CANADIAN THESES ON MICROFICHE

I.S.B.N.

THESES CANADIENNES SUR MICROFICHE

National Library of Canada
Collections Development Branch

Canadian Theses on
Microfiche Service

Ottawa, Canada
K1A 0N4

Bibliothèque nationale du Canada
Direction du développement des collections

Service des thèses canadiennes
sur microfiche

NOTICE

The quality of this microfiche is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us a poor photocopy.

Previously copyrighted materials (journal articles, published tests, etc.) are not filmed.

Reproduction in full or in part of this film is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30. Please read the authorization forms which accompany this thesis.

THIS DISSERTATION
HAS BEEN MICROFILMED
EXACTLY AS RECEIVED

AVIS

La qualité de cette microfiche dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de mauvaise qualité.

Les documents qui font déjà l'objet d'un droit d'auteur (articles de revue, examens publiés, etc.) ne sont pas microfilmés.

La reproduction, même partielle, de ce microfilm est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30. Veuillez prendre connaissance des formules d'autorisation qui accompagnent cette thèse.

LA THÈSE A ÉTÉ
MICROFILMÉE TELLE QUE
NOUS L'AVONS REÇUE

NL-339 (r. 82/08)

Canada

A Thesis

Entitled

FORMATION AND EVALUATION OF LIPOSOMAL DRUG DELIVERY SYSTEMS

by

© Ashokkumar Goundalkar

M.Pharm. (Nagpur University, India)

Submitted to the

Faculty of Graduate Studies of Dalhousie University

in partial filfillment of the requirements for

the degree of

Doctor of Philosophy

College of Pharmacy, Faculty of Health Professions

Dalhousie University, Halifax, Nova Scotia

December 11, 1984.

To my parents

TABLE OF CONTENTS

ABSTRACT

The two major objectives of this project were: to improve liposome encapsulation of triamcinolone acetonide (TRMA) and, to develop and evaluate chemoimmunoliposomes using methotrexate (MTX) as a model drug.

Formulations and methods of preparation were varied to overcome the poor encapsulation of TRMA, with little success. High encapsulation of TRMA in liposomes was finally achieved through chemical modification. A new derivative, TRMA-21-palmitate (TRMA-P), was synthesized using a procedure which resulted in high yield and purity. Encapsulation studies revealed almost complete incorporation of TRMA-P into liposomes.

In the second part of this investigation, MTX was entrapped in liposomes by reverse phase evaporation (REV) and by sonication method (SUV). A new lipid derivative, N-[3-(2-Pyridyldithio) propionyl]stearylamine (PDP-SA), was synthesized and used successfully, for covalent coupling of antibodies to liposomes. In vitro evaluation of REV MTX liposomes against EL_4 lymphoma, kidney carcinoma (Caki) and melanoma (M_{21}) cell lines showed reduced effectiveness of MTX entrapped in liposomes. Coating anti-EL_4 globulin did not improve the efficacy of MTX liposomes against EL_4 cells. The interaction studies with melanoma cells showed increased binding of REV and SUV MTX liposomes when coated with IgG. However, specific binding of targeted liposomes could not be substantiated.

In the in vivo evaluation against murine EL_4 lymphoma, MTX entrapped in REV liposomes showed a marginal improvement in the survival time of tumor bearing mice. Coating the liposomes with antibodies did not improve the efficacy of MTX liposomes. Overall, these results seem to indicate no beneficial effect of MTX entrapped in liposomes whether coated with antitumor antibodies or not.

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AEG	anti-EL_4 globulin, anti-EL_4 IgG
AMG	anti-melanoma globulin, anti-M_{21} IgG
BSA	bovine serum albumin
BSS	balanced salt solution
CHOL	cholesterol
Ci	curie
conc	concentration
cpm	counts per minute
DCP	dicetyl phosphate
DMSO	dimethyl solfoxide
DPPC	DL-α-dipalmitoylphosphatidyl choline
DPPE	dipalmitoyl L-α-phosphatidylethanolamine
dpm	disintegrations per minute
DTT	dithiothreitol
ECDI	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl
EL_4 cells	EL_4 lymphoma cells
EPC	egg phosphatidylcholine
FACS	fluorescence activated cell sorter
FBS	fetal bovine serum
FCS	fetal calf serum
g	gram
x g	gravity
hr	hour
Hz	hertz

IgG	immunoglobulin G
I.P.	intraperitoneal
I.V.	intravenous
IR	infrared
log P	logarithmic partition coefficient
M	molar
M_{21} cells	M_{21} melanoma cells
min	minute
MLV	multilamellar vesicle
mp	melting point
MTX	methotrexate
nm	nanometer
NMR	nuclear magnetic resonance
NRG	normal rabbit globulin, normal rabbit IgG
N.S.	not statistically significant
PBS	phosphate buffered saline
PC	egg phosphatidylcholine
PDP-DPPE	N-[3-(2-pyridyldithio)propionyl]dipalmitoyl L-α-phosphatidylethanolamine
PDP-IgG	IgG substituted with SPDP
PDP-PE	N-[3-(2-pyridyldithio)propionyl]phosphatidylethanolamine
PDP-SA	N-[3-(2-pyridyldithio)propionyl]stearylamine
PE	egg phosphatidylethanolamine
ppm	parts per million
q.s.	quantity sufficient to produce

REV	reverse phase evaporation vesicle
rpm	revolutions per minute
SA	stearylamine
S.C.	subcutaneous
S.D.	standard deviation
SDS	sodium dodecyl sulfate
SPDP	N-succinimidyl-3-(2-pyridyldithio)propionate
SUV	small unilamellar vesicle
TLC	thin layer chromatography
2-TP	2-thiopyridinone
TRMA	triamcinolone acetonide
TRMA-P	triamcinolone acetonide-21-palmitate
UV	ultraviolet
VPC	vegetable phosphatidylcholine

Prefixes for units of measurement:

n	nano (10^{-9})
μ	micro (10^{-6})
m	milli (10^{-3})
k	kilo (10^{3})

ACKNOWLEDGEMENTS

First and foremost, I would like to thank Dr. Michael Mezei for advice and encouragement during the entire course of these investigations and the preparation of this thesis. I am grateful to Dr. Tarun Ghose for the guidance and facilities generously provided for the second part of this investigation. The chemistry aspect of this project could not have been accomplished but for the invaluable guidance of Dr. Patrick Farmer. For that, I am indebted to him.

I owe my thanks to Jane Hilchie, Bruce Cameron, Dr. Isaac Abraham, Sujata Joshi, Dr. Padmaja Kulkarni, Damaso Sadi, Molly Mammen, Helen Cruz and Shashi Uniyal for their help in various aspects throughout these investigations. Special thanks to Elizabeth Foy for keeping me up-to-date with the literature. Last, but not least, thanks are due to Lisa Parsons for the neat typing of this thesis.

The financial assistance in the form of Graduate Fellowship (1980-1984) received from the Faculty of Graduate Studies and the College of Pharmacy of Dalhousie University and also the Pharmacy Research Award Fellowships from Parke, Davis Canada Inc. (1983-84) and Novopharm Ltd. (1984) are gratefully acknowledged.

INTRODUCTION

STATEMENT OF THE PROBLEM

For the past several years attempts have been made to reduce the toxicity and increase the therapeutic activity of drugs. Often the toxicity is due to their disposition at a site where the therapeutic action is not desired. Thus conceptually, if it were possible to achieve a higher concentration of drug at the site of action and a decrease or elimination at the site of toxicity, the potency of the drug could be increased with concomitant reduction in its toxicity. Such a concept is referred to as "drug-targeting". Optimal treatment of any disease involves the targeting of a drug to the site of action.

In topical therapy the untoward side effects are invariably due to percutaneous absorption and consequent systemic action of drugs. Thus optimal topical therapy involves the reduction in systemic action and the increase in the local action of drugs. Despite great strides in the ointment bases, it has not been possible to eliminate the percutaneous absorption of drugs. Use of liposome entrapped drugs has shown promising results in this direction (Mezei and Gulasekharam 1980, 1982).

In cancer chemotherapy the indiscriminate action of antitumor drugs limits their use severely. Here again liposomes have been recently investigated as carriers of these drugs with the hope of achieving selective action.

They are equipped with tumor specific antibodies to further improve their selectivitiy (Toonen and Crommelin 1983, Weinsten and Leserman 1984).

The feasibility of therapeutic application of liposomal drug delivery approach in topical as well as cancer therapy deserves careful assessment. The goal of this project therefore was to study the encapsulation of drugs into liposomes and their evaluation as drug delivery systems.

