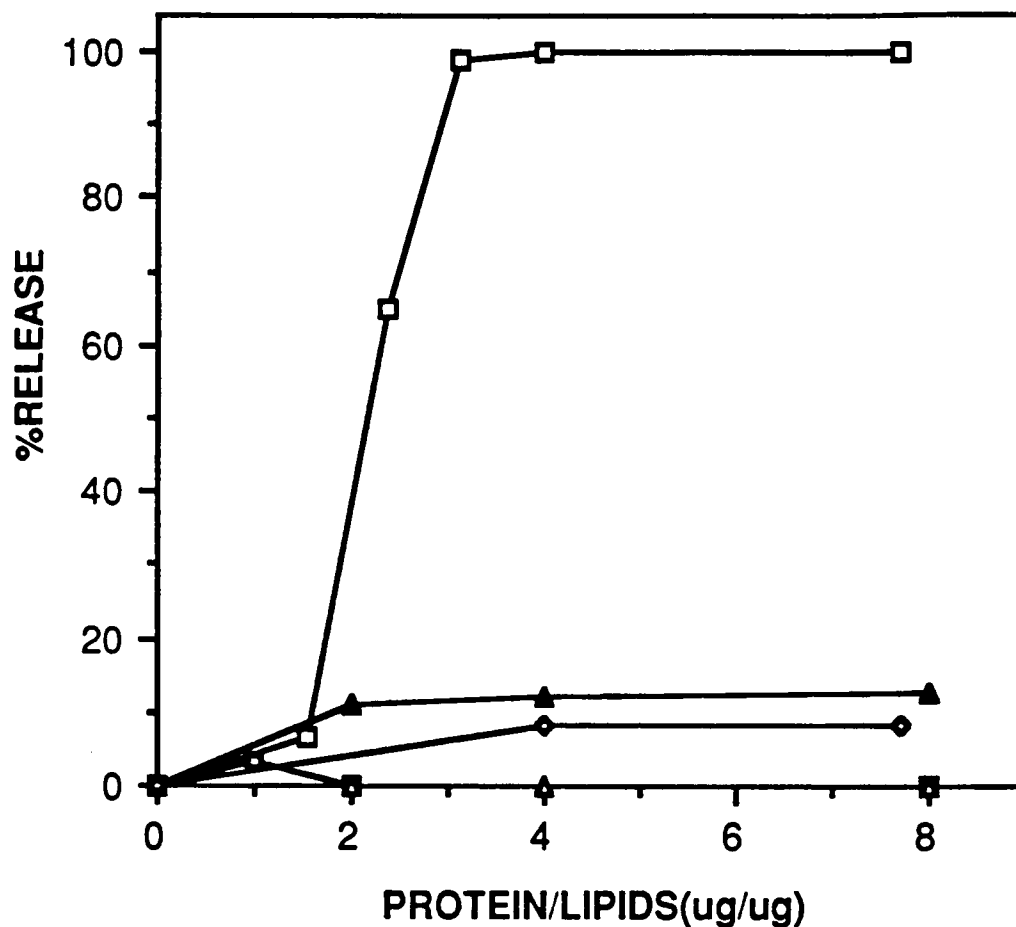


Figure V-1. Effect of serum proteins on liposome stability. Ten microliters of liposomes containing 15 μ g of lipid was added at time zero to 1.99 ml of PBS in a cuvette with constant stirring. Proteins were added at 1 min. Percent release was calculated according to equation one (see Materials and Methods). The fluorescence intensity at 9 min was used as Fx to calculate the release. ($\square$) BSA; ($\blacksquare$) HDL; ($\blacktriangle$) LDL; ($\triangle$) VLDL; ($\diamond$) Igs.

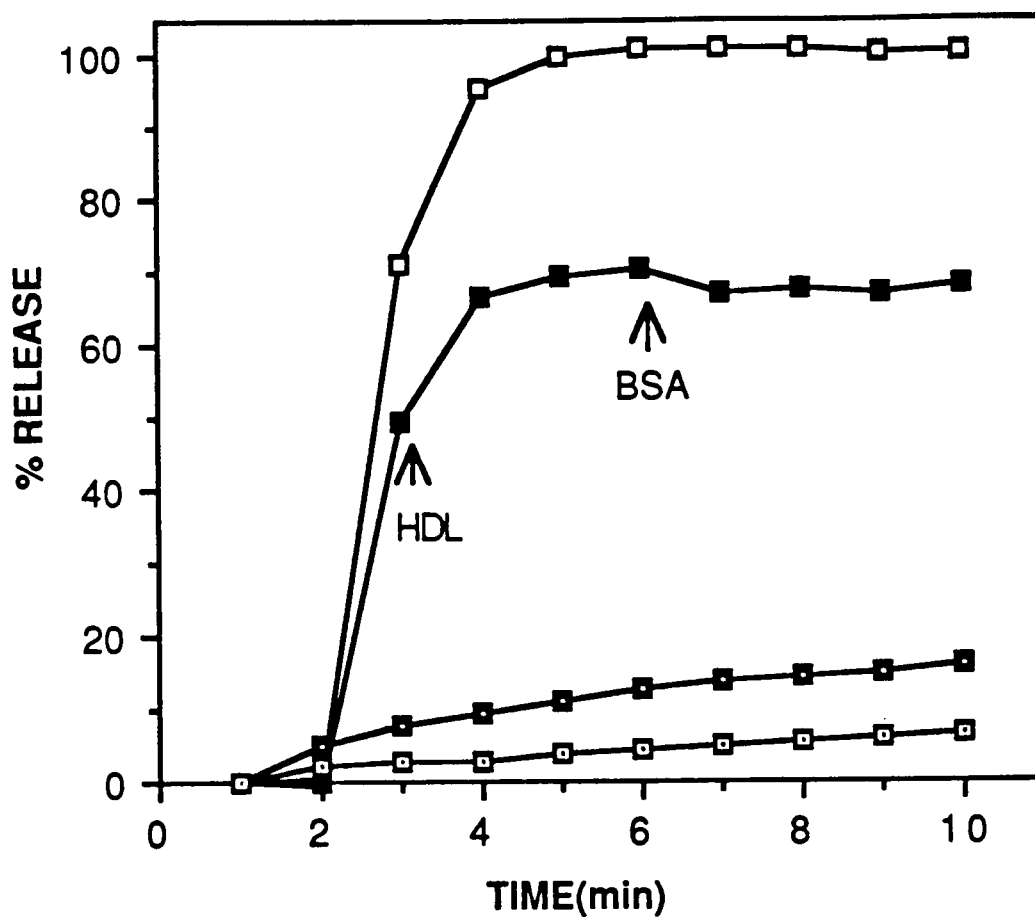


Figure V-2. Effect of HDL on BSA induced lysis of liposome. Ten microliters of liposome containing 15 μg of lipids was added to 1.99 ml of PBS in a cuvette with constant stirring. Proteins were added at 2 min. (□) BSA only (50 $\mu\text{g}/\text{ml}$); (○) HDL only (1.5 $\mu\text{g}/\text{ml}$); (■) BSA (50 $\mu\text{g}/\text{ml}$) plus HDL (1.5 $\mu\text{g}/\text{ml}$); (■) BSA (50 $\mu\text{g}/\text{ml}$) followed by HDL (1.5 $\mu\text{g}/\text{ml}$) and then more BSA were added as indicated by arrows (final concentration of BSA was 2 mg/ml).

not shown). The rapid increase in light scattering, which was BSA concentration dependent, was followed by a slower decrease. This is due to the formation of very large lipid aggregates which gradually settled out of the solution. Addition of both BSA and HDL to liposomes had completely blocked the liposome aggregation (data not shown).

The protective effect of HDL against the BSA induced lysis of liposomes can also be seen by negative-stain electron microscopy (Fig. V-3). Liposomes prepared by sonication were unilamellar and non-aggregative with an average diameter of 120 nm (Fig. V-3A). Addition of BSA had caused the formation of large lipid aggregates such as the one shown in Fig. V-3C. These aggregates were greater than 1 μ m in size and often showed a fine structure of parallel cylinders (Fig. V-3C) which could be related to the hexagonal (H_{II}) phase of DOPE. Treatment with both BSA and HDL showed that liposomes remained unilamellar and small in size (average $d = 120$ nm) (Fig. V-3B). Numerous HDL particles can also be seen in Fig. V-3B. The stabilization activity was not limited to HDL; VLDL had a similar, but somewhat weaker activity (Fig. V-4). LDL only showed a partial stabilization activity at very high concentrations. Igs was without any activity in the concentration range tested.

Removal of OA by BSA from Liposomes in the Presence or Absence of HDL

Albumin is the most abundant protein in serum. It also has a strong affinity to bind amphipathic molecules such as free fatty acids. Removal of OA from liposomes was investigated by including [14 C]OA in

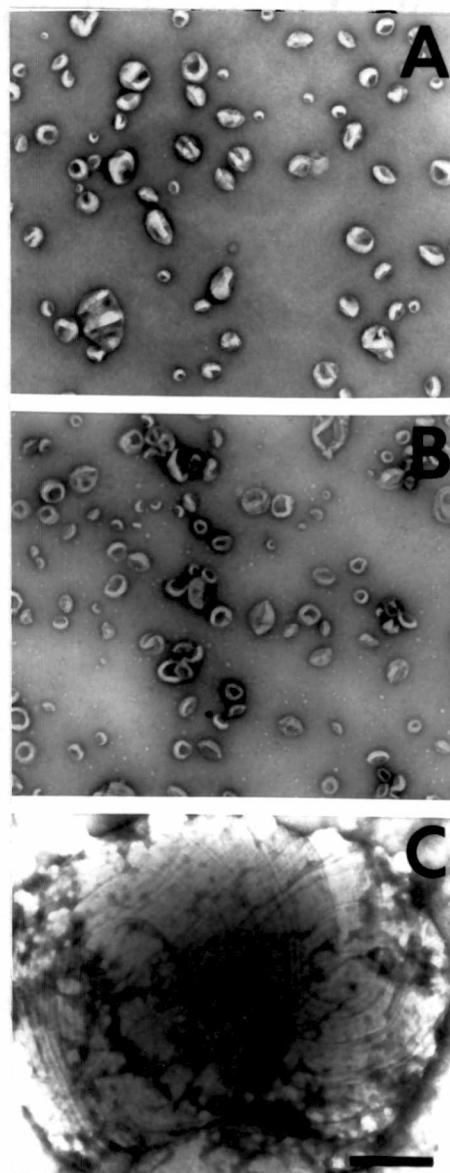


Figure V-3. Electron micrographs of HDL stabilized liposomes. Samples were negatively stained with 1% uranyl acetate. Bar is 0.5 μm .

- A. Liposomes treated with PBS.
- B. Liposomes treated with BSA in the presence of HDL. BSA/Lipid = 22.5, HDL/Lipid = 0.34.
- C. Liposomes treated with BSA (BSA/Lipid = 22.5) in the absence of HDL. All ratios are weight ratios.

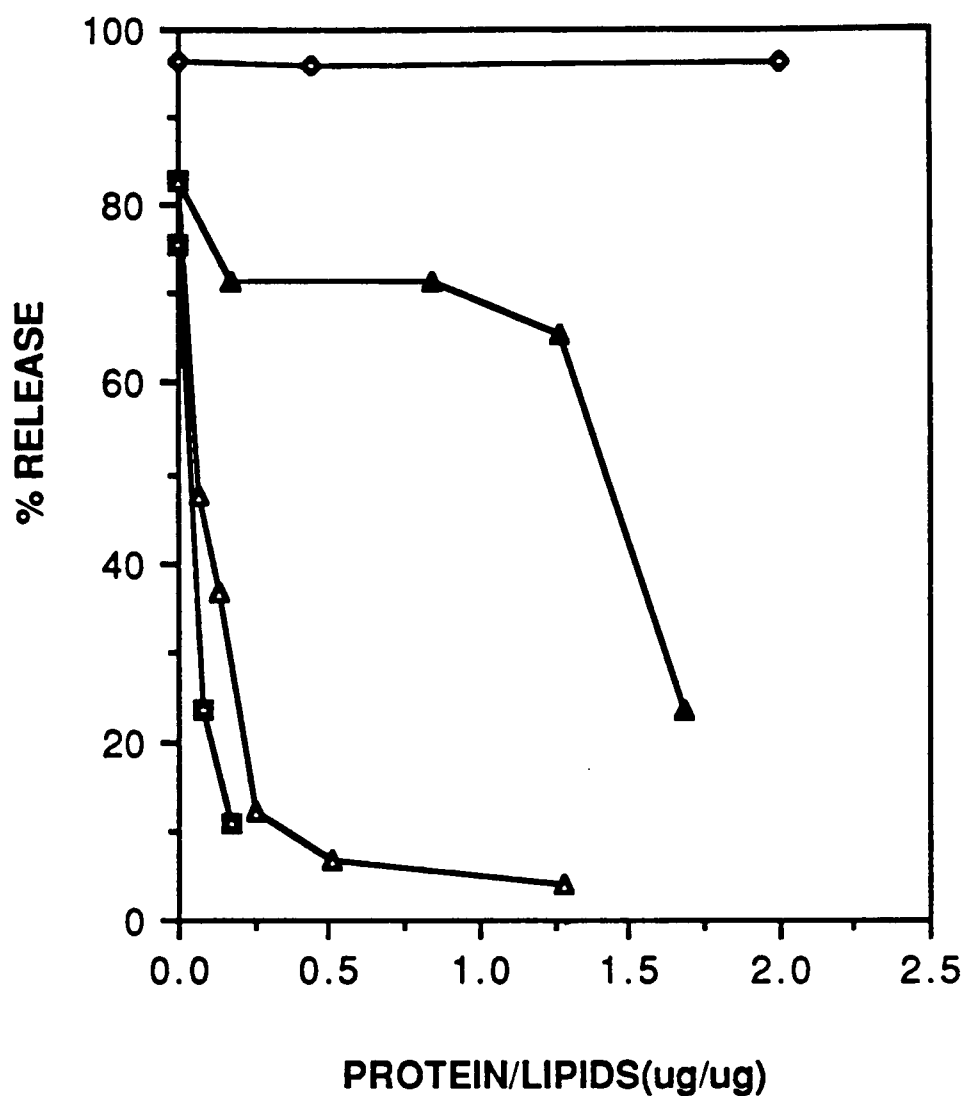


Figure V-4. Blockade of BSA induced release by plasma proteins. The BSA concentration used in this experiment was 0.2 mg/ml which gave a ratio of BSA to lipids of 26 ($\mu\text{g}/\mu\text{g}$). The maximum release was plotted. (■) HDL; (▲) LDL; (△) VLDL; (◇) Igs.

the liposomes. Using gel filtration to separate [^{14}C]OA associated with liposomes and BSA, it is shown in Fig. V-5 that at 5 μg BSA/ μg lipid, approximately 57% OA was removed from liposomes which had caused the complete release of all entrapped calcein, i.e. 0% fluorescence quenching. Higher amounts of BSA removed more OA from liposomes. Thus, the interaction of albumin with small unilamellar liposomes composed of DOPE and OA is to extract OA from liposomes, leading to a rapid destabilization of the liposomes.

One possibility for the stabilization activity of HDL is to prevent the removal of OA from liposomes by BSA. It is clear from the data shown in Fig. V-6 that HDL did not prevent the removal of OA from liposomes by BSA. Approximately 90% of liposomal OA was still transferred to BSA in the presence of HDL which had completely protected the liposomes against the lytic activity of BSA. The liposomes, with only about 10% of the original OA remaining, stayed stable showing 80% calcein fluorescence quenching.