LITERATURE REVIEW

LIPOSOMES

Liposomes can be defined as concentric phospholipid bilayers separated by aqueous compartments (Tyrrell et al. 1976). Pharmaceutically, liposomes can be considered as a type of galenic dosage form. They are microcapsules made up of phospholipids. Historically, the liposomes were first prepared by Bangham and his co-workers in Cambridge (Bangham et al. 1965). Subsequently these liquid crystals (smectic mesophases) were called "Bangosomes" for some time. In the beginning, they were used only as models of biological membranes (Bangham et al. 1965, 1974). However, as their applications became diversified, they have become increasingly popular as drug carriers. A number of reviews, books and several research reports have appeared to substantiate this notion (Tyrrell et al. 1976, Kimelberg and Mayhew 1978, Papahadjopoulos 1978, Gregoriadis 1979,

Ryman and Tyrrell 1979, Gregoriadis and Allison 1980, Juliano and Layton 1980, Kellaway et al. 1980ab, Knight 1981, Rieger 1981, Lelkes 1982, Puisieux and Benita 1982, Yatvin and Lelkes 1982, Gregoriadis 1983, Ostro 1983, Poste 1983, Puisieux 1983, Gregoriadis 1984, Weinstein and Leserman 1984, Whelan 1984).

Although drug entrapment in lipid vesicles has been utilized for almost two decades, the term "chemoliposomes" for liposomes containing drugs is a recent one (Hashimoto et al. 1983). Chemoliposomes when further equipped with an antibody are called "chemoimmunoliposomes" (Hashimoto et al. 1983).

LIPOSOMES AND MICELLES

Micelles are well known in pharmacy mainly as solubilizing systems. There is some similarity between liposomes and micelles. The similarity lies in the arrangement of lipid molecules. In both systems, i.e. liposomes and micelles, the amphiphilic lipid molecules align themselves in a similar manner, however in micelles the leaflet or the lipid layer is monomolecular and in liposomes is bimolecular. Another difference is that micelles do not possess aqueous compartments whereas liposomes do. A more important distinction is that micellar structure is in a dynamic equilibrium state. There is a continuous equilibrium between monomers, micelles and drug solubilizates (Fendler and Romero 1977, Fendler 1984). Such

an equilibrium nature does not exist with liposomes. This unique property makes liposomes more suitable as drug carriers.

COMPOSITION OF LIPOSOMES

Phospholipid is the main component of liposomes. However, one or more other lipids are usually included for specific reasons. For example, the role of cholesterol in membranes to bring about "intermediate fluidity" by modifying the hydrocarbon chains of phospholipids, is well known. This, in effect, produces a tighter packing of lipid molecules in liposomes leading to decrease in permeability.

Cholesterol is therefore included to reduce the leakage of entrapped substances (Fendler 1980) as well as to improve the structural stability of the lipid vesicle in biological fluids (Senior and Gregoriadis 1982). Antioxidants like α- tocopherol (vitamin E) are included to prevent autooxidation of unsaturated phospholipids (Hunt and Tsang 1981, Fukuzawa et al. 1982, Guey-Shuang et al. 1982). Anionic lipids like dicetyl phosphate, phosphatidic acid, phosphatidylserine or stearic acid and cationic lipids such as stearylamine are used to impart either negative or positive surface charge to liposomes. The presence of a charge may prevent aggregation of liposomes (Page Thomas and Phillips 1979); also it may improve the entrapment of the aqueous phase because of increased distance between lipid

layers brought about by electrical repulsion (Puisieux and Benita 1982).

In addition to the lipid phase discussed above the aqueous phase (swelling medium) is essential for the formation of liposomes. Such an aqueous phase could either be water, ionic solution or a solution of drug.

TYPES OF LIPOSOMES

Multilamellar (MLV), Small unilamellar (SUV) and Large unilamellar (LUV) vesicles:

Various types of liposomes exist. The type may depend on size or number of lipid bilayers, on the method of preparation, or on other characteristics.

On the basis of size and the number of lipid bilayers, liposomes are classified mainly into 3 types (Juliano 1981): MLV, LUV and SUV, (Fig. 1)(Schreier 1982). The multilamellar or multicompartment vesicles (MLV) vary in size from 0.05 µm to 10 µm and possess up to 10 lamellae, (Lichtenberg and Markello 1984). The unilamellar or single compartment vesicles, small (SUV) and large (LUV) are of size 0.025 µm to 0.05 µm and 0.1 µm respectively. The SUV has a particle weight of about 2×10^6 daltons and because of its small size cannot efficiently accommodate materials of molecular weight more than 4×10^4 daltons (Szoka and Papahadjopoulos 1980).

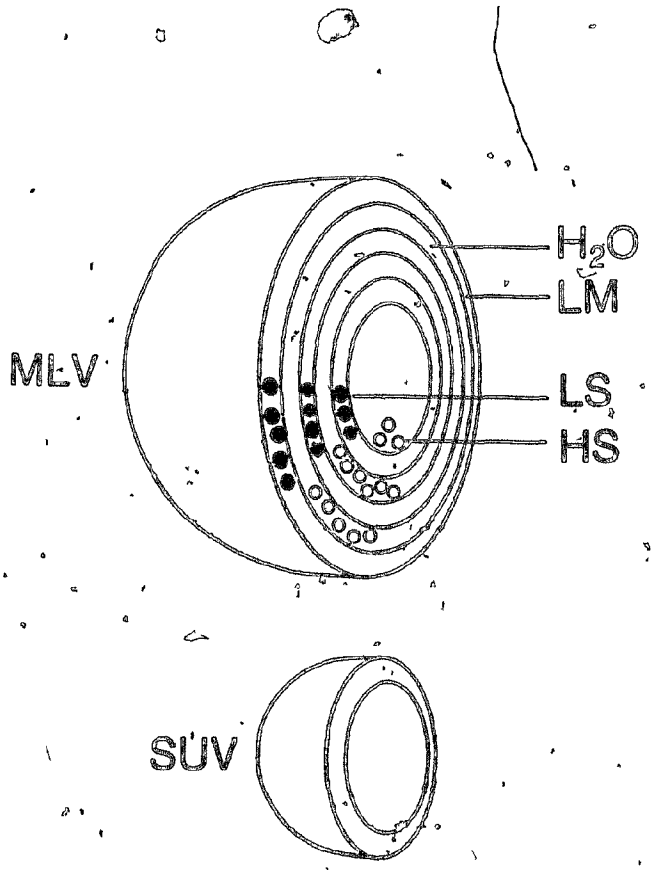


Figure 1. Liposomes (Schreier 1982).

MLV: multilamellar vesicle
SUV: small unilamellar vesicle
LM: lipid membrane
LS: lipid soluble drug
HS: water soluble drug

Reverse phase evaporation vesicles:

Vesicles obtained by the reverse phase evaporation method of Szoka and Papahadjopoulos (1978) are referred to as REVs. These REVs are uni- to oligo-lamellar in nature and larger than LUV. Terms LUV and REV are often used synonymously.

French press and Ether injection vesicles:

The French press vesicles or FPVs are prepared by using an hydraulic press (Hamilton et al. 1980). These are similar to LUV in their characteristics. Ether injection vesicles or EIVs are liposomes resulting from the injection of ethereal solution of phospholipids into warm aqueous solution (Deamer & Bangham 1976). Though they are unilamellar their volume trapping efficiency is almost 10 times superior to SUV.

Cell sized liposomes:

Kim and Martin (1981) have reported the preparation of cell size unilamellar liposomes which have potential for introduction of genetic materials like DNA, chromosomes, etc. into cultured mammalian somatic cells. These have diameters comparable to that of human erythrocyte (7 μm) and a capture volume of up to 144 μl/mg.

Giant liposomes:

Uni- and oligo-lamellar liposomes with a diameter greater than 10 μm are considered as giant liposomes. Oku and MacDonald (1983ab) have reported the formation of giant

liposomes by two different procedures. In one procedure (1983a) they make these liposomes from lipids in chaotropic ion solutions such as sodium trichloroacetate, guanidine hydrochloride and urea. The other procedure (1983b) utilises alkali metal chlorides like KCl or RbCl in conjunction with freezing, thawing and dialysis.

Multivesicular liposomes:

Recently, a novel type of liposome called multivesicular liposomes (MVL) have been described (Kim et al 1983). These were prepared by evaporation of organic solvents from chloroform ether spherules dispersed in water. Diameters of these vesicles range from 29 ± 10 μm to 5.6 ± 1.7 μm. The striking feature is the extremely high percentage of encapsulation of up to 89%.

pH sensitive liposomes:

The pH sensitive liposomes were suggested by Yatvin et al. in 1980 to target anticancer drugs to metastatic lesions. Such liposomes, constructed with a pH sensitive lipid-palmitoylhomocysteine (PHC), were supposed to release their contents when in contact with areas of body in which pH is less than physiological e.g. primary tumors and metastases.

Polymerized liposomes:

They are supposed to be better models for biomembranes. The polymerized liposomes (Buschl et al. 1982, Leaver et al. 1983, Fendler 1984) are made by

using polymerisable lipids e.g. butadiene derivatives, diacetylene containing phospholipids. A recent report (Juliano et al. 1984) relates to photopolymerized liposomes.

Magnetoliposomes:

Immunoliposomes containing ferromagnetic particles (ferrite powder) were proposed by Margolis et al. (1983) for sorting of cells. Associated with cytotoxic drugs they might possibly be of use in cancer therapy as well.

"Solid" and "Fluid" liposomes:

Every liposome has a gel to liquid crystalline phase transition temperature. Above this phase transition temperature liposomes are "fluid" and below it they are "solid". For instance, DPPC liposomes are solid at 37^{o} where as egg PC liposomes are fluid at the same temperature. Transition temperatures of different phospholipids have been tabulated by Szoka and Papahadjopoulos (1980).

A comparison of the properties of some of the above mentioned liposomes is given in Table 1.

METHODS OF PREPARING LIPOSOMES

The type of liposomes, in some respects, depends on the method used to prepare them. To date, several methods have been reported to prepare liposomes. A comparative study of these different methods can be found in excellent reviews by Szoka and Papahadjopoulos (1980,1981), Ryman and Tyrrell (1979) and Hauser (1982). The choice of the method

Table 1[a]
Types of liposomes

NAME	SIZE (μm)	INTERNAL AQUEOUS SPACE[b] (μl/μmol lipid)	ENTRAPMENT EFFICIENCY[c] (%/mg lipids)
SUV	0.025-0.05	<0.5	<1
MLV	0.05-10	>4	5-15
LUV	0.1	10	20
REV	0.5	≥10	20-50
FPV	0.04-0.08	≤10A	10-30
FTV	0.05-0.02	N.A.	20-30
GV	1-100	100-800	N.A.
EIV	0.03-0.1	15	>1
CUL	6.2-12.2	243	15-60
MVL	5.6-29	106	<89

SUV: small sonicated unilamellar vesicles.
MLV: large vortexed multilamellar vesicles.
LUV: large unilamellar vesicles.
REV: reverse phase evaporation vesicles.
FPV: french-pressed vesicles.
FTV: freeze-thaw vesicles.
GV: giant vesicles.
EIV: ether-injection vesicles.
CUL: cell-size unilamellar liposomes.
MVL: multivesicular liposomes
NA: not available.

[a]Updated the table from Yatvin and Lelkes (1982).

[b]Internal aqueous space or capture volume is defined as the volume enclosed by a given amount of lipid and with units liters (or microliters) entrapped per mole (or/micromole) of total lipid ($l\,mol^{-1}$ or $\mu l\,\mu mol^{-1}$). It is independent of lipid concentration.

[c]Entrapment or encapsulation efficiency is defined as the percentage of the compound that becomes entrapped. It is directly proportional to lipid concentration.

has a bearing on the type of liposomes desired for a particular application. Shaking, sonication and reverse phase evaporation are the three most widely used methods.

Bangham method:

This method, also known as shaking method, is the classical method to prepare liposomes. Using a rotary vacuum evaporator, a thin film of lipids is produced on the inner wall of a round bottom or pear shaped flask. The lipid film is then shaken with the aqueous medium at the phase transition temperature of the phospholipid. Liposomes obtained in this way are mainly multilamellar and of heterogeneous size distribution.

Sonication:

This method produces vesicles of fairly uniform size when MLV, obtained as above, are subjected to high energy ultrasonic waves (Juliano 1981). The sonication is usually carried out at the temperature of phase transition of the phospholipid. Either the probe or the bath sonicator could be employed for this purpose.

Reverse phase evaporation:

The disadvantages of the SUV were partly overcome when Szoka and Papahadjopoulos (1978) reported their reverse phase evaporation method. In this procedure the lipids are dissolved in organic solvents (like ether) and then dispersed in aqueous medium to be entrapped. The dispersion is then sonicated to produce a w/o type of emulsion. After

the removal of organic solvents, the aqueous dispersion is shaken at or above the phase transition temperature of the lipid. The resulting liposomes are uni- to oligo-lamellar with a distinct advantage of high capture volume of aqueous phase.

The importance of the method of preparation of liposomes on the encapsulation efficiency of the drug is evident from the study of Tsukada et al. (1982). They observed maximum encapsulation of the poorly soluble drug carboquone when the liposomes were prepared by REV method. Bangham, Ethanol injection and Ether infusion methods were unsuccessful for carboquone encapsulation.

Other methods and their advantages and disadvantages are listed in Table 2. It is of interest to note that even some commercial equipment like Lipoprep ^{R}* and Mini-Lipoprep ^{R}, for preparation of liposomes on the laboratory scale, have started to appear on the market. Lipoprep-CLS ^{R} is suitable for continuous large scale production of liposomes. All these equipment work on the

---

*Lipoprep ^{R}, Mini-Lipoprep ^{R} and Lipoprep-CLS ^{R} are available from Dianorm-Gerate, Postfach 126, D-8000, Munchen 65, FRG. First two cost about 12480 and 945 Swiss Francs respectively.

Table 2
Methods of preparation of liposomes

Method	Liposome obtained	Encapsulation efficiency %	Advantages	Disadvantages	Reference
Bangham method	MLV 0.05-10 µm	5-15	Easy to prepare	Low encapsulation efficiency Heterogeneous size distribution Not suitable for thermolabile substances	Bangham et al. 1965
Sonication	SUV 0.025-0.05 µm	0.5-1	Homogenous size distribution Perhaps better for uptake by cells (Machy & Leserman 1983)	Least encapsulation efficiency Proteins may be denatured	Huang 1969 Huang and Thompson 1974
Reverse phase evaporation	REV 0.12-0.2 µm	20-60	High encapsulation efficiency & large aqueous space Good for certain enzymes (Kurosaki et al. 1981)	Organic solvents may be deleterious to biologically active molecules Heterogenous size distribution	Szoka & Papahadjopoulos 1978 Szoka et al. 1980
Calcium-induced fusion	LUV 0.2-1 µm	10-15	High encapsulation efficiency Suitable for macromolecules including viruses	Restricted to acidic phospholipids like phosphatidylserine Heterogeneous	Papahadjopoulos et al. 1975 Dimitriadis 1978 Papahadjopoulos & Vail 1978 Wilschut et al. 1983
Detergent dialysis & Detergent gel filtration	unilamellar 0.03-0.1 µm		Good for reconstitution of intrinsic membrane proteins Homogeneous size distribution	Low encapsulation efficiency less practical for encapsulation of water soluble drugs	Brummer et al. 1976 Milsmann et al. 1978 Enoch and Strittmater 1979 Zumbuehl and Weder 1981

Table 2 (continued)

Method	Liposome obtained	Encapsulation efficiency %	Advantages	Disadvantages	Reference
French press	SUV 0,04-0.06 µm	10-30	Gentler handling of labile materials vesicles of greater stability Unilamellar vesicles with 10 to 50 times larger aqueous space than sonicated vesicles. Liposomes could be prepared even at lower temperatures like 0°, 25° etc.		Hamilton et al. 1980
Freezing and thawing	0.05-0.2 µm	10-30		Size and entrapment efficiency critically dependent on the ionic strength of the buffer	Pick 1981
Ether injection	unilamaller 0.06-0.13 µm	1	Vol. trapping efficiency is 10-100 times that of sonicated preparations suitable for macromolecules.	Unsuitable for heat or ether labile drugs Low encapsulation efficiency not homogeneous in size.	Deamer & Bangham 1976 Deamer 1978
Ethanol injection	SUV .03-0.11 µm	0.4-1.5		Heterogeneous Produces a dilute dispersion of SUV. Low encapsulation efficiency.	Batzri and Korn 1973 Kremer et al. 1977
Differential high-speed ultracentrifugation	Unilamellar		Homogeneous size distribution No disadvantages of molecular sieve chromatography. Rapid method. Higher vesicles yield without dilution.		Barenholz et al. 1977.

principle of detergent dialysis, reported by Milsman et al. (1978) and Zumbuehl and Weder (1981). Another liposome preparation instrument, a microemulsifier called Microfluidizer M-110**TM, has recently emerged. It works on the principle of fluid flow and produces liposomes of uniform size from 50 nm to 1000 nm.