Association of Serum Proteins with Liposomes

The strong liposome stabilization activity of HDL could be due to the interaction of apolipoproteins with the liposome membrane. To examine this possibility, we have isolated liposomes after incubation with HDL using a Ficoll gradient flotation protocol. The protein composition of the stabilized liposomes was examined by SDS-PAGE using 15% acrylamide under reducing conditions. Fig. V-7 shows the electrophoretic patterns of liposomes. It is clear from lane 5 of the figure that ApoA1 was associated with the HDL-stabilized liposomes. It

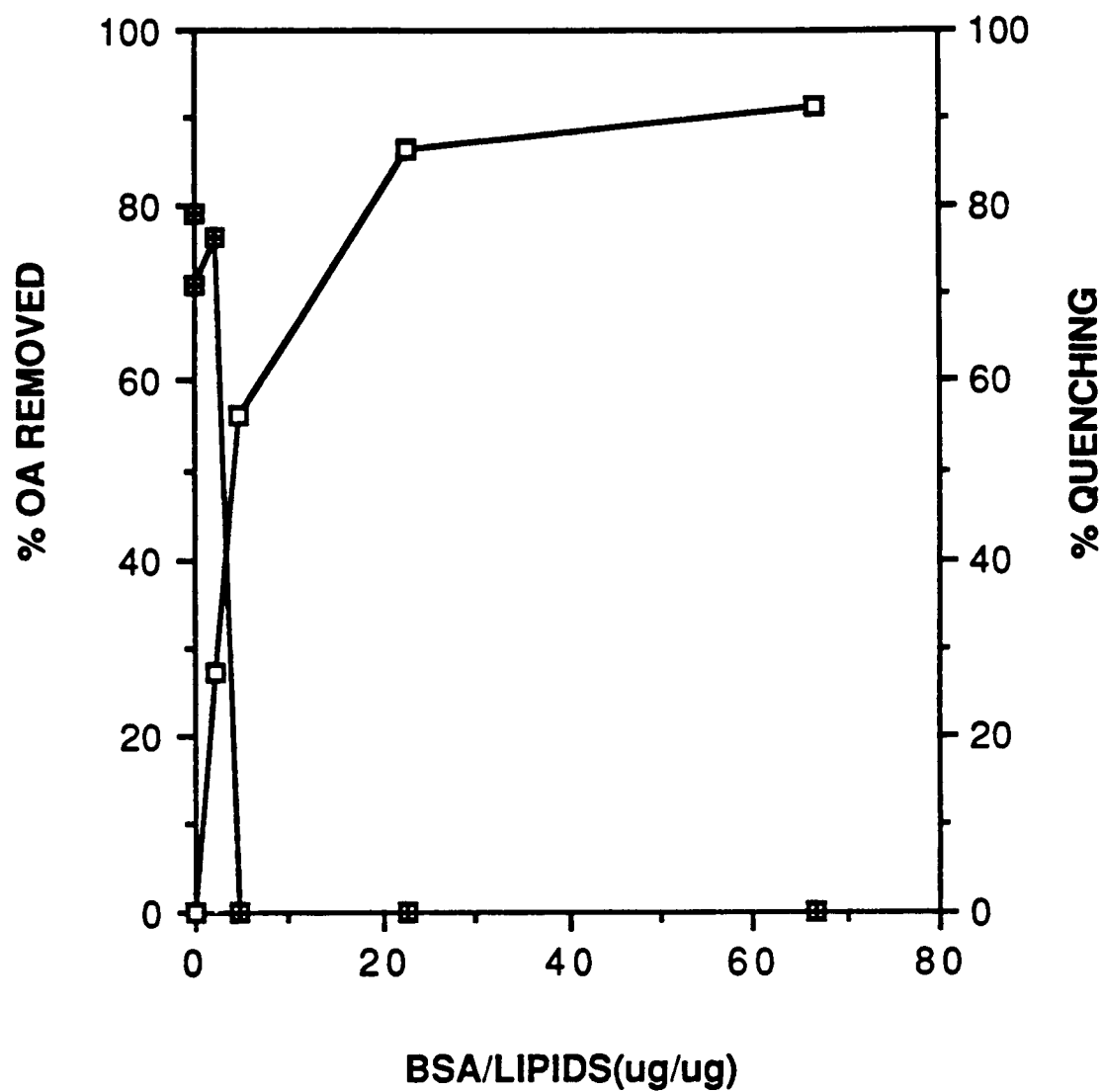


Figure V-5. Removal of OA from liposomes by BSA. (□) % OA removed; (■) % fluorescence quenching.

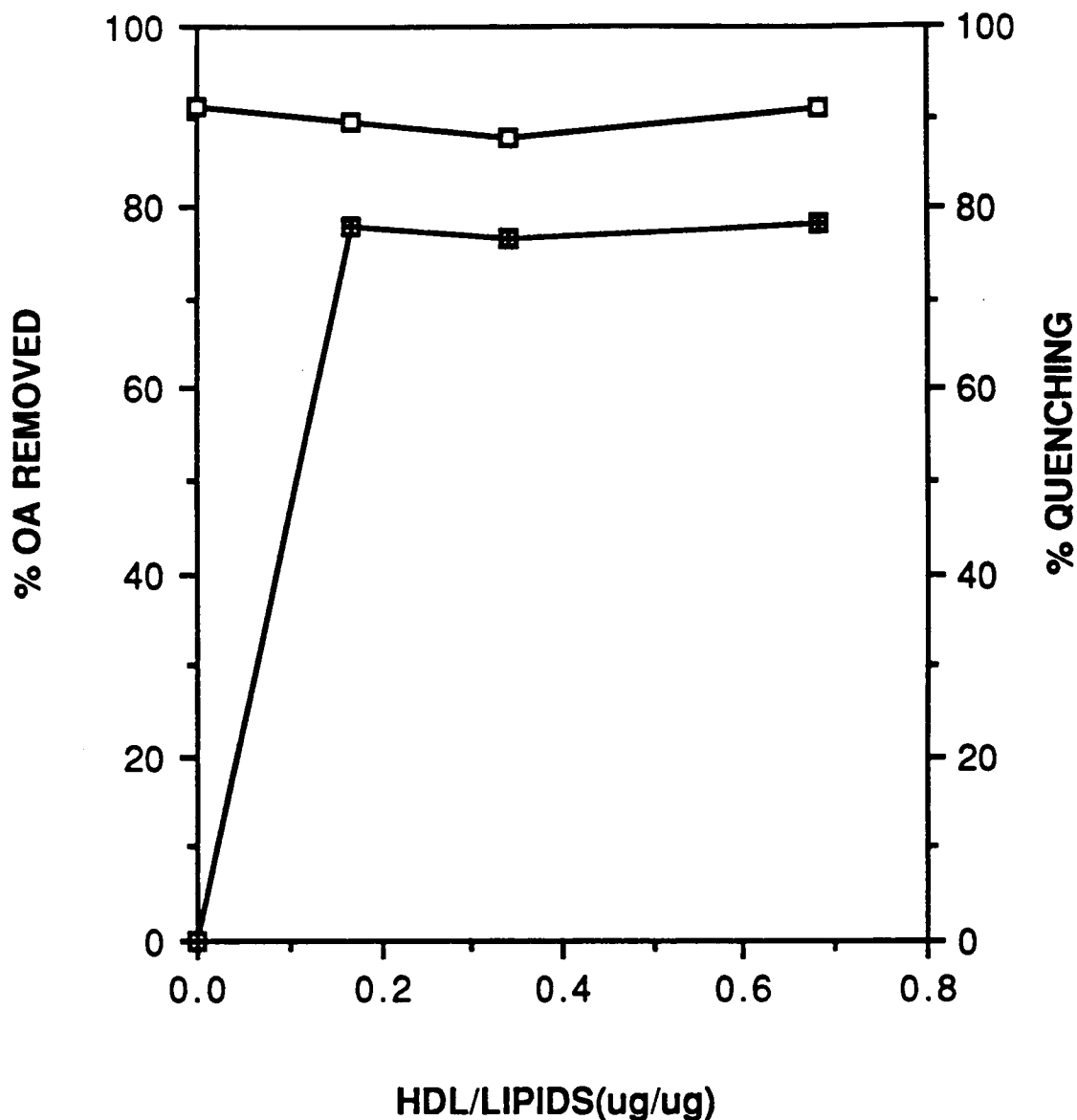


Figure V-6. Removal of OA from liposomes by BSA in the presence of HDL. Liposomes containing trace amounts of [^{14}C]OA were incubated with BSA in the presence of different amount of HDL for 1 hour at 37°C and a BioGel A1.5M column was used to separate BSA extracted OA from liposome associated OA. (□) % OA removed from liposome; (■) % quenching of fluorescence.

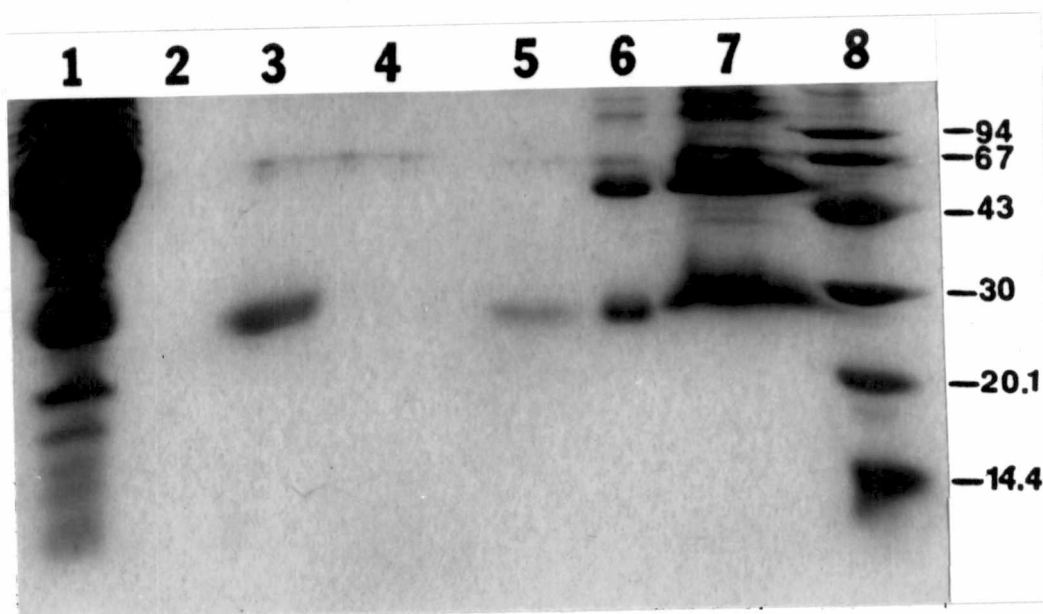


Figure V-7. SDS-PAGE analysis of HDL stabilized liposomes. Lane 1. Whole human plasma, 2. HDL alone floated, 3. HDL, 4. Liposomes floated, 5. Liposomes treated with HDL and then floated, 6. Immunoglobulins, 7. Liposomes treated with immunoglobulins and then floated, 8. Mr standards.

should be noted that ApoA1 is the major apolipoprotein of HDL. Under the electrophoretic conditions used in the experiment, ApoA1 was the only apolipoprotein seen on the gel; no other smaller molecular weight apolipoproteins such as ApoA2 were seen (lane 3). It is also clear that HDL itself did not float in the Ficoll gradient, hence no protein bands could be detected in the floated HDL sample (lane 2). Since the light scattering measurements (data not shown) suggest that Igs may be weakly interacting with liposomes, we have also examined the liposomes reisolated by the floating protocol after incubation with Ig's (lane 7). It is also evident that Ig's were associated with the liposomes. Free Ig's did not float in the gradient (data not shown), hence the bands shown in lane 7 were due to liposome association.

Stabilization Effect of Apo A1 Against the Lytic Activity of BSA

The results of the experiment shown in Fig. V-7 strongly suggest that ApoA1 is responsible for the liposome stabilization activity of HDL. We have thus tested the stabilization of liposomes with purified ApoA1. To rule out the possibility of lipid involvement in the stabilization process, we have also used a mixture of purified lipids resembling the lipid composition of HDL (20). The mixture contained the following lipids (all weight %): cholesterol oleate (32.4%), cholesterol (6.8%), glycerides (13.4%), egg PC (35.2%), lysoPC (1.4%), sphingomyelin (6.3%), phosphatidylserine (0.04%), phosphatidylinositol (1.1%), DOPE (1.5%) and phosphatidic acid (1.0%). ApoA1 added together with BSA blocked the liposome lysis induced by BSA alone (Fig. V-8). Even added 0.5 min after the addition of BSA, ApoA1 still

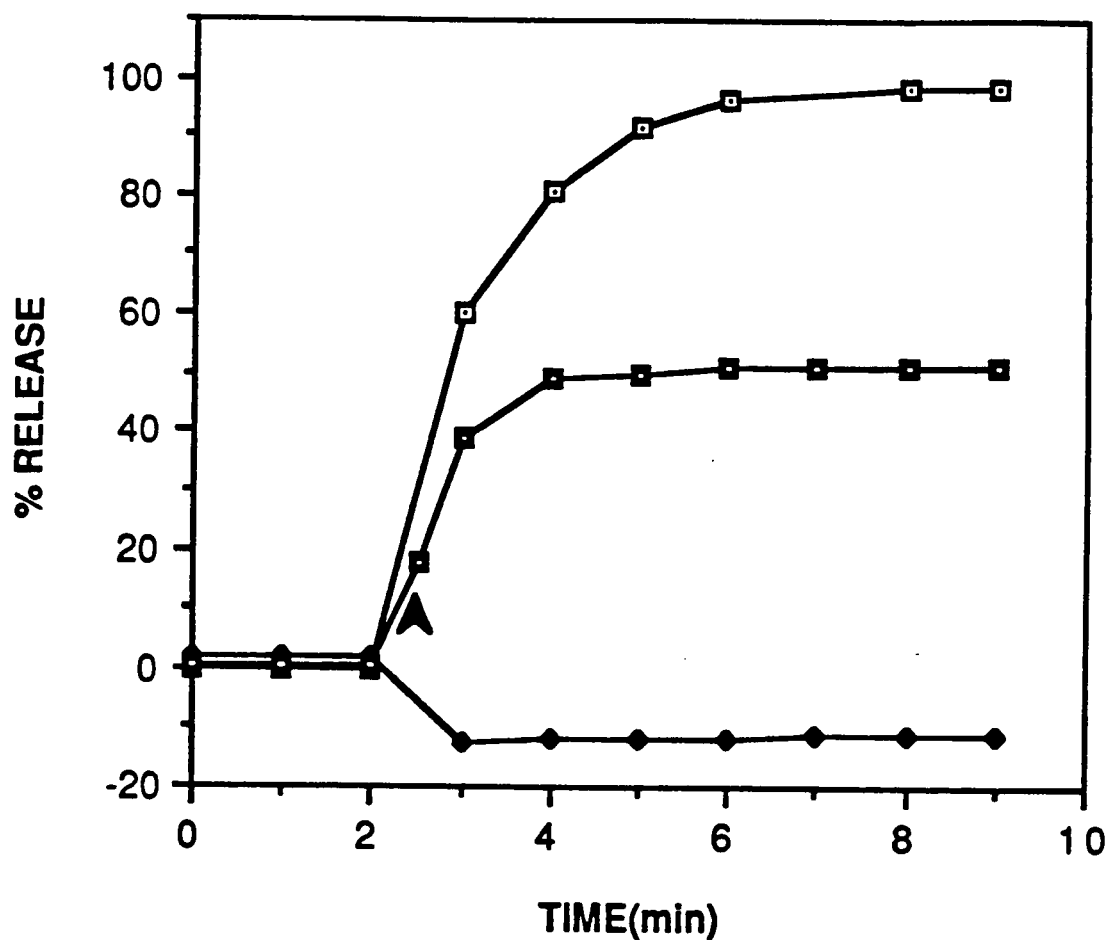


Figure V-8. Blockade of BSA-induced lysis of liposomes by Apo A1. The conditions used in this experiment were the same as described in figure V-2. (□) BSA only (0.2 mg/ml); (◆) ApoA1 plus BSA (ApoA1/Lipid = 0.2 μ g/ μ g); (■) ApoA1 was added 0.5 min after addition of BSA (arrow head).

partially protected the liposomes against the lysis of BSA. The action of ApoA1 completely resembled that of HDL (see Fig. V-2). The stabilization activity of ApoA1 was as strong as that of HDL, giving a complete stabilization at protein/lipid ratios about 0.15 or higher (Fig. V-9). A lipid mixture resembling the lipid composition of HDL had no stabilization effect at the concentration range tested. It also did not interfere with the activity of ApoA1 when it was premixed with the apolipoprotein before adding to the liposomes. These results strongly indicate that ApoA1, not the lipids, is the molecular component in HDL which gives rise to liposome stabilization activity.

Stabilization Effect of Amphipathic Peptides Against the Lytic Activity of BSA

Numerous studies have suggested that the amphipathic helical regions of ApoA1 are responsible for the interaction with the lipids in the HDL particles (21,22). A few peptide analogs of the amphipathic helix of ApoA1 have been tested to see if these amphipathic peptides could stabilize the liposomes against the lytic activity of BSA. As shown in Fig. V-10, peptide 37PA had a strong stabilization activity which was indistinguishable from that of ApoA1 on the basis of mass. However, on the molar basis, peptide 37PA was much more potent in liposome stabilization than ApoA1, since its molecular weight is much smaller. Peptides 37AA and 18A were less potent, requiring about 5-fold higher concentrations for a similar stabilization effect as that of peptide 37PA. Peptide 18R was ineffective over the concentration range tested.