DRUG ENTRAPMENT IN LIPOSOMES

The potential of liposomes as drug carriers was recognised in the early 1970s. Since then the aspect of drug entrapment into liposomes has been addressed by several authors (Fendler and Romero 1977, Stamp and Juliano 1979, Fendler 1980). The entrapment of a drug is accomplished by placing the drug either in the aqueous phase or in the lipid phase during the preparation of liposomes. Drugs that are lipid soluble are treated similar to lipids and those that are water soluble are dissolved in the aqueous phase. Drugs of intermediate polarity could be treated either way.

The distribution of a drug within the liposomes is governed by its polarity. The nonpolar drugs are

---

**MicroFluidics Corporation, a division of Biotechnology Development Corporation, 44 Mechanic Street, Newton, Massachusetts 02164 U.S.A.)

intercalated with the lipid bilayer whereas polar ones are present in the aqueous phase. The drugs with intermediate polarity span both aqueous and lipid phase of the vesicle (Fig. 2).

There is an interplay of several factors affecting the entrapment of drugs within liposomes. These factors are:

Physicochemical properties of the drug
- solubility
- steric hindrance

Properties related to liposomes
- size
- charge
- lipid composition
- capture volume
- ionic concentration, pH of hydration medium

Method of preparation

Miscellaneous

Physicochemical properties of drug:

The drugs that are highly soluble either in water or in lipid are encapsulated well in liposomes. On the contrary, the encapsulation of drugs with only marginal solubility both in water and lipid, e.g. 6-mercaptopurine (Fendler and Romero 1977), is not efficient. Defrise-Quertain et al. (1980) have related the log P values of drugs to their encapsulation efficiency in liposomes. The log P value is the logarithm of octanol

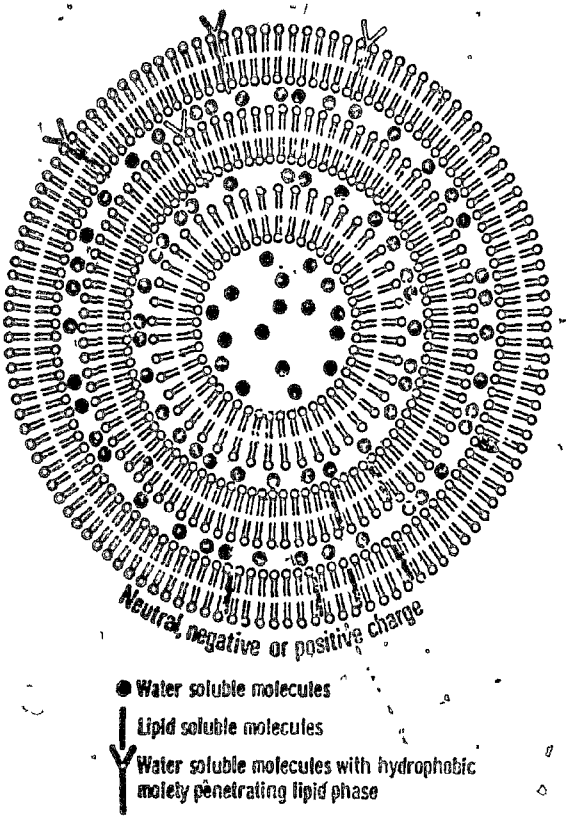


Figure 2. Distribution of drugs within a liposome (Gregoriadis 1976).

water partition coefficient of the drug and is a measure of its solubility. Defrise-Quertain et al. concluded that drugs with log P values of greater than 5 and less than -0.3 were optimum candidates for encapsulation in liposomes. Drugs with log P values between 1.7 and 4 were the worst candidates for this purpose. This finding was supported by the fact that drugs like cortisol palmitate (log P ≈ 9), cortisol octanoate (log P = 4.75) and methotrexate (log P = - 1.85) were encapsulated well in liposomes. On the contrary, the encapsulation of drugs like cortisol (0.59 < log P < 1.93) was very poor (Defrise-Quertain et al. 1980).

The log P values of drugs could be increased by chemical modification such as palmitoylation. This approach has been successfully exploited for enhancing drug encapsulation in liposomes (Shaw et al. 1976, Goundalkar and Mezei 1984). One should, however, be cautious not to reduce the pharmacological activity of the drug as a result of such chemical manipulation. Within a certain limit the liposomal encapsulation of palmitoyl derivative of a drug approaches 100% (Goundalkar and Mezei 1984).

Since in chemotherapeutic applications it is the absolute drug concentration rather than the percentage incorporation, that is the important factor, it is essential to increase the amounts of drug entrapment as much as possible (Fendler and Romero 1977). The palmitoylation not

only provides such increased absolute drug concentration in liposomes but also the encapsulation is complete (i.e. 100%). Thus, the liposomal encapsulation obtained by palmitoylation is unsurpassed by any other method. Hence this approach is worth trying even for water soluble drugs with optimum log P values such as methotrexate. Preparation of drug-phosphatidate compounds and hydrophobic prodrugs are other analogous approaches suggested for improving liposomal encapsulation of drugs (Page Thomas and Phillips 1979, Knight 1981b). The compound, squalene, stands out distinctly as a compound with a high log P value and yet has very poor liposomal encapsulation. This unusual behavior is attributed to the steric hindrance caused by this molecule (Defrise-Quertain et al. 1980). Other miscellaneous principles like ion pairing (Jay and Digenis 1982), charge transfer complex formation (Tsujii et al. 1976, Kano and Fendler 1977) have also been utilized for enhancing drug entrapment in liposomes.

Properties related to liposomes:

Capture volume, size, charge: The higher the capture volume, the higher is the entrapment of water soluble drugs. The same relationship also applies to the size of liposomes, since size and capture volume are often proportional. The presence of a charge on liposomes may lead to increased encapsulation (Alpar et al. 1981). As mentioned earlier, higher entrapment is attributed to

increased aqueous space due to electrostatic repulsion between like charged lamellae of liposomes (Puisieux and Benita 1982).

Lipid compositions Hydration medium: Cholesterol plays a major role as a stabilizing agent for liposomes. It reduces the leakage of drugs out of liposomes. Apart from this, it also contributes to increased encapsulation of drugs like doxorubicin (Yotsuyanagi et al. 1981). Conversely, cholesterol may also interfere with encapsulation (Tsukada et al. 1982). The phospholipid composition could also play a major role in determining the efficiency of drug incorporation in liposomes (Tsukada et al. 1982).

The influence of the pH of hydration medium on the association of doxorubicin with liposomes has recently been reported (Crommelin et al. 1983).

SEPARATION OF FREE DRUG FROM LIPOSOMES

The encapsulation of drugs within liposomes is rarely one hundred percent. A lot of drug remains unentrapped. Separation of untrapped or "free" or "naked" drug is necessary in order to obtain a "pure" liposomal preparation. The methods used for this purpose are as follows:

Gel filtration

Centrifugation

Dialysis

Ultracentrifugation

Other methods

Gel filtration:

When a crude liposomal preparation is passed through a gel filtration (molecular sieve chromatography) column, liposomes come out in the void volume. The free drug appears later in the retarded volume. So, the two can be easily separated. However, problems of mechanical blocking of columns have been experienced with crude MLV and REV liposomes (unpublished observations). Filtration of liposomes through smaller pore size polycarbonate filters may perhaps overcome this problem. Also, to be considered is the non specific adsorption of liposomal lipids to the gel of the column. A way to get around this problem is to presaturate the column with lipids before use (Huang 1969).

The dilution of the liposomal fraction is a main drawback of this method. The diluted preparation can, however, be concentrated by ultrafiltration method, for example, using amicon centriflow ^{R} filters. Various gel filtration media, e.g. Sephadex G-25, G-50, Sepharose 4B, 6B etc., are being used for this purpose. Sepharose CL-4B, CL-2B and controlled pore glass have been used also to separate multilamellar liposomes from unilamellar ones (Chen and Schulle 1979, Yotsuyanagi et al. 1981). An evaluation of different gel-media for their ability to separate liposomes of different size has been reported (Jederstrom and Russell 1981). When the unentrapped

substance is a macromolecule like DNA, protein etc., the choice of proper gel is more important, as routine gels like Sephadex G-50 or G-25 cannot be used in such cases. The upper limit of the fractionation range of gels like Sephadex G-50 or G-25 is less than the molecular weight of the protein.

Centrifugation:

Macrovesicles can be sedimented down by high speed centrifugation. The liposome pellet could further be washed by centrifugation to remove the nonspecifically adhering drug. However, this method of separation cannot be applied to SUV. Also, when the unencapsulated drug is in the form of undissolved crystals this method has little value. Simplicity and absence of dilution effect are the main advantages of this method.

Dialysis and Ultracentrifugation:

Untrapped drug can also be separated from liposomes by exhaustive dialysis. However, leakage of trapped drug out of liposomes may be a problem sometimes. Dialysis has also been used to improve the liposome size distributions (Bosworth et al 1982). The ultracentrifugation method of separating free and encapsulated drug is used mainly for microvesicles, like SUV. Repeated washing of liposomal pellet is needed to remove the adhering free drug as in other centrifugation methods.

Other methods:

The method of differential centrifugation has been used to separate liposome encapsulated nucleic acids from unencapsulated material. Similarly, nuclease digestion followed by gel filtration and floatation of liposomes on discontinuous polymer gradients, have been used for the separation of liposomes from free nucleic acids. The latter method is rapid and well suited for the separation of liposomes under sterile conditions (Fraley and Papahadjopoulos 1981).

FILTRATION OF LIPOSOMES

Filtration of liposomes is done either to reduce the size of liposomes (Morii et al. 1983), or to improve the size distribution of liposomes (Olson et al. 1979).

The filtration of liposomes is usually carried out under positive or negative pressure, using filters made of polycarbonate (Olson et al. 1979). The liposomes obtained by filtration are supposed to be more uniform than unfiltered ones. It is important to note that the "soft" liposomes, those slightly bigger than the pore size of the filter, are able to pass through the filter by deformation with no change in post-filtration liposome size (Brendzel and Miller 1980). Use of elevated temperature is known to facilitate the operation of filtration reducing the chances of clogging of filters (Olson et al. 1979). A combination of reverse phase evaporation and extrusion

through polycarbonate membranes has been shown to produce unilamellar liposomes of intermediate size (0.1-0.2 μm (Szoka et al. 1980, Duzgunes et al. 1983).

CHARACTERIZATION OF LIPOSOMES

The properties of liposomes have profound influences on their behaviour in vitro or in vivo. Therefore, the characterization of liposomes in terms of their size, surface charge, drug release etc. are important.

Size distribution of liposomes:

The average size and the size distribution of liposomes are important parameters affecting the behaviour of liposomes. Optical microscopy has been used by some investigators (Rahman et al. 1974, Gulasekharam 1980) to determine gross size distribution of liposomes especially MLV and REV. Since smaller liposomes are beyond the resolution of an optical microscope, the use of this method is far from satisfactory to obtain an exact size distribution of liposomes. Fluorescence microscopy could be used if a fluorescent lipid is included in the lipid bilayer of liposomes. Electron microscopy seems to be the popular method of assessing liposome size. However, it involves considerable skill, effort and time to prepare samples for electron microscopic analysis.

Gel exclusion chromatography on Sephacryl S-1000 has been used to analyse, as well as to fractionate, liposomes on the basis of their size (Nozaki et al. 1982, Machy

and Leserman 1983, Reynolds et al. 1983). An Agarose Gel TLC method is particularly suited for a quick assessment of the efficiency of sonication, consequently the size reduction of liposomes (Van Renswoude et al. 1980). The sedimentation field flow fraction (SFFF) method described by Kirkland et al. (1982) is rapid and analyses both size and particle weight of liposomes. A laser light scattering method called quasi-elastic light scattering (QELS) has been used by Morri et al. (1983) to obtain average diameter and polydispersity index of liposomes. It is said that this method can detect even very small liposomes down to 30 nm (Rao 1983). A technique called photon correlation spectroscopy has also been used to study vesicle size (Gole and Carlson 1982).

Another simple method of obtaining liposome size distribution is to use an electronic counter i.e. Coulter counter with proper specifications (400-channel analyser, 10-15 µm aperture) as recommended by Rahman (1980) and used by others. It is an extremely rapid method and gives an accurate size distribution. Dilution of the sample is all that is needed to count the liposomes using this instrument. The recently introduced "Coulter Model N4 Analyser" which measures particle size in the range of 3 nanometers to 2 micrometers would perhaps be a better model for size analysis of liposomes. Once the size distributrion of a liposome preparation is known, the other parameters such as

particle number, surface area, trapped volume etc. can easily be derived (Pidgen and Hunt 1981).

Surface charge of liposomes:

It is usually assumed *a priori* that incorporation of a charged lipid gives rise to either positive or negative charge on the surface of liposomes. No tests are usually carried out to check whether this is true. However, the surface potential (quantitative measure of surface charge) of liposomes could be measured in a Zetameter (Bangham *et al*. 1974) or by using a fluorescent pH indicator (Fernandez 1981).

Drug release from liposomes:

The *in vitro* testing of drug release from liposomes could reflect *in vivo* release pattern. Therefore, some investigators have carried out efflux experiments before administering the liposomes *in vivo*. Most of the techniques used to separate free and bound drug could be used to evaluate drug release from liposomes.

The dialysis technique was used by Arakawa *et al*. (1975) to test the release of model water soluble compound from liposomes. Technique of ultrafiltration through Amicon centriflo CF-25 was applied by Tsukada *et al*. (1982) in their release study. In a recent report Arrowsmith *et al*. (1983) used centrifugation technique to study *in vitro* release of steroids from liposomes.

STABILITY AND STORAGE OF LIPOSOMES

In many of the investigations liposomes have been stored in the refrigerator from a few days to a few weeks without apparent instability. However, such short time stability is of little use if liposomes are ever to be used like other pharmaceutical preparations. As a pharmaceutical preparation, liposomes should have a shelf life of at least a year, preferably at room temperature (Rao 1983). Stability and storage studies of liposomes have special significance in this context.

Frokjaer et al. (1982) have investigated the long term storage of liposomes. They concluded that distearoylphosphatidylcholine (DSPC)/cholesterol (CHOL)(2/1) liposomes were stable physicochemically when stored at 4°C for about 10 months. Similarly Rao (1983) has claimed that a Pentostam^{R} liposome product was stable for several months with less than 10% leakage. The formulation or composition of this product, however, was not reported.

Van Bommel and Crommelin (1983) have tried freeze drying and freezing of liposomes for storage. They reported that in most cases the aqueous marker was lost considerably as a result of freeze thaw cycle or freeze drying. However, this was dependent on lipid composition. Best results were obtained with dipalmitoylphosphatidylcholine (DPPC)/ dipalmitoylphosphatidylglycerol(DPPG) 10/1 vesicles. Using additives like lactose improved the stability of these

vesicles to freeze drying. Strauss and Ingenito (1980) have investigated the use of cryoprotective agents (e.g. glycerol) and membrane proteins in stabilizing liposomes to freezing and thawing effect. Effect of phospholipid: cholesterol ratio on the response of liposomes to various rates of cooling to -196°C has been reported by Morris (1982). In the absence of DMSO, the cryoprotectant, liposomes lost the entrapped glucose completely following freezing to and thawing from -196°C. In the presence of DMSO, however, liposomes containing 20 mol% cholesterol exhibited minimum glucose release when subjected to similar treatment at a cooling rate of $1.7^{\circ}C\ min^{-1}$ (Morris 1982).

DRUG TARGETING

Drugs are "double edged swords". They have beneficial effects as well as toxic side effects. Severity of toxic effects may sometimes not be as serious as we think. But, certainly the toxic effects are very severe in case of anticancer drugs. These toxic side effects are, often, due to the non selective drug disposition and therefore the indiscriminate action of the drugs. For instance, anticancer drugs exercise their lethal effect on proliferating cells of both normal type as well as malignant type. There is not much qualitative difference between a cancer cell and a normal dividing cell. Therefore in order to reduce or eliminate the toxic effects, drugs have to be

directed to only the diseased sites (i.e. target sites) to achieve selective action.

The concept of drug targeting has been with us ever since Paul Ehrlich in 1906 dreamt about the "magic bullets" of diphtheria toxin for the treatment of cancer (Magee and Ristow 1983, Poste and Kirsh 1983). Recently there has been dramatic increase in research activities to solve the problem of drug targeting.