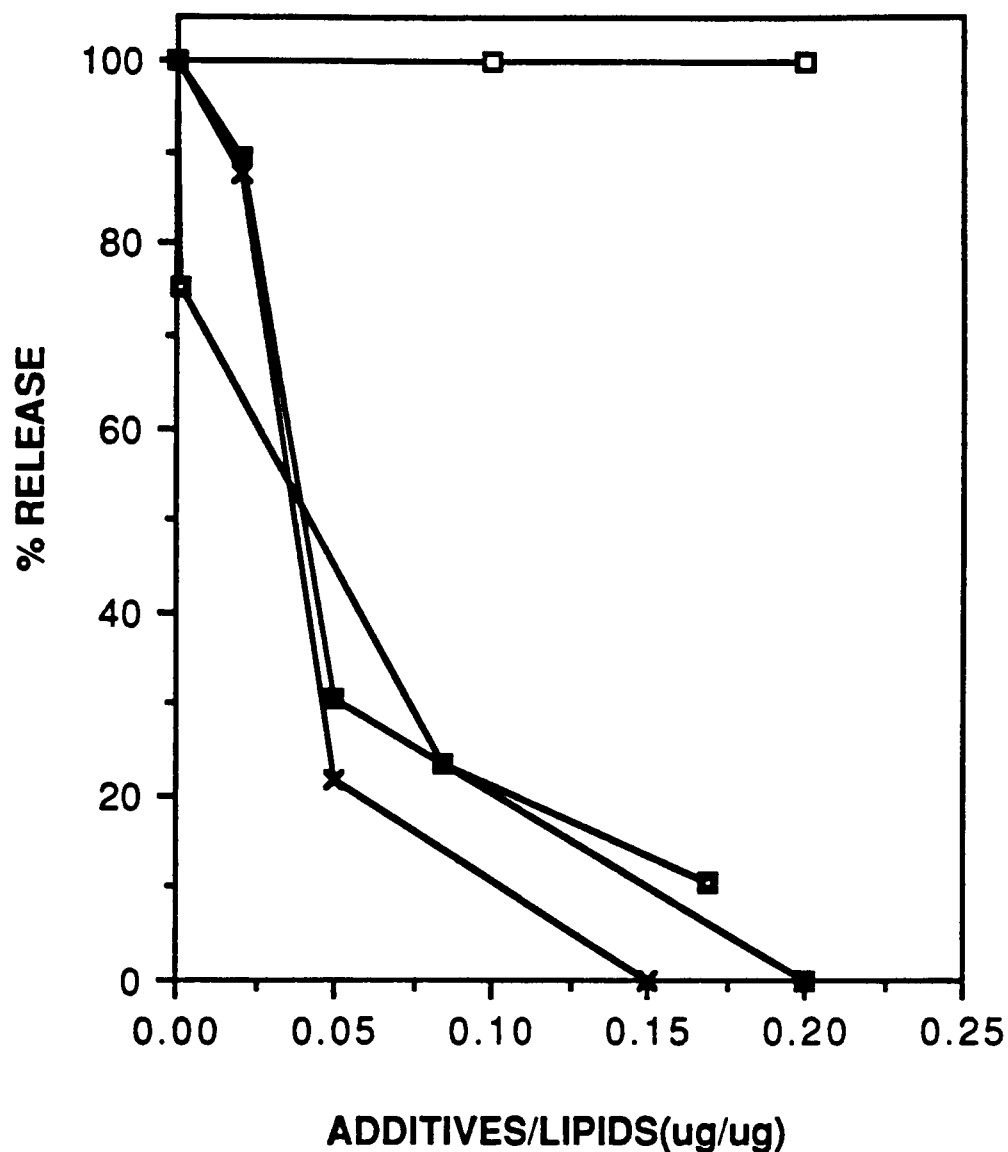


Figure V-9. Comparison of stabilization effect of liposome by HDL or HDL components against BSA induced lysis. The assay was carried out in the same way as described in figure V-4 except that the lipid components of HDL were mixed, the organic solvent was evaporated in a N_2 gas and hydrated for a few hours before an intensive sonication (30 min). The lipid mixture was incubated with ApoA1 for 3 hours at room temperature before the stabilization assay. (■) HDL only; (X) ApoA1; (□) lipid components of HDL (see text for composition); (⊠) mixture of ApoA1 and lipid components of HDL.

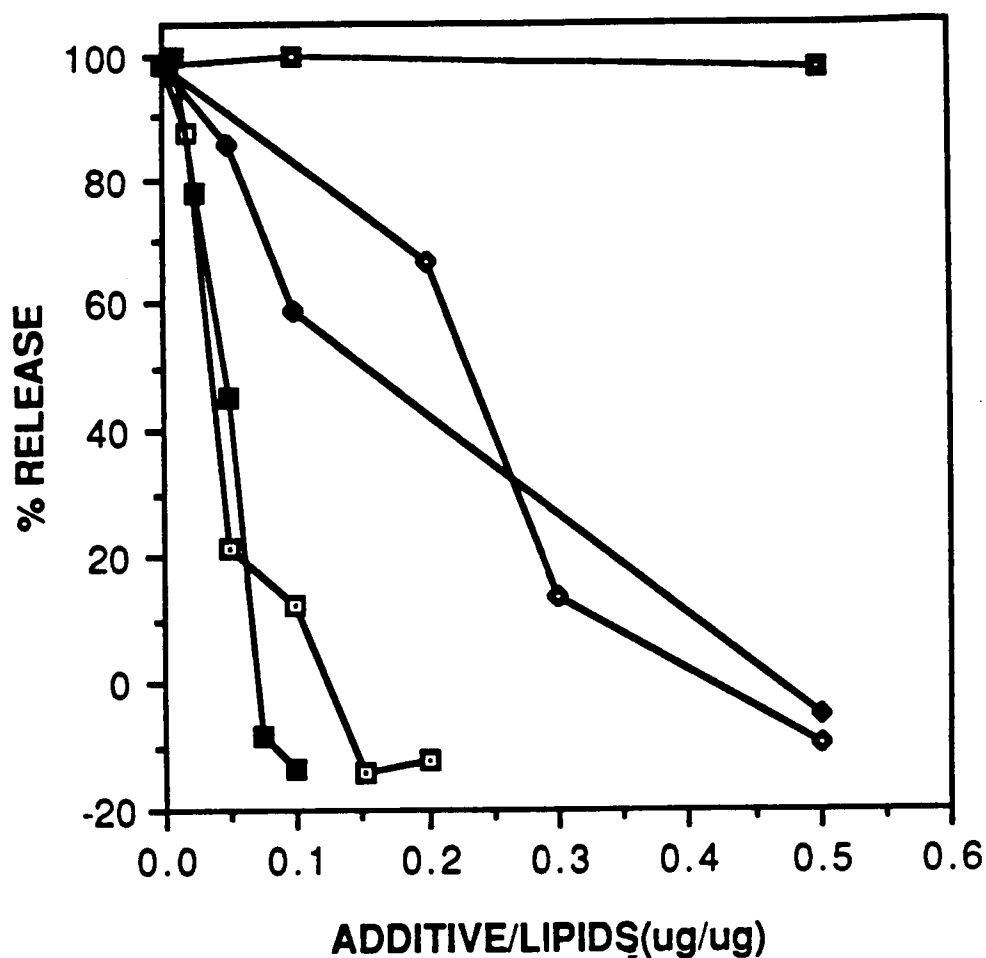
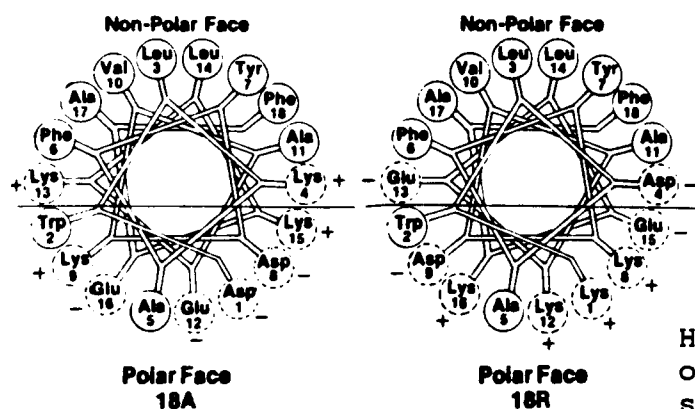


Figure V-10. Blockade of BSA induced release by Apo A1 and peptides. The conditions were the same as described in Fig. V-4. (□) ApoA1; (◆) 18A; (■) 18R; (◇) 37AA; (●) 37PA.



Helical wheel representations of the amphipathic helical structure of peptides.

Discussion

It is known that albumin lyses the pH-sensitive liposomes containing DOPE and OA by extracting OA from the liposomes (23). This lytic activity is at least partially responsible for the instability of large unilamellar liposomes in serum or plasma (24,25). We have shown that inclusion of cholesterol in the large liposomes composed of DOPE and OA improves the stability of liposomes. The enhanced stability is not due to the absence of OA removal from liposomes, because the same rates of OA transfer are observed from liposomes with and without cholesterol (25). The effect of serum or plasma on the small unilamellar liposomes ($d \leq 200$ nm) of the same composition is quite different from that of the larger liposomes. The small liposomes, with or without cholesterol, are stabilized by some serum component(s) such that the liposomes are stable even after most of OA is extracted by albumin (11). The molecular component(s) of the serum stabilizing factor was not identified in the previous work. The present studies utilizing purified serum proteins have helped to elucidate the identity of the factors.

It is not surprising that BSA showed a rapid destabilization activity to the small liposomes composed of DOPE:OA (Fig. V-1, V-4). It caused release of entrapped calcein (Figs. V-1, V-2 and V-5), extraction of OA (Fig. V-5), and formation of H_{II} phase-like aggregates (Fig. V-3). These activities are best explained by the removal of OA from liposomes, which leaves DOPE by itself, and the transition of DOPE from a bilayer phase to H_{II} phase. The temperature

of $L\alpha \rightarrow H_{II}$ phase transition of pure DOPE is approximately 10°C at pH 7 and physiological ionic strength (26). Thus, the equilibrium phase of DOPE at room temperature is the reverse-micellar, H_{II} phase. However, the presence of OA has stabilized the bilayer phase by providing a net negative surface charge (27) such that liposomes can be stabilized. Liposomes composed of DOPE and OA are acid-sensitive in that at mildly acidic pH rapid bilayer destabilization and vesicle fusion take place (28). This is the basis for using such liposomes for efficient cytoplasmic delivery following the endocytosis of liposomes by the target cells (27,29). Removal of OA would cause the liposomes to lose their negative surface charge and promote interliposome juxtaposition upon collision, which drives a rapid conversion into the H_{II} phase.

The emphasis of the present work is not on the liposome destabilization by BSA, but rather to use the BSA-induced liposome lysis as a method to study the stabilization effect of serum lipoproteins. We have used experimental conditions mimicking the condition of serum incubation. The key aspect is to look for the stabilization event which proceeds at a rate much faster than that of the albumin-induced liposome lysis. Our data strongly indicate that HDL shows such stabilization activity (Figs. V-2,V-3,V-4). Liposomes treated with HDL and BSA did not release calcein (Fig. V-2,V-4) and stayed non-aggregated (Fig. V-3). The effect of HDL was concentration dependent (Fig. V-4) and proceeded at a very fast rate (Fig. V-2). Even added to liposomes 1 min after the addition of BSA, substantial amounts of liposomes were already stabilized (Fig. V-2). The stabilization activity of HDL is potent; at as low protein/lipid ratios as

0.1-0.3, which is roughly equivalent to 0.3 to 1.0 $\mu\text{g/ml}$ in concentration, HDL had completely stabilized the liposomes. Note that the concentration of HDL in normal human serum is 3.6 (2.90-7.70) mg/ml (30) which is 3600-12000 fold higher than the minimal concentration required for liposome stabilization. Thus, it is highly likely that HDL is a major serum component which stabilizes the small unilamellar liposomes composed of DOPE and OA, a peculiar phenomenon previously observed by us (11).

VLDL showed a similar but somewhat weaker stabilization activity (Fig. V-4). However, the concentration of VLDL in normal human serum (0.1 mg/ml) is lower than that of HDL. Its contribution to the serum (or plasma) stabilization activity for liposomes is probably not as important as that of HDL. Igs also showed a weak stabilization activity when assayed by the enhancement of light scattering induced by BSA (data not shown).

The data shown in Fig. V-7 clearly indicate that ApoA1 of HDL had transferred to liposomes during the incubation. Data in Figs. V-8 and V-9 also indicate that ApoA1, not the lipid components, of HDL is responsible for the liposome stabilization activity. The activity of ApoA1 was as potent as that of HDL, effective at a protein/lipid ratio of about 0.15 or higher. Thus, ApoA1 is likely a major molecular species in the normal human serum or plasma which has brought about the liposome stabilization activity. Since ApoA1 is not found in VLDL (20) which has a similar stabilization activity as HDL (Fig. V-4), other apolipoproteins may also be involved in the stabilization reaction. This possibility will be investigated further.

Apolipoproteins contain amphipathic helices which interact strongly with phospholipids to form discoidal structures which resemble the structure of native lipoprotein particles. The peptide analogs of the amphipathic helix in ApoA1 have been studied in detail (16, 31). They contain positively charged lysine and arginine residues at the interface between the hydrophilic and hydrophobic surfaces and negatively charged residues at the center of the hydrophilic face (16). Due to the relatively bulky and hydrophobic side chains of lysine and arginine, the overall shape of the helix cylinder when lying on the surface of a phospholipid membrane looks like a wedge, i.e. the volume of the hydrophobic surface is bulky near the interfacial region and the volume is relatively small near the center of the surface. It has been proposed that such a wedge-shaped amphipathic helix is essential for maximal interaction with phospholipids (16,31,32).

As shown in Fig. V-11, DOPE contains a relatively small and weakly hydratable head group (26) and two cis-unsaturated, bulky acyl chains. The overall shape of the molecule is that of an inverted cone, i.e. a shape opposite to that of a wedge. This is the primary reason that DOPE has a high tendency to aggregate into the reverse-micellar, H_{II} phase. In small unilamellar liposomes of high curvature, DOPE is well suited to locate in the inner monolayer, because it closely resembles the packing of the H_{II} phase. The lipids in the outer monolayer, on the other hand, are likely not packed well, resulting in the exposures of hydrophobic surfaces to the aqueous environment. This energetically unfavorable situation is the major driving force for the aggregation

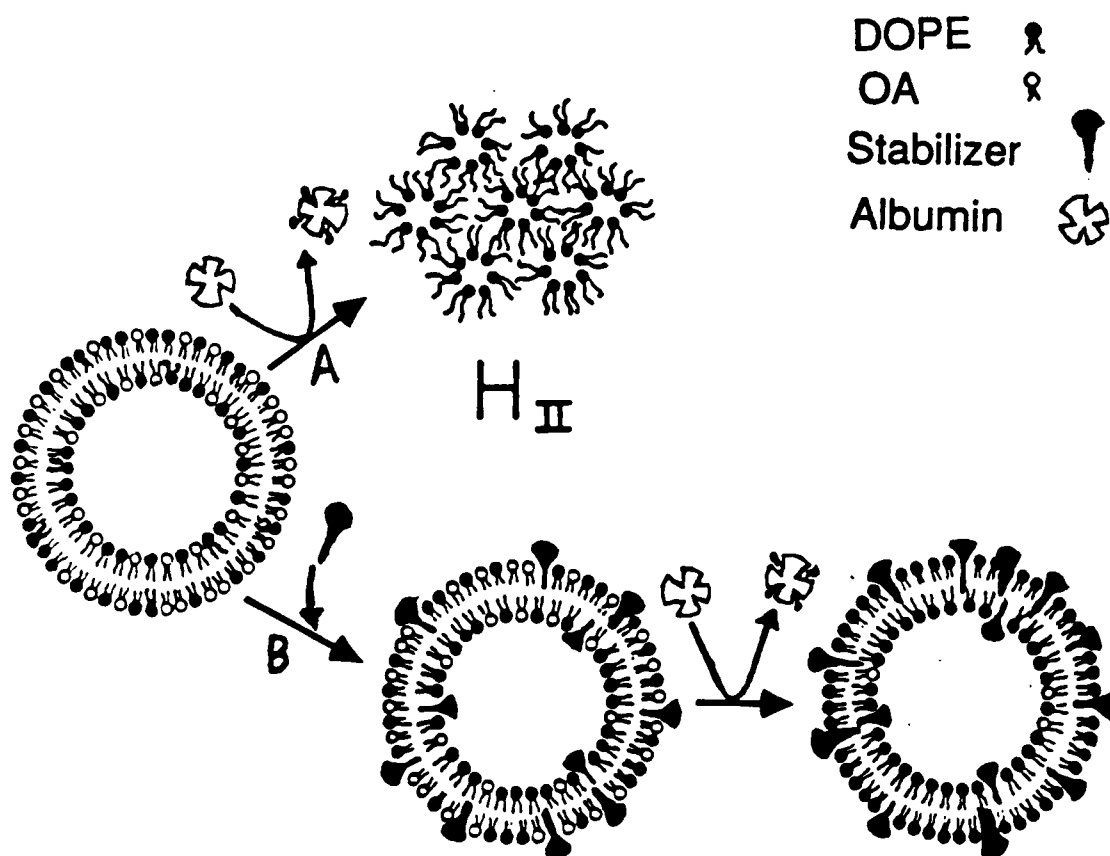


Figure V-11. Schematic presentation of hypothetical mechanisms of liposome stabilization:

- A. Liposomes are disrupted by removal of OA and DOPE form H_{II} phase.
- B. Liposomes are stabilized by inserting part of the stabilizers into bilayer. Bilayer is kept even OA is removed.

and fusion of the small unilamellar liposomes incubated in a simple buffer at 37°C (11). On the other hand, a wedge-shaped amphipathic helix such as the ones found in ApoA1 can readily insert into the hydrophobic voids in the outer monolayer of the small unilamellar liposomes and reduce the free energy state of the system. The resulting proteoliposomes are stable even when OA is removed by BSA.

Data in Fig. V-10 support this model. Of the amphipathic peptides tested for liposome stabilization, 37PA showed the strongest activity. This peptide contains two amphipathic helices of 18 amino acids each, joined together with a proline residue to allow bending of two helices (16). It resembles the amphipathic helices found in ApoA1 (31) and its stabilization activity is stronger than that of ApoA1 on a molar basis (Fig. V-10). Peptide 37AA is an analog of 37PA (33). It contains two amphipathic helices but is connected by an alanine residue which does not allow bending of the two helices. Activity of 37AA is considerably lower than that of 37PA (Fig. V-10), indicating that motional flexibility of the two joining helices is important for maximal interaction with the highly curved surface of a liposome. The activity of peptide 18A, a single amphipathic helix, is comparable to that of 37AA, but less than that of 37PA, suggesting some cooperativity between the two joining helices in the interaction with liposome membrane. Peptide 18R is a single amphipathic helix with the negatively charged residues located near the hydrophobic-hydrophilic interface and positively-charged residues at the center of the hydrophilic surface, a situation opposite to that of peptide 18A. The molecular shape of helix 18A is that of a wedge, but the shape of helix

18R is not. Data shown in Fig. V-10 clearly indicate that peptide 18R has no liposome stabilization activity in the concentration range tested. Thus, the wedge shape of an amphipathic helix, which is shared by peptides 18A, 37AA and 37PA, is important for the interaction with DOPE liposomes. This is consistent with the model described above.

This model also predicts that other amphipathic helices of wedge shape could also mediate the stabilization of liposomes. This could be the reason why the stabilization activity is also found in VLDL. The apolipoproteins in VLDL undoubtedly contain amphipathic helices which could be suitable for interaction with small unilamellar liposomes described here.

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CHAPTER VI

EFFECT OF DIFFERENT LIPID COMPONENTS ON THE BIODISTRIBUTION OF
SONICATED LIPOSOMES COMPOSED OF PRIMARILY UNSATURATED
PHOSPHATIDYLETHANOLAMINE

Abstract

"Stealth" liposomes are those liposomes which show reduced affinity to the reticuloendothelial system (RES) and thus stay in the circulation for relatively long periods of time. We have used a basic lipid composition of dioleoylphosphatidylethanolamine (DOPE)/oleic acid (OA)/X (10:5:1 molar ratio) and examined the effect of adding a third lipid component X on the biodistribution of sonicated, small unilamellar liposomes ($d \sim 100$ nm) after intravenous injection into Balb/c mice. The results indicate that gangliosides GM₁ and GT_{1b}, high melting-temperature lipids distearoyl phosphatidylcholine (DSPC) and sphingomyelin, provide excellent stealth property to liposomes. The RES/blood ratios of these liposomes at 5 hours after injection were significantly lower than that of DOPE/OA (10:5, molar ratio). On the other hand, sulfatide and phosphatidylserine significantly increased the RES/blood ratio of the liposomes. The stealth activities of GT_{1b} were concentration dependent. The activity of DSPC did not seem to relate to its relatively long chain length; shorter chain disaturated

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phosphatidylcholines had a similar activity. The stealth activity of the liposomes did not correlate with the rigidity of the lipid membrane which was measured by fluorescence depolarization of a lipid probe, diphenylhexatriene (DPH). However, liposomes composed of rigid bilayers showed generally higher stealth activity than those with less rigid bilayers. Since sulfatide is known to be a stealth lipid and GT_{1b} has opposite activity for liposomes composed of primarily phosphatidylcholine (Gabizon and Papahadjopoulos, Proc. Natl. Acad. Sci. USA 85 (1988) 6949-6953), the inconsistency of our results with those of others suggests the complexity of the mechanism underlying the liposome interactions with RES in vivo.

Introduction

Liposomes, or phospholipid vesicles, have been extensively studied for their potential use as drug carriers (for recent reviews, see 1). Systematically administered liposomes are rapidly taken up by the reticuloendothelial system (RES), primarily by the resident macrophages in liver and spleen. Although such a property is an advantage for delivering drugs to the macrophages, it is a problem for those delivery efforts with targets other than the RES. Strong affinity to RES is particularly problematic for liposomes designed to bind with the target cells via attached targeting ligands such as antibody (2,3,4). Several investigators have shown that immunoliposomes are just as rapidly taken up by the RES as the nontargeted liposomes, such that only a small

fraction of the liposomes binds to the target cells (for review, see 4).

Recently, efforts have been made to reformulate the liposome composition for a reduced affinity to RES. Such liposomes have been called "stealth liposomes,"¹ because they are not readily taken up by the macrophages in the RES and hence stay in the circulation for relatively long periods of time ($t_{1/2}$ in circulation up to 10-12 hours) (6,33). The stealth liposomes are generally small in size with an average diameter of 100 nm or smaller (5,7,33). They contain high levels of cholesterol (5-7,33) and are usually composed primarily of phospholipids with high melting temperatures, e.g. distearoyl-phosphatidylcholine and sphingomyelin (6,7,33). Most importantly, the stealth liposomes contain glycolipids such as ganglioside GM₁ (5,6,7,33) and hydrogenated phosphatidylinositol (HPI) (6). The carbohydrate residues on the liposome surface are supposed to reduce the coating of liposomes by serum opsonins (6). Although the molecular nature of opsonins has not been identified, they are probably responsible for the rapid uptake of liposomes by macrophages (6,7). The importance of the stealth liposomes has been demonstrated by the observations that their uptake by solid tumor is considerably higher than that of the ordinary liposomes. Presumably, liposomes with longer circulation half-lives stand a better chance to penetrate the leaky vasculature of the solid tumor (6).

¹"Stealth liposomes" is a registered trademark of Liposome Technology, Inc.

Enhanced tumor accumulation of liposomes, however, does not necessarily ensure an enhanced cellular delivery of the entrapped drug. This is because most of the surface bound liposomes are endocytosed by the tumor cells and the entrapped drug is delivered to the cellular degradative compartment, i.e. lysosomes, for destruction (8). It has been shown, for example, that cytosine arabinoside encapsulated in liposomes is less cytotoxic to tumor cells than the free drug (9). To overcome this problem, pH-sensitive liposomes composed of primarily unsaturated PE have been developed. These liposomes undergo a bilayer-to-H_{II} phase transition at an acidic pH such as the pH in the cellular endosomes and lysosomes (10-13). Destabilization of liposomes at the acidic pH activates the fusion activity of the liposomes and significantly enhances the cytoplasmic delivery efficiency of the entrapped drugs (14-16). Antitumor drugs (14), toxins (15), and DNA (16) have been efficiently delivered to tumor cells using this type of liposomes.

Thus, it is important to examine if stealth liposomes can be constructed with lipid compositions consisting of primarily unsaturated PE. The previously developed stealth lipid compositions contain primarily PC. Since PC does not undergo the bilayer-to-H_{II} phase transition under physiological conditions, these stealth liposomes are unlikely to be effective for the cytoplasmic delivery of drugs to the tumor cells. In this report, we describe an effort to search for the stealth lipid compositions which consist of primarily unsaturated PE. We have compared the circulation time of the PE-containing liposomes

with those of the stealth liposomes composed primarily of PC. Since some reported stealth lipid compositions contain lipids of high melting temperatures, the membranes of the stealth liposomes are likely to be more rigid. The fluidity of the lipid membrane of the stealth liposomes described here has also been examined by measuring the fluorescence depolarization of a hydrophobic fluorescent probe, diphenylhexatriene.

Materials and Methods

Materials

DOPE, egg PC, PS, hydrogenated phosphatidylinositol (HPI), dicapryl PC (DCPC), dimyristoyl PC (DMPC), dipalmitoyl PC (DPPC) distearoyl PC (DSPC), diarachidoyl PC (DAPC), and sphingomyelin were purchased from Avanti Polar Lipids; ganglioside GM₁ and GT_{1b} were from Calbiochem; bovine brain sulfatide was from Supelco; DPH, cholesterol, glycophorin A, and oleic acid were from Sigma. N-[N-acetylneuraminyl-lactosyl]-DOPE (NANA-DOPE), N-[lactosyl] DOPE (Lac-DOPE) and N-[galactosyl] DOPE (Gal-DOPE) were gifts from Y.S. Park of this laboratory. [¹²⁵I]tyraminyl inulin was synthesized according to Sommerman et al. (17).

Liposome Preparation

Liposomes were prepared by hydrating a thin lipid film with an isotonic solution of PBS (pH 8.0) containing [¹²⁵I]tyraminyl inulin (2 mg/ml) (8). In the case of high melting temperature lipids, hydration was carried out at 65°C for about 3 hours. The lipid suspension was

then sonicated in a bath type sonicator (Laboratory Supplies, Hicksville, NY) and the pH was maintained at 8.0 during sonication. Liposomes were left at room temperature overnight for DOPE/OA containing liposomes and at 65°C for DSPC/cholesterol containing liposomes for 3 hours and then room temperature overnight. Unencapsulated [125 I]tyraminyl inulin was removed by gel filtration of the liposome suspension on a BioGel A0.5M column. Liposome size was determined by dynamic light scattering using a Coulter N4SD instrument.

Biodistribution Studies

Six to eight week old Balb/c mice (male) (Harlan Sprague Dawley, Indianapolis, IN) were injected i.v. with liposomes containing about 1 μ mole lipids in 150 μ l per mouse. Five hours after injection, mice were bled at the retro orbital sinus and sacrificed by cervical dislocation. All organs were weighed and their radioactivities were quantitated in a γ -counter. The total radioactivity in the blood was determined by assuming that the total volume of blood is 7.3% of the total body weight (19). The combined counts in liver and spleen were regarded as the counts in RES.

Fluidity of Liposome Membranes

The membrane fluidity of liposomes was measured as previously described (18). Liposomes containing 0.25% (mol/mol) of DPH were prepared and treated with either PBS or human plasma for 1 hour at 37°C and free serum components were separated by gel filtration (BioGel A1.5M). Fluorescence was measured with excitation at 360 nm and emission at 430 nm. A trace amount of hexadecyl [3 H]cholestanyl ether

was included in the lipid to monitor the lipid. The anisotropy and microviscosity were calculated according to Shinitzky and Barenholz (20).

Results

Effect of Various Lipids on the Biodistribution of DOPE:OA (10:5)

Liposomes

We have previously shown that pH-sensitive liposomes can be prepared with a lipid composition of DOPE/OA (10:5). These liposomes which are prepared by sonication showed an unusual stability in plasma or serum. The biodistribution studies in mouse have revealed a partial stealth activity of these liposomes (18). In order to improve the stealth activity of the liposomes, we have undertaken a comprehensive study of examining the biodistribution of a series of liposome compositions. All compositions had a basic formulation of DOPE/OA/X (10:5:1), with X being varied as a potentially stealth lipid component. The liposomes were prepared by sonication to entrap an aqueous phase marker [^{125}I] tyraminylinulin, a non-degradable marker which is widely used by many investigators (5,7,17,18,33). The size of the sonicated unilamellar liposomes was examined by quasi-elastic light scattering to be about 100 nm or less in diameter. The mice were killed 5 hours after injection and the ^{125}I -cpm was measured in all major organs and blood. Table VI-1 shows the results of these experiments. Data are listed in the approximate order of the RES/blood ratio which is a useful parameter describing the stealth activity of the liposomes (6).

Table VI-1. Distributions of liposomes (DOPE/OA/X = 10:5:1) in blood and RES.

X	Experiment ^a	Size (nm)	% Injected			RES/Blood	Recovery ^b
			Blood	Spleen	Liver		
GM ₁	1	75	27.2±4.5	3.8±0.4	25.8±1.8	1.1±0.2	66.8±5.4
	2	67	28.5±3.4	1.9±0.1	23.1±1.9	0.9±0.1	69.2±4.5
GT _{1b}	1	75	25.3±3.0	2.9±0.4	22.2±2.4	1.0±0.2	56.1±1.7
	2	82	20.2±2.3	2.9±0.4	26.3±2.8	1.5±0.1	65.6±2.4
DSPC	1	91	23.3±3.2	6.4±0.5	25.0±2.2	1.4±0.2	66.0±5.1
	2	76	27.4±2.4	4.5±0.4	42.5±1.8	1.7±0.2	85.9±1.8
Sphingomyelin	1	82	18.5±5.2	5.5±0.4	33.3±3.6	2.2±0.7	68.5±2.9
	2	74	15.9±3.1	3.4±0.3	41.4±4.7	2.9±0.3	74.2±8.1
Glycophorin A ^c	1	83	21.8±0.5	3.4±0.2	39.3±4.4	1.9±0.4	71.7±3.4
	2	80	12.4±2.1	5.0±1.6	41.7±3.2	3.8±0.7	74.6±8.4
NANA-DOPE	1	67	13.3±3.0	5.6±0.9	40.3±4.5	3.5±0.5	70.5±7.5
	2	142	11.6±0.2	6.2±0.4	45.2±2.9	4.4±0.2	72.4±3.3
Gal-DOPE	1	84	10.5±1.3	4.6±0.7	37.3±3.1	4.1±0.5	58.8±4.0
	2	109	8.3±2.1	5.2±0.6	51.4±4.0	6.9±1.9	73.7±1.1
Egg PC	1	95	7.4±0.8	6.8±0.9	41.3±5.4	6.5±0.7	66.0±10.6
	2	70	12.6±0.4	3.7±0.3	44.6±1.9	3.9±0.2	71.5±1.0
None	1	119	7.8±0.9	3.4±0.2	40.8±0.6	5.5±0.5	58.3±2.3
	2	126	7.2±0.9	2.8±0.6	39.3±2.3	5.6±0.5	60.3±4.9
Cholesterol	1	74	6.6±2.5	3.1±0.2	46.3±3.3	7.5±2.7	68.5±6.8
	2	93	7.0±1.3	3.3±0.7	47.2±5.6	7.3±1.1	65.7±5.0
HPI	1	95	5.3±0.4	5.9±0.2	47.2±1.5	10.1±0.8	62.1±0.5
	2	72	11.8±0.4	3.4±0.4	50.9±2.5	4.6±0.3	75.2±1.4
Lac-DOPE	1	67	4.5±0.7	2.9±0.1	48.1±3.4	11.4±2.5	58.3±3.3
	2	92	5.1±0.1	3.2±0.3	57.3±3.7	12.0±0.7	74.6±5.6
PS	1	102	3.2±0.2	9.4±0.7	53.3±5.6	19.6±2.0	74.4±2.7
	2	73	7.1±0.3	7.2±0.7	46.1±1.1	7.6±0.4	70.4±1.5
Sulfatide	1	96	4.5±0.5	5.9±0.4	52.7±6.0	13.3±2.3	70.5±2.4
	2	91	3.3±0.2	4.1±0.0	60.1±4.1	18.8±0.4	73.7±3.3

^an=5 and 3 for experiment 1 and 2, respectively.

^bPercent recovery was calculated from the ¹²⁵I counts of the blood, RES, intestine, lung, heart, and kidney.

^c2 mg of glycophorin A was used per 5 μ mole of lipid.

Among all the lipids tested, some had made the liposomes more stealthy than the reference DOPE/OA liposomes (RES/blood ratio ~ 5.5), i.e. smaller RES/blood ratios; whereas other lipids had made the liposomes less stealthy, i.e. larger RES/blood ratios. Among the lipids which decreased the RES/blood ratio, many of them were sialoglycolipids. For example, GM₁ and GT_{1b} are natural gangliosides containing one and three sialic acid residues, respectively. NANA-DOPE is a synthetic glycopospholipid which also contains a terminal sialic acid residue. Glycophorin A is a glycoprotein which contains 10-20 sialic acid residues (32). Another class of lipid which decreased the RES/blood ratio is the lipids of high melting temperature. DSPC ($T_c = 55^\circ\text{C}$) and sphingomyelin ($T_c \sim 40^\circ\text{C}$) belong to this group. Lipids which caused a significant increase in the RES/blood ratio of DOPE/OA liposomes include sulfatide > PS > lactosyl DOPE. With the exception of PS, lipids in this group are glycolipids which do not contain sialic acid residues. There were also several lipids which did not significantly modify the stealth activity of DOPE:OA liposomes. These were galactosyl-DOPE, egg PC, HPI and cholesterol.

Comparison with the Biodistribution of Liposomes of Other Compositions

The biodistribution of liposomes of the above compositions was compared to those of the published stealth and "unstealth" compositions (6). Table VI-2 shows the biodistribution of 4 liposome compositions: DSPC/cholesterol (10:5), DSPC/cholesterol/GM₁ (10:5:1), DSPC/cholesterol/sulfatide (10:5:1) and DSPC/cholesterol/GT_{1b} (10:5:1). These compositions gave various RES/blood ratios indicating different

Table VI-2. Distribution of liposomes (DSPC/chol/X = 10:5:1) in blood and RES.

X	Experiment ^a	Size (nm)	% Injected			RES/Blood	Recovery ^b
			Blood	Spleen	Liver		
GM ₁	1	93	32.9±5.5	2.7±0.2	13.7±0.2	0.6±0.1	61.3±2.9
	2	111	24.1±0.4	3.0±0.3	24.2±2.2	1.1±0.2	63.9±3.3
None	1	140	16.6±1.4	3.3±0.0	28.9±2.4	1.9±0.3	62.0±0.2
	2	112	13.3±1.4	2.3±0.5	29.9±0.4	2.3±0.2	56.7±0.1
Sulfatide	2	164	13.0±4.3	5.9±0.2	36.0±3.8	3.6±0.7	61.7±7.3
GT _{1b}	1	76	3.8±0.4	4.4±0.2	23.3±3.6	7.3±1.0	40.8±5.6

^an=5 and 3 for experiment 1 and 2, respectively.