The various approaches for drug targeting could be chemical, physical or biological. Prodrugs, the drugs which can be converted into active form at target sites, are examples of chemical approaches (Henderson 1983). Physical approaches embrace such wide areas as emulsions (Kreuter 1983b), polymers (Henderson 1983) microspheres, magnetically responsive or otherwise (Illum and Davis 1982, Widder et al. 1982), nanoparticles (Illum et al. 1983, Kreuter 1983abc), red blood cells (RBC) (Pitt et al. 1983) and RBC ghosts (Ihler 1979) and liposomes (Gregoriadis 1981, Rogers 1982, Widder et al. 1982, Banker et al. 1983, Gregoriadis 1983b) and so on. The biological approaches include the use of biomolecules such as deoxyribonucleic acid (DNA) (deDuve 1980, Trouet et al. 1981, lipoproteins (Counsell and Pokland 1982), and antibodies (Gregoriadis 1983b, Gregoriadis et al. 1982). Antibodies, liposomes, and the combination will be discussed

in more detail since this is one of the main topics of this thesis.

ANTIBODY MEDIATED DRUG TARGETING

The idea of using antibodies as drug carriers was conceived by Paul Ehrlich as early as 1906. However, it was not until 1958, that this idea was first put to application by Mathe et al. (1958). Since then several studies have been reported on cytotoxic drugs as well as diagnostic agents linked to antibodies. Comprehensive review articles have covered these studies (Ghose and Blair 1978, Rankin and McVie 1983, Rowland 1983).

Most investigations of antibodies as drug carriers have been conducted with heterologous (polyclonal) antibodies raised in rabbit, goat, etc. The procedure of extensive absorption of the xenogenic antisera with normal tissues (liver, spleen, kidney, RBC) has been used to improve the specificity of such polyclonal antibodies. This procedure, however, sometimes takes away the specificity of the antibodies and other times leaves toxic antinormal antibodies, resulting in an antibody preparation which is either toxic or non specific. The monoclonal antibodies of recent origin may be an answer for such major drawbacks of polyclonal antibodies. Further, the advent of hybridoma (hybrid myeloma) technology has made possible the production of monoclonal antibodies in adequate amounts (Levy and Miller 1983). These monoclonal antibodies have exceptional

specificity and therefore they are displacing the polyclonal antibodies in their role as carriers of drugs and/or diagnostic agents. Recent reviews signify such a trend (Baldwin 1983, Baldwin and Pimm 1983, Davis and Illum 1983, Lutz 1983, Obrist 1983, Poste and Kirsch 1983, Conner 1984).

LIPOSOMAL DRUG TARGETING

Targeting of drugs using unmodified liposomes is referred to as passive targeting of liposomes (Poste 1983, Poste and Kirsh 1983). Liposomes, like other colloidal particles, are rapidly scavenged by organs like liver, spleen and bone marrow which are rich in reticuloendothelial cells. The capillaries in the lung can physically trap aggregates of liposomes. These properties have made liposomes selective drug carriers in diseases like leishmaniasis where target sites are liver cells. Many anticancer drugs have also been tried in liposomes for targeting or to reduce side effects (Kaye 1981, Yatvin and Lelkes 1982). Weinstein et al. (1979) applied hyperthermia to cause selective release of methotrexate (MTX) from liposomes. Strategies like this, in which the drug-carrier complex releases the drug only when exposed to specific microenvironment changes such as pH or temperature, have been referred to as physical targeting by Poste and Kirsh (1983). This category also includes magnetic drug carriers which could be modulated by external magnetic fields. In many instances liposomes have improved the

effectiveness of the entrapped drug for reasons other than targeting. Those reasons are - protection of drug from biological degradation, (Weinstein and Leserman 1984), reduction of toxic effects (Forssen and Tokes 1981), improved transport of drugs across transport deficient tumor cells (Patel et al. 1982, Todd et al. 1982, Deliconstantinos et al. 1983) depot effect (Kaye et al. 1981).

However, in many instances liposomal entrapment of drugs has not brought about any significant beneficial effect, rather in some cases it has led to increased toxicity. Most of these negative effects have been attributed mainly to the uptake of liposomes by reticulo endothelial system (RES). Efforts were therefore concentrated to overcome this situation. Use of antibodies conjugated to liposomes is a result of such efforts.

As mentioned earlier, the properties of antibodies which enable them to interact specifically with complementary antigens has made them suitable as site specific drug carriers. However, there are some major concerns for using antibodies alone as drug carriers - one is that, the carrier capacity of antibodies is limited. If too much drug is loaded on an antibody, its reactivity is at stake. Secondly, administration of a large quantity of antibody-drug conjugate, to compensate for the limited carrier capacity, could lead to allergic reactions.

Perhaps the combination of the higher carrier capacity of liposomes and target seeking capability of antibodies may be the answer for some of the problems outlined above. As a result this field of active targeting of liposomes is getting increased attention these days (Zaharko et al. 1979, Gregoriadis 1982, Magee and Ristow 1983). The term "active targeting of liposomes" is, however, not restricted to liposome-antibody complexes but includes all liposomes bearing any ligand that will "recognize" molecular or macromolecular determinants of the surface of the "target" cells (Poste and Kirsh 1983).

BINDING OF ANTIBODIES TO LIPOSOMES

In order to realize the potential of active or immunospecific targeting of liposomes, the two entities, namely, antibody and liposomes, have to be somehow associated or conjugated. Initial studies resorted to physical binding of antibodies to liposomes. This means that the antibodies were bound by methods like incubation with preformed liposomes or during liposome preparation by co-sonication of immunoglobulins and liposomes (Gregoriadis and Neerunjun 1975). Two main criticisms have been levelled at this kind of binding. The force of association may be too weak to keep the two entities together for sufficient time until the liposomes reach the target sites. Secondly, the amount of antibody that is bound to vesicles may be too small to serve its needed purpose.

Significant improvements over these drawbacks came about when chemical or covalent binding methods were introduced. The advent of covalent binding methods were partly due to the existence of such linking procedures in areas such as protein modification, enzyme-antibody conjugation, hapten-carrier conjugation, cytoxic agent-antibody conjugation (Carraway and Koshland 1972, Wold 1972, Bauminger and Wilchek 1980, Erlanger 1980, O'Sullivan and Marks 1981, Blair and Ghose 1983, Ghose et al. 1983).

Among the several methods available to date, some are of particular interest because of their high efficiency of binding. The method reported by Barbet et al. in 1981 has a binding efficiency of more than 40%. In this method the antibody has to be modified with the heterobifunctional reagent N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) and then reduced with dithiothreitol before coupling to liposomes. Another method by Martin et al. (1981) also has a high efficiency of coupling (14-26%). Both these methods require the presence of a reactive disulfide group in the liposome membrane. Reactive liposome lipids for this purpose are synthesized from reaction between SPDP and amine containing phospholipids like phosphatidylethanolamine (PE), dipalmitoylphosphatidylethanolamine (DPPE) etc. The binding of antibodies to liposomes in both cases is brought about by an exchange reaction between the thiol group of the antibody

and disulfide group present in the liposomal membrane. The difference in the two methods lies in the way the thiol group is generated in the antibody. In the method described by Martin et al. (1981) the thiol antibody (Fab'-SH) is obtained by the reduction of $F(ab')_2$, whereas in the other method immunoglobulin G (IgG) is first modified using SPDP by the method of Carlsson et al. (1978). Modified IgG is then treated with dithiothreitol to produce thiol IgG which then reacts with liposomes. In both the methods, homopolymerization and/or intramolecular cross linking are kept to a minimum because thiol-disulfide reaction is faster than thiol-thiol interaction. The method described by Martin et al. is time consuming, as it involves many steps like digestion of IgG, protein A sepharose 4B and sephacryl S-1000 column chromatography to separate $F(ab')_2$. Methods similar in principle to that of Barbet et al. have been used to conjugate antibodies or proteins to sendai virus particles (Tomasi et al. 1982) and to red blood cells also (Godfrey et al. 1981, Jou et al. 1983).

There is a drawback in using a disulfide linkage to bind antibodies to liposomes. Disulfide bonds could be cleaved by thiols such as cysteine present in the serum. An improved method was reported by Martin and Papahadjopoulos (1982) to overcome such unstable linkage. The improved method utilizes a maleimide derivative of

phosphatidylethanolamine and introduces an essentially irreversible thio-ether bond between antibody (Fab') and liposomes. Using this same principle, Hashimoto et al. (1983b) have linked subunits of IgM antibodies, a different class of antibodies, to liposomes. Immunoliposomes bearing IgM fragments, being divalent, may have a superior antigen binding property compared to Fab' bearing liposomes.