^bPercent recovery was calculated from the ¹²⁵I counts of the blood, RES, intestine, lung, heart and kidney.

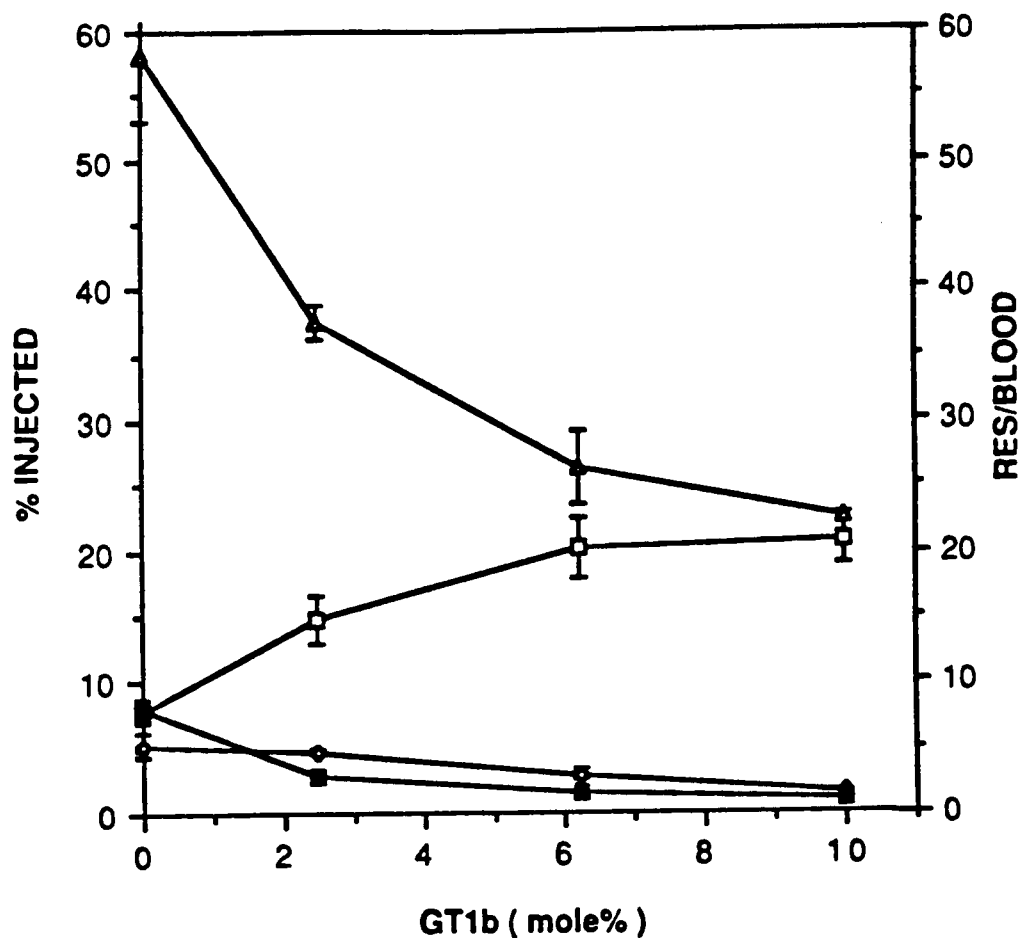


Figure VI-1. Effect of the GT_{1b} concentration on the biodistribution of DOPE/OA based liposomes. 150 μ l of liposomes containing ~ 1 μ mole of lipids per mouse was administered i.v. and the mice were sacrificed and organs were collected after 5 hours. Each point represents the average of 5 mice. ($\square$) blood, ($\diamond$) spleen, ($\triangle$) liver, ($\blacksquare$) RES/blood.

stealth activities. Liposomes containing GM₁ gave lowest RES/Blood ratio, followed by DSPC/cholesterol and sufatide containing liposomes. Liposomes containing GT_{1b} showed the highest RES/blood ratio, which was much higher than that of the liposomes composed of DOPE/OA/GT_{1b} (Table VI-1).

Effect of the Amount of GT_{1b} on the Biodistribution of DOPE/OA Liposomes

Since GT_{1b} showed a strong stealth activity, we have examined the concentration dependence of this glycolipid on the biodistribution of DOPE/OA liposomes. The ratio of DOPE to OA was kept at 2:1, but the amount of GT_{1b} in liposomes was varied from 0 to 10% (Fig. VI-1). Statistically significant increases in the percent injected dose in blood were observed for liposomes containing as little as 2.5% GT_{1b}. There was a concomitant drop in the liver and spleen uptake of liposomes containing GT_{1b}. The RES/blood ratio slightly decreased with increasing GT_{1b} concentration. Thus, the stealth activity of GT_{1b} increased with its concentration in liposomes, with a saturation effect observed at 6.25% GT_{1b}.

Effect of DSPC Concentration on the Biodistribution of DOPE/OA Liposomes

Similar experiments were done to examine the concentration dependence of DSPC on the biodistribution of the DOPE/OA liposomes. Table VI-3 shows that with as low as 5 mol % DSPC the amount of liposomes in the blood had increased significantly, with a concomitant

Table VI-3. Effect of DSPC concentration on biodistribution of DOPE/OA containing liposomes^a (n=5).

DSPC (mole %)	% Injected			
	Blood	Spleen	Liver	Recovery ^b RES/Blood
0 ^c	7.8±0.9	3.4±0.2	40.8±0.6	58.3±2.3 5.5±0.5
5	15.1±1.9	3.3±0.3	33.5±0.2	60.3±0.5 2.6±0.3
10	14.8±1.1	3.7±0.0	29.2±1.9	55.8±1.7 2.2±0.1
20	19.0±0.8	2.1±0.3	30.2±0.7	59.5±1.1 1.7±0.1
40	18.0±0.2	4.3±0.5	30.6±1.2	62.0±2.1 2.0±0.2

^aDOPE/OA = 10:5. Liposome diameter was 73 ± 4 nm.

^bPercent recovery was calculated from ¹²⁵I counts in blood, RES, intestine, lung, heart and kidney.

^cData were taken from Table 1.

decrease in the liver uptake of the liposomes. The RES/blood ratio (2.6) of liposomes containing 5% DSPC is significantly lower than that (5.5) of the liposomes without DSPC. However, further increases in the DSPC content up to 40 mole% did not further decrease the RES/blood ratio significantly. Thus, the stealth activity of DSPC was not strongly concentration dependent.

Effect of Disaturated PC on the Biodistribution of DOPE/OA

Liposomes

DSPC is a disaturated PC. We wondered if other disaturated PC would also show the stealth activity. The biodistribution of DOPE/OA (2:1) liposomes containing saturated PC with chain length varying between 10 and 20 carbons was studied. It is clear from Fig. VI-2 that the stealth activity was not limited to DSPC; other saturated PC with shorter or longer chain length were also effective. Dicapryl PC (10 carbons) and dimyristoyl PC (14 carbons) are not high melting temperature lipids; yet their stealth activities were comparable to those of high melting temperature lipids. These data suggest that there is not a correlation between the stealth activity and the melting temperature of the lipid in the liposomes.

Relationships Between Lipid Fluidity and Biodistribution of Liposomes

Liposomes prepared with primarily the high melting temperature lipids contain quite rigid membranes, i.e. the rotational and translational diffusions of the lipids in the liposome membrane are restricted at the physiological temperature. Since previous reports on

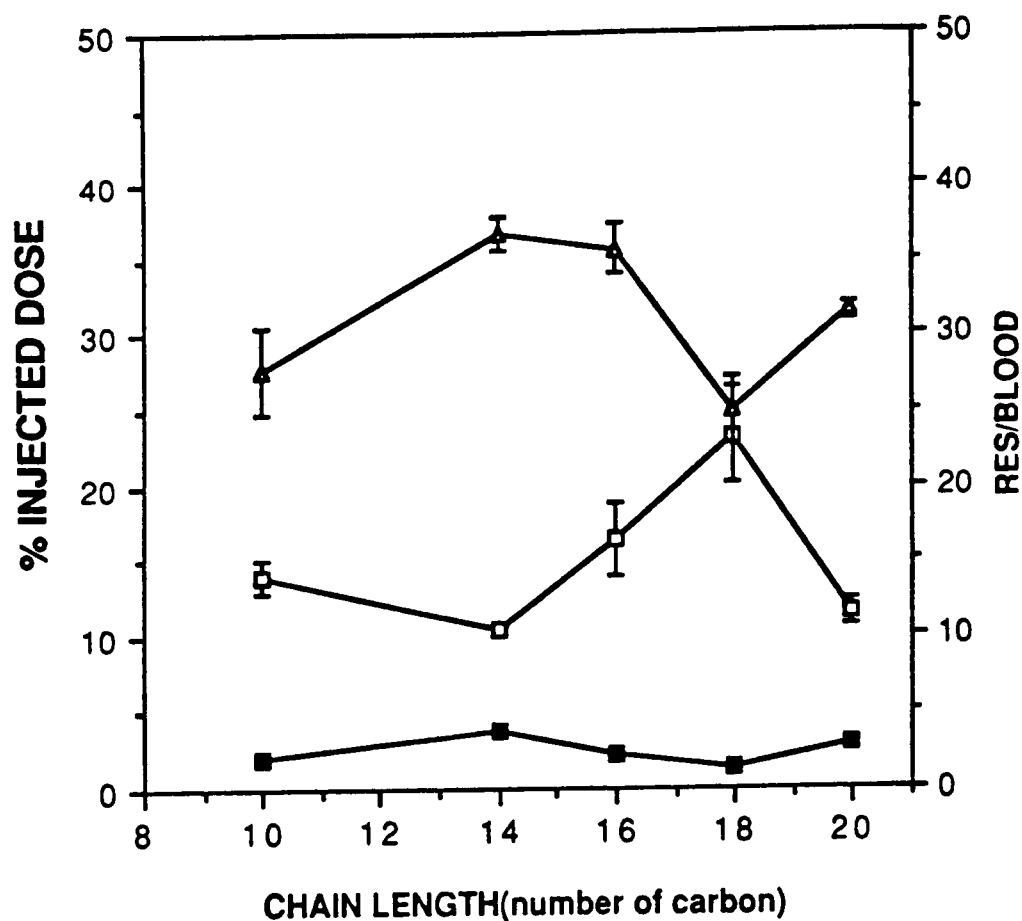


Figure VI-2. Effect of chain length of the disaturated PC on the biodistribution of DOPE/OA based liposomes. 6.25 mol % of disaturated PC with different chain length was included in the lipid composition. Other conditions were the same as described in Fig. VI-1 legend. ($\square$) blood, (Δ) liver, ($\blacksquare$) RES/blood.

the stealth liposome composition conclude that liposomes of low membrane fluidity generally show better stealth activities (6,7,33), we decided to measure the membrane fluidity of some of the liposomes used in this study. This was done by measuring the fluorescence depolarization of a hydrophobic probe, diphenylhexatriene, incorporated into the lipid core of the liposome membrane (18,20). The microviscosities of the lipid membrane can be calculated from the values of fluorescence depolarization (20). Furthermore, the fluorescence depolarization was measured for liposomes suspended in PBS, as well as for those which had been treated with 50% human plasma at 37°C for 1 hour and then separated from excess plasma components by column chromatography. This is because the liposome composition undergoes a significant change when exposed to plasma or serum (18,30). Table VI-4 lists the microviscosity values for 8 different liposome compositions, some of which are stealth and some of them are unstealth (Tables VI-1,2, and 3). It is clear that there was no strong correlation between the stealth activity and the microviscosity of the liposomes. Although the DSPC/cholesterol/GM₁ (10:5:1), a stealth composition, showed a high microviscosity value (19.029 poise), other stealth compositions such as DOPE/OA/DSPC (10:5:1) and DOPE/OA/GM₁ (10:5:1) showed low microviscosity values (0.737 and 0.807 poise, respectively). Furthermore, the microviscosity of the liposome membrane changed significantly after the plasma treatment (Table VI-4). Liposomes with low microviscosity in PBS showed increased microviscosity after the plasma treatment. Plasma treatment had also significantly decreased the microviscosities of those originally rigid

Table VI-4. Fluorescence depolarization of DPH in liposomes with different lipid composition.

Composition (molar ratio)	Treatment ^a			
	PBS		Human plasma	
	Anisotropy ^b	Microviscosity (poise) ^c	Anisotropy	Microviscosity (poise)
DOPE/OA (10:5)	0.087±0.003	0.761±0.038	0.120±0.008	1.194±0.115
DOPE/OA/GM ₁ (10:5:1)	0.091±0.005	0.807±0.054	0.126±0.007	1.290±0.114
DOPE/OA/DSPC (63.3:31.7:5)	0.085±0.000	0.737±0.005	0.121±0.002	1.211±0.036
DOPE/OA/DSPC (60:30:10)	0.094±0.005	0.848±0.065	0.124±0.003	1.251±0.042
DOPE/OA/DSPC (53.3:26.7:20)	0.128±0.007	1.308±0.119	0.151±0.006	1.714±0.113
DOPE/OA/DSPC (40:20:40)	0.199±0.004	2.929±0.133	0.189±0.003	2.632±0.090
DSPC/chol (10:5)	0.327±0.000	22.154±0.340	0.258±0.002	5.991±0.125
DSPC/chol/GM ₁ (10:5:1)	0.321±0.003	19.029±1.458	0.262±0.002	6.312±0.170

^aLiposomes (10 mM) containing 0.25 mole % DPH were incubated with equal volume of either PBS or human plasma for 1 hour at 37°C, followed by chromatography on a Bio-Gel A1.5M column.

^bMeasured in 5 determinations.

^cCalculated according to Shinitzky and Barenholz (20).

liposomes such as DSPC/cholesterol (10:5) and DSPC/cholesterol/GM₁ (10:5:1). In general, plasma treatment showed a "buffer" activity on the microviscosity of liposomes; it made the originally large difference in microviscosity among the different lipid compositions much smaller.

Discussion

The purpose of our undertaking this study is to look for a lipid composition(s) composed of primarily unsaturated PE, and which composition(s) allows liposomes to stay in the circulation for a relatively long period of time. Since such compositions have already been reported for liposomes composed of primarily PC (5,6,7,33), there are many candidate lipids which are potentially effective to prolong the circulation time of the PE-based liposomes. Of the long list of lipids and one protein (glycophorin A) tested, glycolipids containing sialic acid residues were most effective in enhancing the stealth property of the liposomes. Apparently, the number of sialic acid residues on the molecule is not a critical factor because monosialoglycolipids (GM₁ and NANA-DOPE), trisialoglycolipid (GT_{1b}) and oligosialoglycoprotein (glycophorin A) were all effective. GM₁ (5,6,7,33) and glycophorin A (22) have been shown to significantly increase the circulation time of the PC-based liposomes. However, in the study of Gabizon and Papahadjopoulos (6) GT_{1b} did not show any stealth activity. In fact, liposomes composed of PC/cholesterol/GT_{1b} (9:5:1) were among those compositions which showed the shortest

circulation time and highest RES/blood ratio. Other discrepancies between the results of Gabizon and Papahadjopoulos (6) and those of our work are the activities of HPI and sulfatide. These two compounds are among the list of highest stealth activity in their work (6). But the data in Table VI-1 clearly indicate that both compounds have made the liposome's RES/blood ratios higher, and therefore belong to the group of "opsonins" (21). The difference between the two pieces of work may be due to the fact that Gabizon and Papahadjopoulos had used PC and cholesterol as the major lipid species in the lipid composition and we had used DOPE and OA as the major species. This is evident because the composition DSPC/cholesterol/GT_{1b} (10:5:1) showed the highest RES/blood ratio in the experiment described in Table VI-2, indicating that the stealth activity of GT_{1b} may depend on the rest of the liposome composition. A much lower stealth effect of sulfatide and HPI was also reported by other investigators (7).

There are other differences in the experimental conditions between the two pieces of work. Gabizon and Papahadjopoulos had prepared their liposomes by an extrusion method (6) and the liposomes used in the experiment described in Tables VI-1 - VI-3 were prepared by sonication. Although the average sizes of the liposomes prepared by the two different methods are very similar, liposomes prepared by sonication may be less homogeneous than the extruded liposomes. Furthermore, among the sonicated liposomes there could be an uneven distribution of the lipid components due to the high curvature of the small liposomes. Other subtle differences in the bilayer packing density and the lateral compressibility could also lead to different interactions of liposomes

with serum components which are supposed to be responsible for the rapid uptake of liposomes by RES (6,7,33). While the actual reason(s) for the apparent discrepancy remains unclear, it is likely that the molecular mechanism underlying the liposome interactions with the RES is very complex.

Not all of our results are inconsistent with those of Gabizon and Papahadjopoulos. The observation that saturated PC and sphingomyelin are effective in reducing liposome's affinity to RES (Table VI-1) agrees well with the conclusions of other investigators (5,6,7,33). However, this activity is apparently not related to the high melting temperature of the lipid, as previously suggested (6,7). Saturated PC's of shorter chain length were also effective (Fig. VI-2). For example, dicapryl PC (10 carbon chains, t_c presumably below 0°C) gave liposomes a RES/blood ratio of 2.1, which is very similar to the value 2.7 given by DSPC (18-carbon chains, $T_c = 55^\circ\text{C}$ (23)) under the same conditions. Furthermore, the stealth activity of DSPC was not strongly dependent on its concentration in the liposome membrane; the RES/blood ratio (2.0) of liposomes containing 40% DSPC was similar to that (2.6) of liposomes containing 5% DSPC (Table VI-3). Since high melting temperature lipids generally give more rigid liposome membranes, these results would suggest that the stealth property of liposomes is not directly related to the microviscosity of the liposome membrane. Data in Table VI-4 support this notion. There is no apparent correlation between the stealth activity of a given composition and the microviscosity of the liposomes. For example, DSPC/cholesterol/GM₁

(10:5:1) and DOPE/OA/GM₁ (10:5:1) are both stealth compositions (Tables VI-1 and VI-2), but their microviscosities are approximately 25-fold different (Table VI-4). Even after exposure to plasma, DSPC/cholesterol/GM₁ composition still showed a much higher microviscosity value (6.312 poise) than DOPE/OA/GM₁ composition (1.290 poise) (Table VI-3). Thus, we conclude that membrane rigidity is not a necessary requirement for the stealth liposomes, although rigid liposomes are often stealthy.

Opsonins are not limited to proteins. Data in Table VI-1 indicate that the presence of PS in the DOPE/OA liposomes significantly increased the RES uptake of the liposomes. Similar results have been reported by other investigators (6,21). It has been proposed that PS is directly recognized by receptors on the surface of macrophages (24). Aged erythrocytes showed higher amounts of PS on the outer leaflet of the plasma membrane and are more readily taken up by macrophages than the young erythrocytes (24). Metastatic tumor cells also showed 7-8 fold higher PS content on the surface membrane and are thus more readily attacked by macrophages than the normal cells (25). Another lipid opsonin used in our study is Lac-DOPE (Table VI-1). This synthetic glycopospholipid contains a terminal galactose residue which is potentially recognizable by the galactose receptor of the liver cells (26). Whether the apparent opsonin activities of sulfatide are related to any saccharide receptors of RES is not clear.

All reported stealth liposome compositions contain high concentrations of cholesterol (5,6,7). Cholesterol functions to provide stability to the liposomes in the blood (27,28). The presence

of cholesterol effectively reduces interactions of the liposome membrane with serum lipoproteins, particularly HDL (29). Data in Table VI-1 clearly show that DOPE/OA liposomes can be made stealthy without the presence of cholesterol. Also, inclusion of cholesterol in the liposomes did not significantly change the liposome's affinity to RES (Table VI-1). This is because the DOPE/OA liposomes with a diameter $\leq$ 200 nm are quite stable in serum (30). Apolipoprotein A1 and possibly other apolipoproteins rapidly interact with these liposomes and provide a strong stability to the liposome membranes (31). Thus, we conclude that cholesterol is not a necessary component for the stealth liposomes, provided that other stabilization mechanisms are available.

In summary, we have demonstrated that stealth liposomes can be prepared with lipids composed of primarily unsaturated PE. Their stealth activity is comparable to that of DSPC based liposomes. Since these liposomes are easy to prepare and extremely stable in plasma or serum, their drug delivery activity should be rigorously evaluated. The stealth activity of the liposomes does not directly correlate with the rigidity of the liposome membrane, although rigid liposomes often show better stealth activity. The molecular mechanism of the stealth liposomes is likely to be very complex.

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CHAPTER VII

**pH-SENSITIVE, PLASMA-STABLE LIPOSOMES WITH RELATIVELY PROLONGED
RESIDENCE IN CIRCULATION**

Abstract

Acid-sensitive liposomes composed of unsaturated phosphatidyl-ethanolamine (PE) are efficient vehicles for cytoplasmic delivery of the target cells. We have recently shown that liposomes composed of dioleoyl PE (DOPE) and dipalmitoyl succinylglycerol (DPSG) retain acid-sensitivity after exposure to human plasma. In the present work, we have extended these observations to investigate the role of ganglioside GM₁ on the blood residence time of these liposomes. Small (d ~ 100 nm) unilamellar liposomes composed of DOPE and DPSG (4:1, molar ratio) became progressively less acid-sensitive when increasing amounts of GM₁ were included in the lipid composition. However, partial sensitivity to acid (40-50% release of entrapped contents at pH 4) could be retained up to 5% GM₁, even for liposomes which had been exposed to human plasma. Inclusion of GM₁ in the lipid composition only slightly increased the release of entrapped contents in the presence of human plasma. The biodistribution of i.v. injected GM₁-containing liposomes was studied by following the entrapped [¹²⁵I]tyraminyl inulin marker in Balb/c mice. Inclusion of up to 5%

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GM₁ showed a transient increase in the blood level and a concomitant decrease of liver and spleen uptake of liposomes. Thus, these liposomes are pH-sensitive, plasma-stable and show a relatively prolonged residence time in circulation. They are potentially significant drug carriers in vivo.

Introduction

Liposomes, or phospholipid vesicles, have been extensively studied for their potential use as drug carriers (for a recent book, see 1). pH-sensitive liposomes composed of unsaturated phosphatidylethanolamine (PE) and weakly acidic amphiphiles are effective in the cytoplasmic delivery of the entrapped contents to the target cells (2-7). These liposomes become destabilized and fusion-competent when they encounter the acidic environment in the cellular endosomes and lysosomes, resulting in the release of the entrapped contents into the cellular cytoplasm (2,3,4). Antitumor drugs (4), toxin (7) and DNA (6) have been efficiently delivered to the target cells using these liposomes.

Although the pH-sensitive liposomes show great promise as a drug carrier, their poor in vivo stability has been problematic for further developments. We have previously shown that large unilamellar, pH-sensitive liposomes composed of DOPE:OA (2:1) are not stable in serum or plasma, primarily due to the rapid extraction of OA from the liposome membrane by albumin. Small ($d \leq 200$ nm) unilamellar liposomes of the same composition, on the other hand, show a striking stability in serum or plasma (8,9). This is due to the fact that insertion of serum apolipoproteins into the liposome membranes of high

curvature, i.e. the membranes of small liposomes, can give rise to a remarkable stability of the liposome even after the extraction of OA by albumin (8,9). Unfortunately, the serum-stabilized small liposomes are no longer pH-sensitive; no release of the entrapped contents can be observed as low as $\text{pH} = 3$ (9). Therefore, these liposomes are unlikely to be useful for the cytoplasmic delivery in the target cells.

Recently, we (10) and others (11) have shown that small unilamellar liposomes composed of DOPE and a double-chain amphiphile, dipalmitoyl succinylglycerol (DPSG), are pH-sensitive and relatively serum/plasma-stable. Furthermore, these liposomes remain partially acid-sensitive after exposure to serum (10). It has been hypothesized that the serum stability of these liposomes arises from the resistance of DPSG to albumin extraction, although no definitive evidence is given (10).

Another important development in the liposome field is the search for liposome compositions which allow the liposomes to stay in circulation for a relatively long period of time (12,13,14). These liposomes have been called "stealth" because of their relatively low uptake by the reticuloendothelial system (RES) in liver and spleen (13). One of the important ingredients for "stealth" liposomes is the ganglioside GM_1 (12,13,14). Inclusion of up to 6.25% GM_1 significantly increases the circulation time of liposomes and reduces the RES uptake such that the RES/blood ratio of the "stealth" liposomes is 8.5 fold smaller than that of the ordinary liposomes composed of PC and cholesterol (12). Furthermore, the uptake of the stealth liposomes by

solid tumor is significantly enhanced, suggesting that the prolonged circulation time can lead to a greater penetration of liposomes to the vesculatures of the tumor (12).

The therapeutic potential of liposomes as drug-delivery vehicles depends on the efficient delivery of liposome-entrapped compounds to cells. A number of studies (2-7) have demonstrated in culture cells that pH-sensitive liposomes show a high efficiency of delivery. However, these liposomes have not been successfully used in animals due to the poor stability of liposomes in buffer (8) and in serum (16), the loss of pH sensitivity after exposure to serum (8) and the rapid uptake by RES system (9). In the present study, we have attempted to overcome these problems by optimizing the lipid composition of the liposomes. Our goal is to prepare liposomes which are acid-sensitive, plasma-stable and relatively "stealthy" for their potential use as drug carriers.

Materials and Methods

Materials

DOPE, DOPC and DPSG were purchased from Avanti Polar Lipids. GM₁ was obtained from Calbiochem. Calcein was purchased from Sigma Chemical Co. ¹²⁵I-labeled tyraminyl inulin was prepared according to Sommerman et al. (15).

Liposome Preparation

Phospholipid and DPSG (molar ratio 4:1) with different amounts of GM₁ were mixed and evaporated free of CHCl₃ with N₂ gas. The dried

lipid films were vacuum desiccated and suspended in either 50 mM calcein in PBS, pH 8.0, or [^{125}I]tyraminyl inulin (2 mg/ml in PBS, pH 8.0). The samples were then incubated 5 hours at room temperature. The samples were sonicated in a bath type sonicator (Laboratory Supplies, Hicksville, NY) and the pH was maintained at 8.0 during sonication. Liposomes were left at room temperature overnight to facilitate the annealing process. Free calcein or inulin was separated from liposomes by passage over a Bio-Gel A 0.5M column equilibrated with PBS (pH 7.6) which was made isotonic to calcein solution by adding 10X PBS. The final osmolarity of these solutions was equal to 320 mOsm, the osmolarity of mouse plasma. The size of liposomes was measured by a dynamic laser light scattering using a Coulter NS4D instrument. To monitor the lipid concentration after chromatography, a trace amount of hexadecyl [^3H]cholestanyl ether (0.8 $\mu\text{Ci}/\text{mmol}$ lipid) was included in the samples prior to CHCl_3 evaporation. Lipid compositions are expressed as mol % or molar ratios.

Liposome Stability Assay

The stability of liposomes was monitored as previously described (16). Percent leakage was calculated using the equation:

$$\% \text{ Release} = \frac{(F_t/F) \cdot F_x - F_o}{F_t - F_o} \quad (\text{equation 1})$$

where F_o is the fluorescence intensity of the liposomes in PBS at room temperature at time zero. F_t is the total fluorescence intensity of liposomes in PBS after addition of DOC. F_x is the fluorescence intensity of the liposomes incubated in plasma at 37°C at time X. F is

the total fluorescence intensity of the liposomes in plasma after addition of DOC. F_t/F is a correction term which is used to minimize the pipetting and other systematic errors in the measurements. The value of F_t/F ranged from 0.95 to 1.05.

pH-Sensitivity Assay

Ten microliters of liposomes containing about 3 nmole lipid were added to a cuvette containing 1.99 ml of PBS at the desired pH, incubated for 5 min., and then adjusted to pH 8.0 by adding appropriate amounts of NaOH. Equation 1 was used to calculate % release. F_o was the fluorescence intensity at pH 8.0 and F_x was the fluorescence intensity at pH 8.0 after incubation at different pH. F_t and F were the total fluorescence intensities of liposomes incubated at pH 8.0 and desired pH after lysis of liposomes with DOC.

Treatment of Liposomes with Plasma

Liposome suspensions containing 1 μ mole lipid (in 100 μ l) were incubated with an equal volume of human plasma for 2 hours at 37°C. Excess plasma proteins were separated from liposomes by chromatography on a Bio-Gel A 1.5M column.

In vivo Biodistribution

One hundred fifty microliters of [125 I]tyraminyl inulin containing liposomes (1 μ mole lipid, $1-5 \times 10^6$ cpm) were administered intravenously by tail vein injection into Balb/c mice (6-8 weeks old, male). At the desired time, mice were anaesthetized with diethyl ether and bled by eye puncture. Blood was collected and weighed. The mice

were killed by cervical dislocation. Organs were collected and counted for ^{125}I -counts in a gamma counter. The total radioactivity in the blood was determined by assuming that the total volume of blood was 7.3% of the total body weight (17). The counts in liver and spleen were regarded as the counts in RES (12).

Results

We have used a basic lipid composition of DOPE:DPSG (4:1) throughout the study. This composition gives rise to liposomes which are acid-sensitive and relatively plasma-stable (10). Small unilamellar liposomes of average diameter ($d \leq 120$ nm) prepared by sonication are used because it has been shown that the circulation time of small liposomes is generally longer than that of larger liposomes (18,19).

Effect of GM_1 on pH Sensitivity of Liposomes

Increasing amounts of GM_1 in DOPE:DPSG (4:1) liposomes have decreased the sensitivity of liposomes to acid treatment. This is shown by the release of the entrapped calcein from liposomes at different acidic pHs (Fig. VII-1). As can be seen in Fig. VII-1A, release of calcein at pH 4-6 was progressively reduced when increasing amounts of GM_1 were included in the lipid composition. Only about 40% release of calcein was observed at pH 4 with the liposomes containing 5% GM_1 , as compared with 100% release from liposomes containing no GM_1 at the same pH. Also shown in Fig. VII-1B is the pH-sensitivity of the same liposomes which had been treated with 50% normal human plasma and

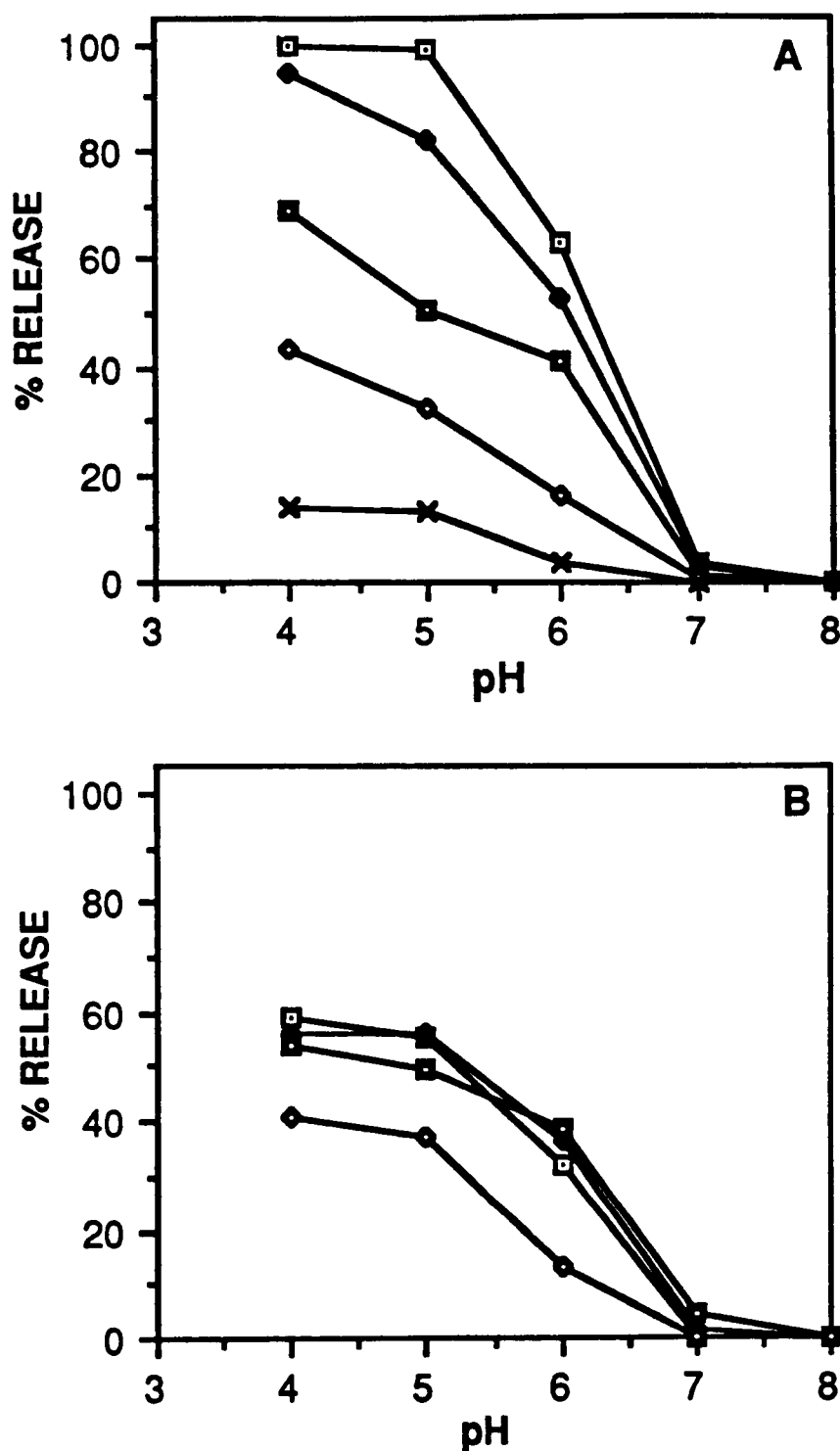


Figure VII-1. Effect of GM₁ on pH sensitivity of liposomes composed of DOPE and DSPG. A: PBS treated; and B: plasma treated liposomes composed of DOPE and DSPG. (□) without GM₁; (◆) with 1% GM₁; (■) with 2.5% GM₁ and (◇) with 5% GM₁. (X) pH-insensitive liposomes composed of DOPC and DSPG (4:1).

reisolated by chromatography. The release of calcein at acidic pH was reduced after the plasma treatment for liposomes containing 0 to 2.5% GM₁. However, the acid sensitivity of liposomes containing 5% GM₁ remained approximately the same after the plasma treatment. In fact, the calcein release curves for all plasma-treated liposomes were very similar to each other, indicating that a partial acid-sensitivity of liposome can be preserved in plasma regardless of the GM₁ content (up to 5%) of the liposome. Higher amounts of GM₁ (7.5 and 9%) further reduced the acid sensitivity of the liposomes, no matter whether the liposomes were treated with plasma or not (Fig. VII-2). Thus, the effect of plasma treatment on the acid sensitivity of liposomes diminishes as the GM₁ concentration increases. At high concentrations of GM₁ ($\geq 5\%$), the acid sensitivity of liposomes was independent of plasma treatment (Fig. VII-2). The lack of acid sensitivity of liposomes composed of DOPC:DPSG (4:1) is also shown in Fig. VII-1A, indicating that the H⁺-induced destabilization of liposome membrane is a characteristic feature of the PE containing liposomes.