The detergent (deoxycholate) dialysis method, a method to prepare liposomes, has also been used to anchor antibodies to liposomes. Variations of the method have been reported (Harsch et al. 1981, Shen et al. 1982). Whatever the variation, a derivative of the antibody has to be made with palmitic acid prior to binding. This is usually done by first preparing an activated ester of palimitic acid using N-hydroxysuccinimide and then reacting this ester with the antibody. Based on percent binding, the detergent dialysis method may appear to have a high efficiency of binding. However, in fact, the absolute amount of antibody bound to liposomes, which is perhaps more important, is low in these cases. Furthermore, the majority of liposomes did not bind the antibody. Goldmacher (1983) attributes this to the saturaced palmitoyl chain. The rotations about the C-C bond give a coiled conformation to the saturated hydrocarbon chain, making it sterically difficult to penetrate into the liposomal membrane. He, therefore, advocates the use of linoleil derivative of

protein rather than palmitoyl derivative for binding to the liposomes. All the detergent dialysis methods suffer from a drawback that the entrapped drug may leak out of liposomes during the procedure of antibody incorporation.

Carbodiimides can also be used to cross link antibodies to liposomes. They react with free carboxyl groups of IgG to form a reactive intermediate, which further reacts with primary amino groups provided by lipids such as PE, DPPE in liposomes. These methods, though rapid, suffer from low amount of binding and reduced efficiency. Moreover undesirable homocoupling and intramolecular crosslinking in antibodies, liposomes or both cannot be ruled out. The principle, binding parameters and the chemistry of different binding methods are given in Tables 3 and 4.

Ideally the choice of a method should meet the following requirements: 1) a sufficient quantity of antibody must be bound with the liposomes; 2) the liposome-antibody bond should be stable; 3) the antibody specific binding properties should remain unchanged; 4) the liposome integrity during the immobilization procedure should be preserved.

LIPOSOME CELL INTERACTIONS IN VITRO

Some indication of *in vivo* performance of liposomes could be obtained by studying their interaction with cells *in vitro*. Such studies necessitate the prior

Table 3
Binding of antibodies to liposomes

Method and Reference	Binding parameters: % binding of antibody at certain protein conc.	Binding parameters: µg antibody /µmole lipid	Binding parameters: No. of antibody molecules/vesicle	Principle of binding
1) SPDP method Martin et al. (1981)	14% at 12.5 mg/ml to 26% at 1 mg/ml	100 to 600 µg Fab'/µmole phospholipid	6000 Fab'/0.2 µm vesicle	2-pyridyldisulfide derivative of PE is prepared using SPDP. $F(ab')_2$ ie Fab'-s-s-Fab' is reduced with DTT to get Fab'-SH which then reacts with PDP-PE in liposomes. Thiol disulfide interchange links Fab' to liposomes via disulfide bridge.
2) SPDP method Leserman et al. (1980) Barbet et al. (1981) Leserman et al. (1981)	>40%		1-10 antibody molecule/580A° vesicle	Using SPDP, 2-pyridyldisulfide groups are first introduced into both IgG and PE. 2-pyridyldisulfide derivative of IgG is then reduced with DTT and reacted with PDP-PE in liposomes. Thiol disulfide interchange links IgG to liposomes via disulfide bond.
3) SMPB method Martin & Papahadjopoulos (1982)	20-30%	70-584 µg Fab' /µmol phospholipid at 0.5-4.0 mg/ml	3000 Fab' at 340 µg/µmol /0.2 µm vesicle.	4-(p-maleimidophenyl) butyryl derivative of phosphatidyl ethanolamine (MPB-PE) is prepared using SMPB. $F(ab')_2$ is reduced with DTT to give Fab'-SH, which then reacts with maleimide moiety of MPB-PE in liposomes. Alkylation reaction cross links Fab' to liposomes via thioether bridge.

Table 3 (continued)

Method and Reference	Binding parameters: % binding of antibody at certain protein conc.	Binding parameters: µg antibody /µmole lipid	Binding parameters: No. of antibody molecules/vesicle	Principle of binding
4) MBPE Method Hashimoto et al. (1983b)	34-68% at 2 - 0.5 mg/ml	31-61 µg IgG protein/µmole phospholipid at 0.5-2 mg/ml		N-(m-maleimidobenzoyl) derivative of DPPE (i.e. MBPE) is obtained using m-maleimidobenzoyl-N-hydroxysuccinimide (MBS). IgM is reduced with cysteine to get SH-bearing subunits which is reacted with MBPE in liposomes. IgM subunits link to liposomes through F_c portion via thioether bridge.
5) Detergent dialysis (palmitoylation) method Huang et al. (1980) Harsch et al (1981) Huang et al. (1982) Shen et al. (1982)	80% or more	100 µg/mg phospholipid	48 IgG/1000A° liposome	Antibody is derivatized with palmitic acid using an activated ester of N-hydroxysuccinimide. Palmitoyl antibody is incorporated into liposomes by detergent (deoxycholate) dialysis method.
6) Carbodiimide method Johnson et al. (1966) Dunnick et al. (1975) Endoh et al. (1981)	0.125%	19-20 µg IgG /µmole PC at 10 mg/ml IgG.		Water soluble carbodiimide (EDCI) activates carboxyl groups on IgG to react with nucleophilic groups such as NH_2 on phosphatidylethanolamine present in liposomes. Amide bond links IgG to liposomes.

Table 3 (continued)

Method and Reference	% binding of antibody at certain protein conc.	Binding parameters: µg antibody /µmole lipid	No. of antibody molecules/vesicle	Principle of binding
7) Citraconylation method Jansons and Mallett (1981)		167-500 µg $F(ab')_2$/µmole phospholipid		First citraconylation of $F(ab')_2$ is done to protect its ϵ-NH_2 groups. Using carbodiimide, phosphatidyl ethanolamine derivative of $F(ab')_2$ is prepared. Amide bonds are formed between carboxyl groups of $F(ab')_2$ and NH_2 group of PE. $PE(Fab')_2$ is incorporated into liposomes by 24 hr incubation
8) Glutaraldehyde method Torchilin et al. (1979)	40-60%		1-2 antibody molecule/10000 lipid molecules	Amino group of PE in liposomes were activated using glutaraldehyde. Activated liposomes were coupled to NH_2 groups of antibodies by overnight incubation.
9) Periodate oxidation method Heath et al. (1980) Heath et al. (1981)	20%	100-200 µg protein/µmole lipid	140 molecules/ 0.2 µm vesicle	The aldehyde groups are produced by periodate oxidation of vesicles containing lipids bearing vicinyl hydroxyls (glycosphingolipids). These aldehyde groups react with amino groups on the protein to form imine (Schiff base) which is then stabilized by $NaBH_4$ reduction.

Table 3 (continued)

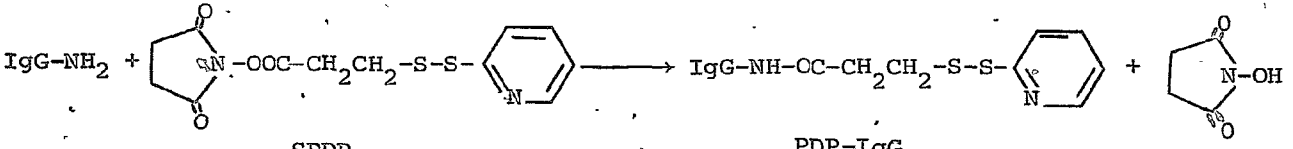
Method and Reference	Binding parameters: % binding of antibody at certain protein conc.	Binding parameters: µg antibody /µmole lipid	Binding parameters: No. of antibody molecules/vesicle	Principle of binding
10) Iodo ADLPE method Sinha & Karush (1979,1982)	~33%	100 µg Fab' /µmole total lipid		Sulfhydryl reactive, phospholipid containing alkylating reagent, N-(N^{α}-iodoacetyl, N^{ε}-dansyllysyl)-phosphatidyl ethanolamine (iodo ADLPE) is synthesized. Fab'-SH obtained by reduction of $F(ab')_2$ using mercaptoethanol is alkylated with iodo-ADLPE. Fab'-ADLPE is then incorporated into liposomes by incubation.

Table 4

Chemical reactions involved in covalent binding of antibodies to liposomes.