Dissociation of DPSG from Liposomes Upon Plasma Treatment

[¹⁴C]DPSG was used to study the dissociation of DPSG with liposomes during plasma treatment. Liposomes containing [¹⁴C]DPSG were separated from the plasma components by gel filtration using a BioGel A1.5m column. Liposomes were eluted in the void volume fractions as indicated by the quenched calcein fluorescence in these fractions. The plasma components including lipoproteins and albumin were eluted in the included volume fractions. Table VII-1 shows the

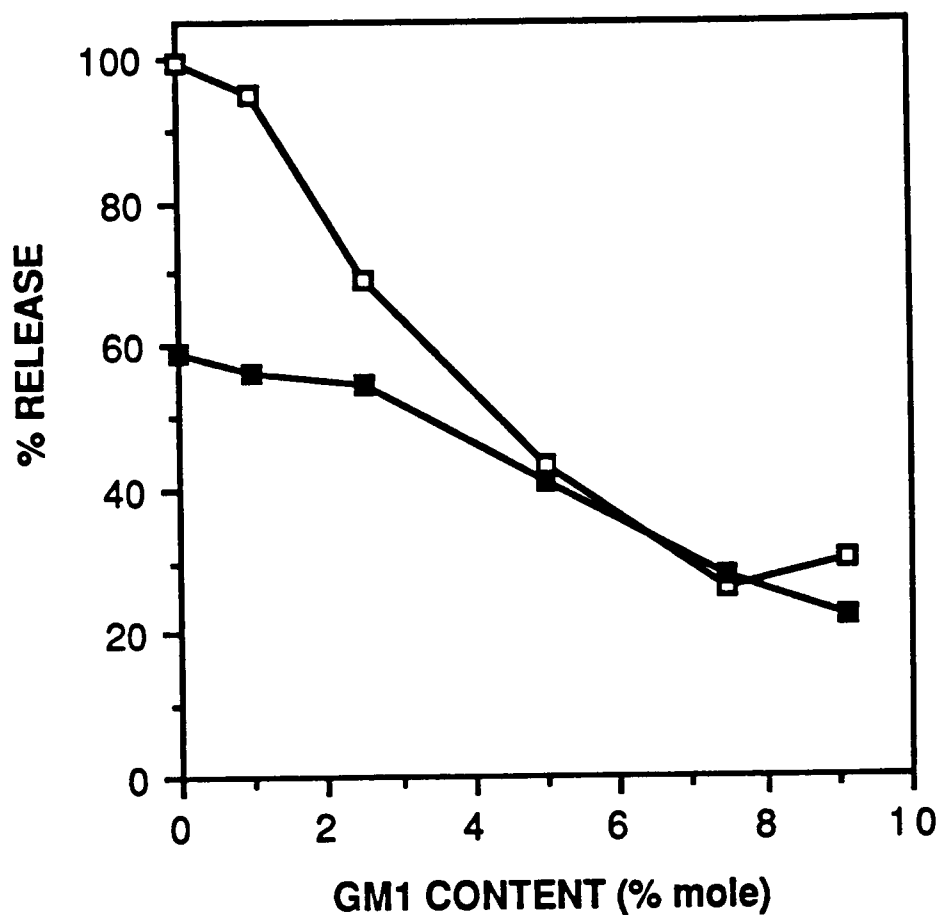


Figure VII-2. Percent calcein release of liposomes composed of DOPE/DPSG with different amounts of GM₁ at pH 4.0. (□) PBS treated; (■) plasma treated.

Table VII-1. Dissociation of DPSG from liposomes upon plasma treatment.

Lipid Composition	Treatment	% [^{14}C]DPSG in	
		excluded vol.	included vol.
DOPE:DPSG (4:1)	PBS	100	0
DOPE:DPSG (4:1)	plasma	62.5	37.5
DOPE:DPSG:GM ₁ (76:19:5)	plasma	60.7	39.3

[^{14}C]DPSG (2×10^5 cpm/mg) was included in the liposome membrane. Liposomes were incubated with 50% normal human plasma for 2 hours at 37°C before fractionation on a Bio-Gel A 1.5M column. Liposomes were eluted in the excluded volume, and lipoproteins and other plasma components were eluted in the included volume.

distribution of [^{14}C]DPSG in the column fractions from liposomes with and without 5% GM_1 . The results clearly indicate that DPSG was partially (~40%) transferred to plasma components during the incubation, and that the transfer was not influenced by the inclusion of GM_1 in the liposome membrane.

Effect of GM_1 on the Stability of Liposomes in Plasma

Allen et al. (20) have reported that GM_1 improves the stability of liposomes in serum. The liposomes used in that study were mainly composed of PC. It is thus important to examine the effect of GM_1 on the plasma stability of PE-containing liposomes. The release of the entrapped calcein from liposomes was measured during incubation with 90% human plasma at 37°C (Fig. VII-3). Both DOPC- and DOPE-containing liposomes were studied. Calcein release from liposomes composed of DOPC:DPSG (4:1) was significantly reduced when 2.5 and 5% GM_1 were included in the lipid composition (Fig. VII-3B), confirming the observations of Allen et al. (20). However, the effect of GM_1 on the liposomes composed of DOPE:DPSG (4:1) was a slight increase of calcein release during the incubation with plasma (Fig. VII-3A). The reduction of plasma-stability of the PE-containing liposomes by GM_1 was quite small. Liposomes composed of DOPE:DPSG: GM_1 (76:19:5) only released about 15% entrapped calcein after 4 hour incubation in plasma at 37°C. Although this level of content release was reproducibly higher than that of the same liposomes containing no GM_1 (~5% release at 4 hours), both liposome types were essentially plasma stable.

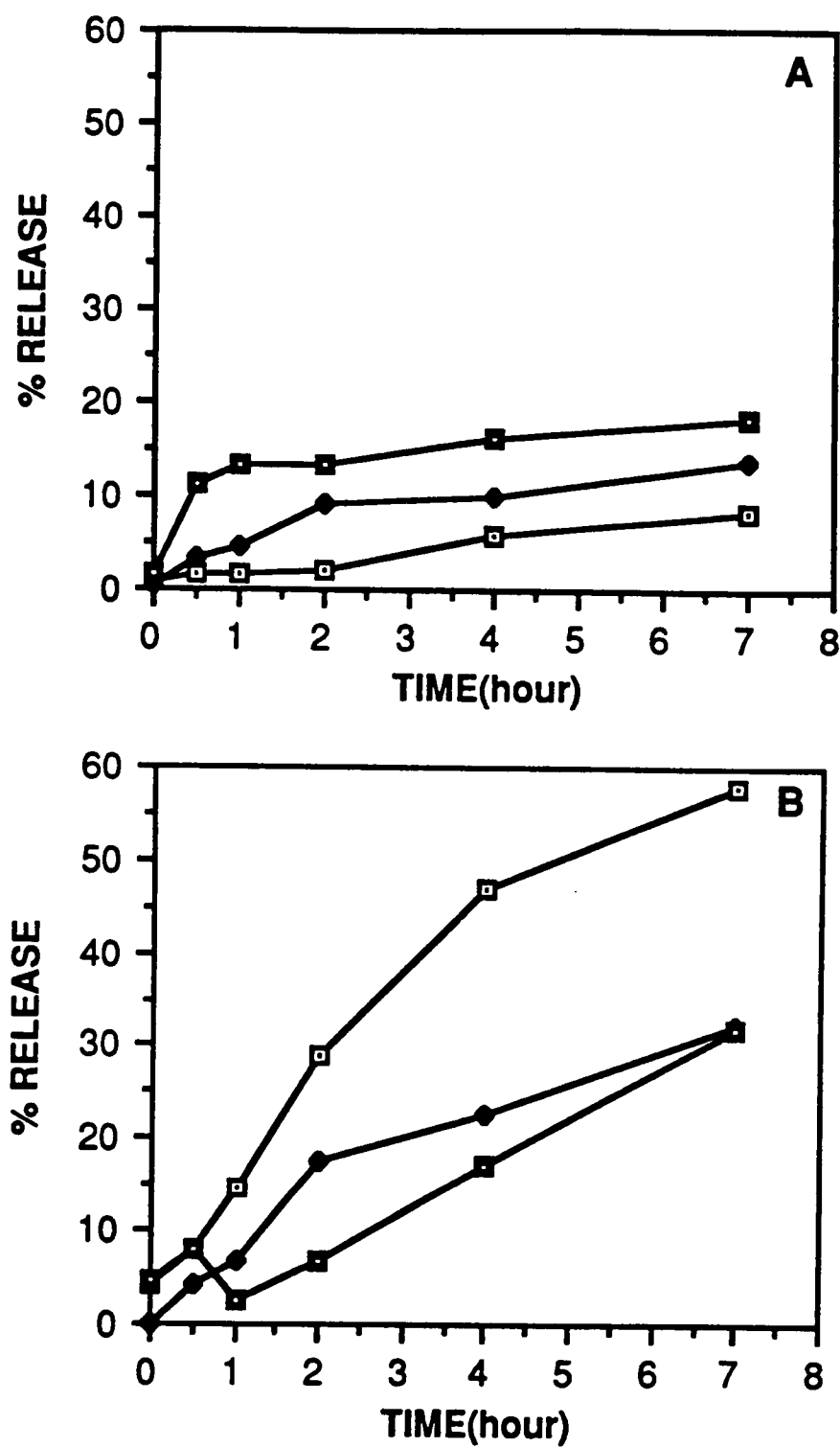


Figure VII-3. Effect of GM₁ on stability of DPSG containing liposomes. A: DOPE/DPSG based liposomes; B: DOPC/DPSG based liposomes (□) without GM₁; (◆) with 2.5% GM₁; (■) with 5% GM₁.

Effect of GM₁ on the Biodistribution of Liposomes in Mouse

The biodistribution of GM₁-containing liposomes in the Balb/c mice was measured at different time periods after i.v. injection.

[¹²⁵I]tyraminyl inulin was used as a non-metabolizable aqueous marker for liposomes. Any free marker leaked from the liposomes is rapidly excreted from the animal with a half-time of a few minutes (data not shown). Thus, the marker retained in the blood represents intact liposome structures. Inclusion of GM₁ in the liposomes composed of DOPE:DPSG (4:1) had significantly enhanced the amounts of liposomes in the circulation up to 2 hours after injection (Fig. VII-4). For example, there was 37.9 ± 3.4 % of injected dose in the blood 30 min after injection for liposomes containing 5% GM₁. This is to be compared with 17.1 ± 0.1 % for liposomes containing no GM₁ under the same condition. The difference in blood level among the liposomes containing different amounts of GM₁ diminishes at longer time periods (5 and 24 hours).

The uptake of liposomes by liver and spleen of the mice was also measured at different time points (Fig. VII-5). Statistically significant decreases in the RES (liver plus spleen) uptake of liposomes were found in the early time points for liposomes containing GM₁. There was 33.1 ± 2.1 % of injected dose found in the combined liver and spleen uptake (mainly liver) at 1 hour after injection for liposomes containing 5% GM₁, as compared to 47.6 ± 0.6 % for liposomes containing no GM₁ at the same period of time. This decrease in the RES uptake of liposomes was GM₁ dose-dependent, as the values for liposomes containing 2.5% GM₁ generally fell in between the values for liposomes

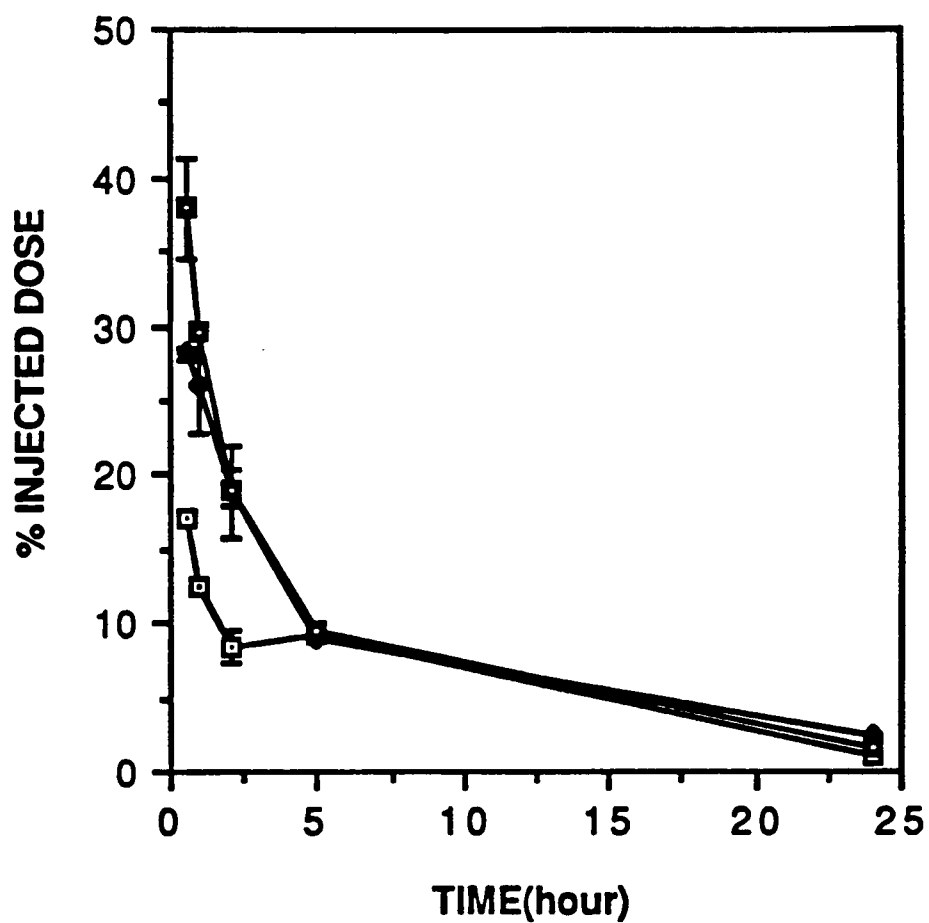


Figure VII-4. Retention in blood of liposomes composed of DOPE/DPSG with different amounts of GM₁. (□) without GM₁; (◆) with 2.5% GM₁; (■) with 5% GM₁.

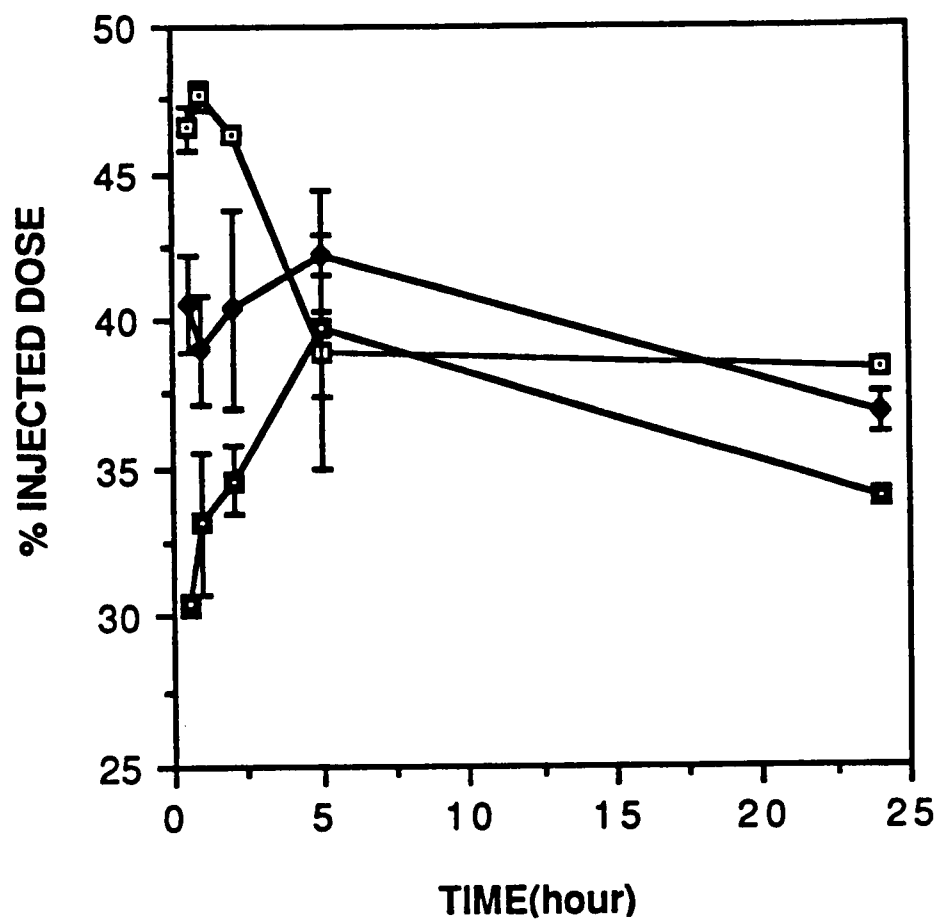


Figure VII-5. Time dependent accumulation of liposomes in RES.
($\square$) without GM₁; ($\blacklozenge$) with 2.5% GM₁; ($\blacksquare$) with 5% GM₁.

containing 0 and 5% GM₁. At longer time periods (5 and 24 hours), the differences in the RES level of liposome uptake diminished.

The data indicate that ganglioside GM₁ can significantly reduce the affinity of PE-containing liposomes for RES cells and concomitantly increase the level in the blood. However, these effects appeared to be transient, as the effect of GM₁ diminished at the longer periods of time.

Discussion

The equilibrium phase of unsaturated PE at physiological conditions is the inverted hexagonal (H_{II}) phase (21). However, the bilayer (L α) phase of unsaturated PE can be stabilized by the presence of a weakly acidic amphiphile (2,3,11,22,23). Protonation of the amphiphile brings about a rapid L α →H_{II} phase transition which destabilizes the liposomes (2,11,22,23,24). One of the intermediate states of the phase transition involves membrane contact and the formation of "inverted micellar intermediate" (IMI) structures at the contact area (25,26,27). These intermediate structures give rise to a high fusion activity of the lipid membrane, which is probably the basis of high cytoplasmic delivery activity of the pH-sensitive liposomes after being endocytosed by the target cells. Addition of a second amphiphile such as GM₁, which is not protonated at the weakly acidic pH, further stabilizes the liposome against acidification. We have previously shown that GM₁ itself is a potent bilayer stabilizer for unsaturated PE (28,29). Indeed, data shown in Fig. VII-1A and VII-2 clearly indicate

that GM₁ diminishes the liposome's sensitivity to acid in a dose-dependent manner. Therefore, the goal of this study is to search for an intermediate level of GM₁ such that the acid sensitivity of the liposome is partially preserved, yet the liposomes are still stable in plasma and, more importantly, show a reduced affinity to the RES cells in vivo.

We (10) and others (11) have shown that pH-sensitive liposomes composed of unsaturated PE and a double-chain amphiphile are relatively stable in serum or plasma. DPSG is a synthetic lipid which falls in the category of a weakly acidic amphiphile with an ability to stabilize DOPE liposomes against the lysis induced by serum or plasma (10,11). It was previously assumed that DPSG is not extracted from liposomes by serum albumin, and thus stays in the lipid membrane to stabilize the bilayer phase of DOPE. Our data (Table VII-1), on the other hand, show that DPSG is also transferred to plasma components during incubation with plasma. The degree of transfer was, however, lower than that of a single-chain amphiphile such as oleic acid. Transfer of approximately 70-80% of oleic acid has been reported under conditions similar to those used in this study (8). Thus, although about 40% of DPSG is extracted from liposomes by plasma, the remaining DPSG is still enough to keep the liposome stable. Furthermore, plasma lipids and/or proteins must also be inserted into the liposome bilayer to help stabilize the liposomes. This is because the acid sensitivity of the plasma-treated liposomes was reduced from that of the original liposomes. Addition of proteins and lipids into DOPE:OA liposomes has resulted in a complete loss of acid sensitivity of the liposomes (8),

and these liposomes are very stable in plasma due to the insertion of plasma components into the liposome membrane (8). Thus, the situation of DOPE:DPSG liposomes is similar to that of DOPE:OA liposomes, except that the degree of modification of the liposomes by plasma components is less severe in the former case.

The partial acid sensitivity displayed by DOPE:DPSG:GM₁ liposomes is probably sufficient for an effective cytoplasmic delivery of their contents to the target cells. It has been shown that pH-sensitive liposomes containing cholesterol also display a partial acid sensitivity (16), yet these liposomes are efficient intracellular delivery vehicles for a fluorescent dye (5) and DNA (6).

Addition of GM₁ to the PE-containing liposomes has slightly, but reproducibly, reduced the stability of liposomes in plasma (Fig. VII-3A). This could be due to the proposed activity of GM₁ in reducing the interactions of serum proteins with the liposome membrane (12). Although the serum proteins involved in such interactions are not yet identified, they are likely to be at least a part of the opsonin system which confers the ordinary liposomes with high affinity to the RES cells (for a recent review see 30). Reduction of protein interactions with liposome membrane is probably the mechanism of improved plasma or serum stability of PC-containing liposomes, as previously studied by Allen et al (20), and also shown here in Fig. VII-3B. Interactions of plasma components with PE liposomes stabilize the liposomes, whereas the same interactions result in a rapid lysis of the PC liposomes (8). Thus, GM₁ reduces the plasma stability of DOPE:DPSG liposomes, probably

by reducing the interactions of the liposomes with plasma components. Nevertheless, liposomes composed of DOPE:DPSG:GM₁ were still reasonably stable in the plasma (Fig. VII-3A). The proposed reduction of plasma protein interactions with the liposomes by GM₁ could also be the basis of the observation that the acid sensitivity of liposomes containing high amounts of GM₁ ($\geq 5\%$) was determined by GM₁ and not by the plasma treatment (Fig. VII-2).

Data shown in Figs. VII-4 and VII-5 clearly indicate that GM₁ at least transiently reduces the liposome's affinity to the RES cells and enhances the liposome concentration in the blood. Thus, the "stealth" activity of GM₁ also works for PE-containing liposomes, although the activity is restricted to the initial few hours after injection. Other reported "stealth" liposome compositions show longer periods of blood residence (12,14). It is not clear at the present time why the compositions described here only transiently retard the liposome's uptake by RES. Nevertheless, it is likely that even a small delay in the clearance of liposomes from circulation is sufficient to enhance the level of uptake by the target cells, because liposome binding to the target cells is a relatively rapid process. For example, immunoliposomes specific for the lung endothelium accumulate in the lung in approximately one minute after i.v. injection (Maruyama et al., unpublished data). Clearance of circulating mouse red blood cells in rats by injecting liposomes covalently coated with anti-mouse red blood cell antibody takes place as rapidly as 10 min after injection (31). Binding of injected immunoliposomes containing anti-erythrocyte antibody with erythrocytes in the circulation also occurs rapidly (< 15

min after injection) (32). Thus, a delay of about 2 hours in liposome clearance in GM₁-containing liposomes should enhance the chance of liposome binding to the target cells, provided that the target cells are readily accessible by liposomes. Since the DOPE:DPSG:GM₁ liposomes are stable in plasma and are still partially acid-sensitive, they may be an efficient carrier for drugs which need to be delivered to the cytoplasm of the target cells. These hypotheses can be evaluated in suitable animal models.

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CHAPTER VIII

SUMMARY AND FUTURE PERSPECTIVES

Summary

A number of studies have demonstrated that pH-sensitive liposomes are superior to the pH-insensitive liposomes in delivering their aqueous contents to target cells in vitro. Thus, the in vivo application of pH-sensitive liposomes in an animal model becomes an essential step toward the clinical application of liposomes as drug delivery agents. The results described in this dissertation take the first step in this direction. The project was initiated by studying the interactions of pH-sensitive liposomes with human serum or plasma. It was demonstrated that pH-sensitive liposomes composed of DOPE/OA prepared by the detergent dialysis method were rapidly lysed when exposed to plasma at 37°C. The inclusion of cholesterol in the liposomes increased the stability of liposomes in plasma, but the pH-sensitivity was compromised. A well-known fact that small liposomes circulate longer in the blood than larger ones has led us to study the behavior of small liposomes in the plasma. The stabilization of small unilamellar liposomes composed of DOPE/OA by a plasma component(s) was discovered as a surprise, because it was inconsistent with the conventional understanding of the PC-based liposomes. One of the significant findings about the stabilization effect is the demonstration of different behavior of liposomes composed of PE versus PC. The size-dependent stabilization of liposomes composed of DOPE/OA by plasma was

another surprise, but the observation is important because it is a common belief that the stability of liposomes in the blood is a key for any successful liposomal drug delivery. Characterization of the plasma-stabilized liposomes described in Chapter III and IV shows that serum proteins and/or lipids are associated with the bilayer of the liposomes, and that oleic acid, which serves as a stabilizer for the original DOPE liposomes, is removed from the liposomes. As a result, the original composition of the liposomes has been altered, leading to the loss of pH sensitivity and the gain of resistance to some extreme conditions such as low and high pH (pH 3.0 and 10.0, respectively) and high salt concentration (0.5 M KCl). Further experiments with purified serum proteins show that albumin lyses the liposomes composed of DOPE/OA by a rapid removal of OA from the liposomes, leading to the formation of the hexagonal phase of DOPE. Stabilization assays using BSA as a lytic reagent helped to identify that lipoproteins, particularly HDL, are responsible for the stabilization activity of the serum or plasma.

Another significant contribution of the stabilization assays using BSA as a lytic reagent is the direct demonstration that amphipathic molecules exhibiting a wedge shape can stabilize the bilayer of small liposomes. Small DOPE/OA liposomes might be an excellent liposome system for the membrane reconstitution studies. It has been demonstrated that amphipathic molecules of different molecular weight ranging from phospholipid such as lysoPC to membrane proteins such as the human glycoporphin A, show high stabilization activity against the BSA induced lysis of liposomes (data not shown). It was hypothesized

that the void space existed on the outer monolayer of the small DOPE/OA liposomes can be filled by these different amphipathic molecules, resulting in the stabilized liposomes.

The significance of the studies reported in Chapter VI relates to the increased chance of liposome binding to targets other than RES in vivo. The demonstration of the effects of GM₁, GT_{1b} and DSPC on prolonging the circulation time of the PE-based liposomes will definitely promote the in vivo applications of liposomes of this novel type. However, the drug delivery activity of the liposomes still needs to be demonstrated in animal model.

The results described in Chapter VII represent an attempt to combine all of the factors which influence the efficiency of liposome drug delivery. New types of liposomes have been designed which maintained the pH-sensitivity after exposure to serum or plasma. The liposomes are stable and exhibit a relatively long circulation time in the blood. Liposomes composed of DOPE/DPSG (8/2) additionally containing 5 mole % GM₁ are partially pH-sensitive after the plasma treatment. They are quite stable and show a prolonged circulation time in mouse.

Future Perspectives

It is believed that two of the major problems in the area of drug delivery using liposomes as a carrier are the stability of liposomes in blood and the RES uptake of liposomes when administered into animals. The work by Allen et al. (1), by Gabizon and Papahadjopoulos (2), and the ones presented in this dissertation have provided some solutions to

these problems. However, a few critical considerations are still essential before liposomes can be used as a drug carrier in the clinical medicine.

One of the critical aspects regarding drug delivery in vivo using liposomes as a drug carrier is to understand the interaction of liposomes with blood components. It is the first time in this dissertation that the stabilization effect of plasms on liposomes is addressed. The insertion of plasma components into the bilayer of liposomes is most likely a common phenomenon for liposomes when they are exposed to blood. The amount of insertion or the degree of stabilization may vary with lipid composition and the size of liposome. The assay system described in Chapter V using BSA as a lytic reagent for liposomes composed of DOPE and OA will be very helpful for the identification of the plasma components which exhibit certain stabilization activity. Fractionated or purified plasma proteins or lipids could be systematically tested for their stabilization activity using this assay. These types of studies will not only help to answer the question of whether there are any factors other than the lipoproteins which stabilize the small liposomes composed of DOPE/OA, but also it will be essential for a better design of liposomes for drug delivery in vivo.

The second consideration concerns the mechanism of RES uptake of liposomes in vivo. The effect of GM1 on decreasing RES uptake is very prominent. The longest reported halflife of GM₁-containing liposomes in circulation is about 11 hours (2). This is accomplished with a composition of DSPC/chol/GM1 (10:5:1). The in vivo halflife of this

type of liposome is much longer than that of the ordinary liposomes, such as PC/chol (10:5), but it is still very short comparing with the halflife (90 days) of the red blood cells in the circulation. Nevertheless, the stealth activity of GM1 offers a good opportunity to explore the mechanisms of liposome uptake by the RES. The immediate experiments would be the investigations of the role of blood components called opsonins in the RES uptake of liposomes. Studying the binding and uptake of liposomes to Kupfer cells in vitro in the presence of fractionated blood components may be very helpful to identify the putative opsonins. Furthermore, new formulations of stealth liposomes are needed because the stealth activity of GM1 is only observed on small liposomes (less than 200 nm in diameter) which are not favorable in terms of their low loading capacity for the water soluble drugs (1,2).

The liposome property regarding the drug delivery efficiency is another important consideration. The results described in this dissertation clearly show that protein insertion into the liposome bilayer and lipid transfer from the liposomes to serum components are two major factors affecting the physical properties of the liposomes. A better design might be the liposomes containing titratable lipids with two long fatty acyl chains. The longer fatty acyl chain may increase the van der Waals interaction between lipids in the bilayer and become less removable by the extraction action of the serum components. The liposomes described in Chapter VII are certainly an advance in this direction. The potential of this type of liposome is

obvious in terms of the intracellular drug delivery via the endocytic pathway. The significance regarding the direct drug delivery to cancer cells is also evident. It is known that the interstitial fluids of a number of tumors in human and animals exhibit an ambient pH that is considerably lower than that of the normal tissue (3,4,5). For example, it has been demonstrated that the pH of efferent blood from rat Walker carcinoma 256 and heptaoma 5123 tumors has a lower average pH (as much as 0.18 units) than that of the afferent blood. The pH of tumor interstitial fluids is about 6.9 and can be further depressed by administration of either glucose or NaHCO_3 , or CO_2 inhalation. In such cases, the pH drops as low as 6.2. At the same time, the pH of the bulk blood is either unchanged or slightly elevated (3,4). In regions of inflammation and infection, the exudate pH drops to 6.5, 60 hours after the start of the inflammation reaction, and capillary permeability is increased in both tumors and inflamed sites (6). In addition, it has also been demonstrated recently in this laboratory (unpublished data) and by Gabizon and Papahadjopoulos (2) that elevated liposome accumulation in tumors can be achieved by increasing the stealth activity of liposomes. Therefore, the preserved pH sensitivity and prolonged circulation time of liposomes composed of DOPE/DPSG/ GM_1 may be highly useful for an efficient delivery of the antitumor drugs in vivo. The low pH in a tumor site will promote the release of the liposome encapsulated drug either when the liposomes are passing through the region or when they are accumulated in that area. If the microscopic domain of metastasis also has a low pH, these vesicles could be of particular benefit in cancer chemotherapy. If it proves

possible to obtain specific release of drugs from pH-sensitive liposomes in the animal tumor models, such liposomes may become a clinical tool of drug delivery.

The intracellular delivery ability of liposomes composed of DOPE/DPSG/GM₁ after plasma treatment can be directly demonstrated in a simple in vitro system. A fluorescence marker can be entrapped in liposome (DOPE/DPSG/GM₁) and the delivery efficiency can be monitored by the fluorescence distribution of the recipient cells, such as L-929 cells, after incubation of the plasma treated liposomes. The results of these experiments may supply some information relating to the role of GM₁ and plasma components associated with liposomes in the release of the liposome contents in the endocytosis pathway.

One of the important advancements in liposome technology is the conjugation of antibody molecules to liposomes, but the amount of antibody required for an efficient binding of liposomes to the target cells in vivo is fairly large (7). This large amount of antibody on the liposomes will undoubtedly affect the properties of liposomes such as the pH sensitivity of the liposomes. One of the options will be to use an indirect targeting strategy. For example, a biotin-avidin bridge system may be used in which a biotinated antibody will be first administered into the animal followed by the injections of avidin and liposomes containing biotinated lipids such as biotin-PE. The small amount of biotin-PE in the bilayer would have much less effect on the properties of the liposomes, yet binding of liposomes to the target cells could still be achieved.

It is known that not all cells are equally endocytotic and the binding of the liposomes to cells via specific receptors may not always lead to liposome endocytosis. The type of liposomes used to deliver drugs may depend on the target cells and the specific receptor on the cell surface. The target-sensitive liposomes (8-10) developed in this lab may be very useful for the situation in which no rapid endocytosis occurs.

Furthermore, additional effort must be placed on the optimization of the ability of liposomes to reach target cells in vivo following their administration. At present, even the smallest liposomes of 15-20 nm in diameter cannot penetrate the tight junctions of the endothelial cells lining the vessel walls. Hence, liposome targeting via the i.v. route to organs other than the cells in the blood circulation is not yet feasible, except for liver and spleen where the fenestrated vessels exist.

Given the considerations discussed here, it is not unreasonable to believe that liposome research has now reached a stage such that progress in other fields will have a great impact on liposome targeting technology. Particularly, as we begin to understand the mechanism(s) of immunorecognition and the process of transcytosis of particles across the endothelial cell barrier, we will be able to design liposomes which are capable of serving as an ideal drug carrier.

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