1. SPDP methods (Leserman et al. 1980, 1981, Barbet et al. 1981, Martin et al. 1981)

Obtaining thiolated antibody:



SPDP

PDP-IgG

DTT

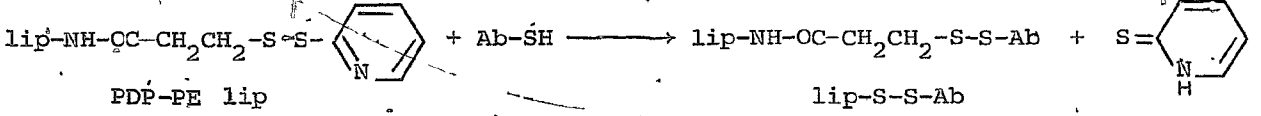
IgG-NH-OC-CH$_2$CH$_2$-SH

Ab-SH

$F(ab')_2 \xrightarrow{DTT}$ Fab'-SH

(Ab-SH)

Coupling antibody to liposomes:



PDP-PE lip

lip-S-S-Ab

Table 4 (continued)

2. SMPB method (Martin and Papahadjopoulos 1982)

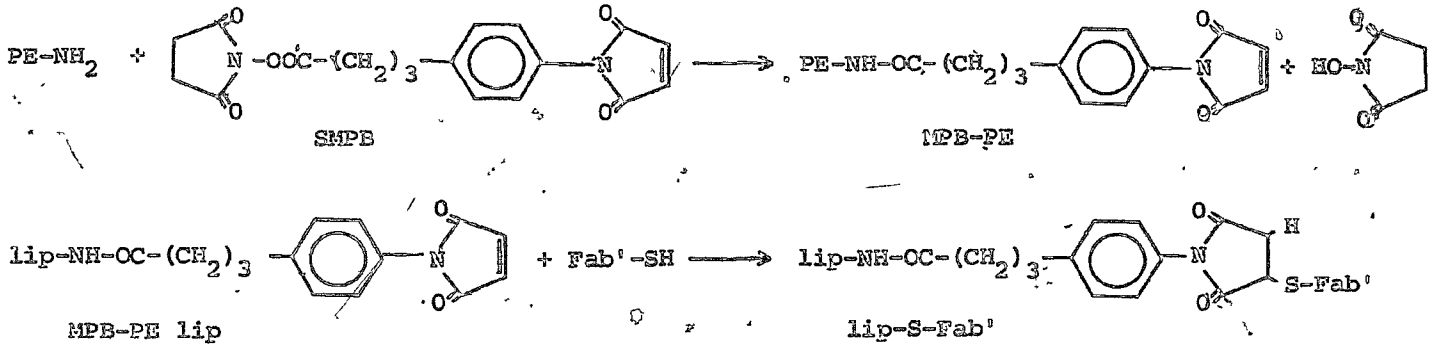
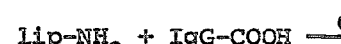


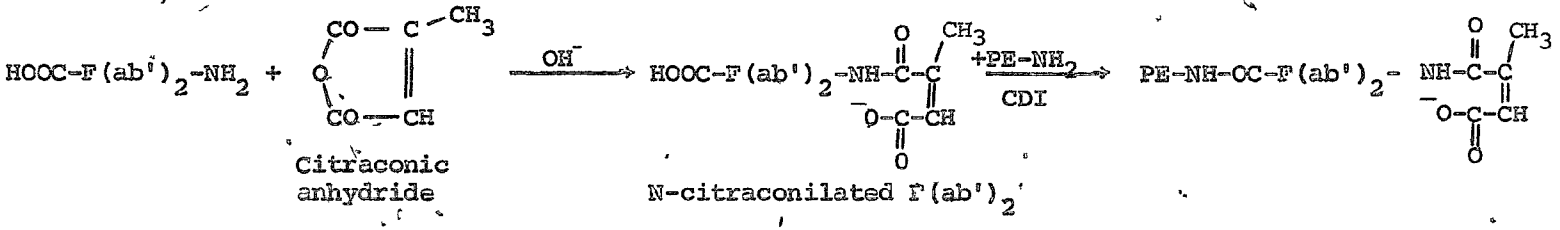
Table 4 (continued)

3. Carbodiimide methods (Johnson et al. 1966, Dunnick et al. 1975, Endoh et al. 1981, Jansons and Mallett 1981)



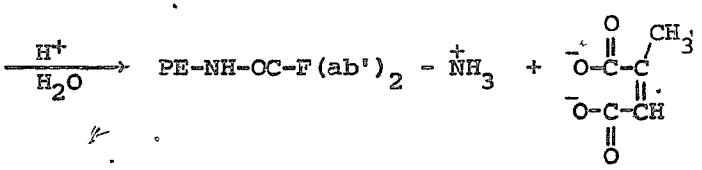
$$\text{lip-NH}_2 + \text{IgG-COOH} \xrightarrow{\text{CDI}} \text{lip-NH-OC-IgG}$$

using citraconylation to block ϵ-NH_2 groups of $F(ab')_2$:



$$\text{HOOC-F(ab')}_2\text{-NH}_2 + \text{Citraconic anhydride} \xrightarrow{OH^-} \text{HOOC-F(ab')}_2\text{-NH-C(CH}_3\text{)=CH-COO}^- \xrightarrow[\text{CDI}]{+\text{PE-NH}_2} \text{PE-NH-OC-F(ab')}_2\text{-NH-C(CH}_3\text{)=CH-COO}^-$$

N-citraconilated $F(ab')_2$



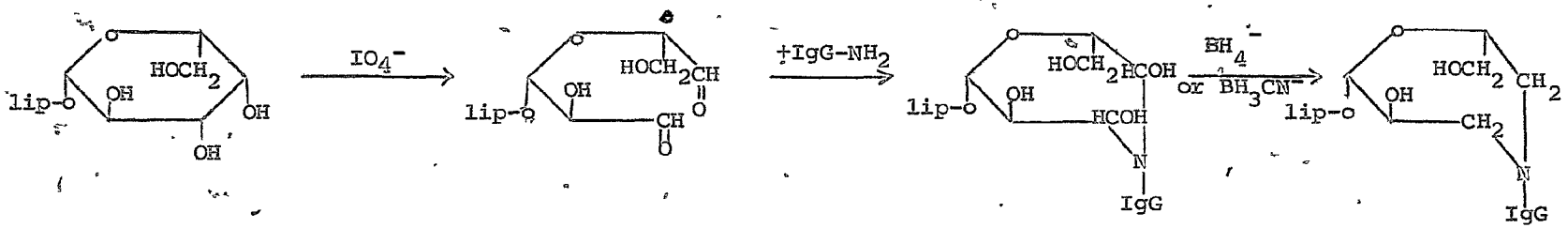
$$\xrightarrow[H_2O]{H^+} \text{PE-NH-OC-F(ab')}_2 - \overset{+}{\text{N}}\text{H}_3 + \text{Citraconic acid}$$

$PE\text{-}F(ab')_2$

$$\text{PE-F(ab')}_2 \xrightarrow{\text{lip}} \text{lip PE-F(ab')}_2$$

Table 4 (continued)

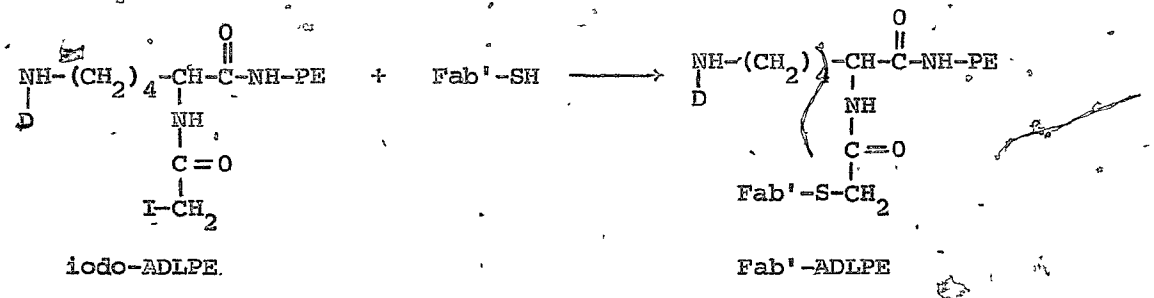
4. Periodate oxidation method (Heath et al. 1980,1981)



lip-glycolipid

Table 4 (continued)

5. iodo-ADLPE method (Sinha and Kazush 1979,1982):



iodo-ADLPE

Fab'-ADLPE

D = dansyl

= 5-dimethylaminonaphthalene-1-sulfonyl

Fab'-ADLPE $\xrightarrow{\text{lip}}$ lip ADLPE-Fab'

6. Glutaraldehyde method (Torchilin et al. 1979):

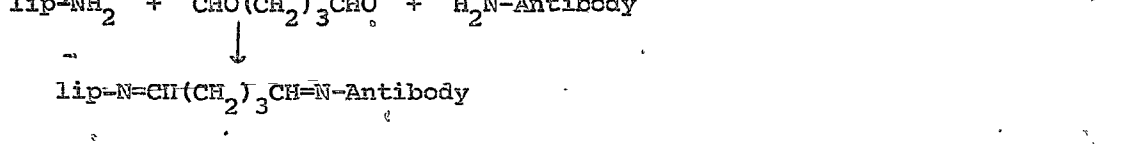
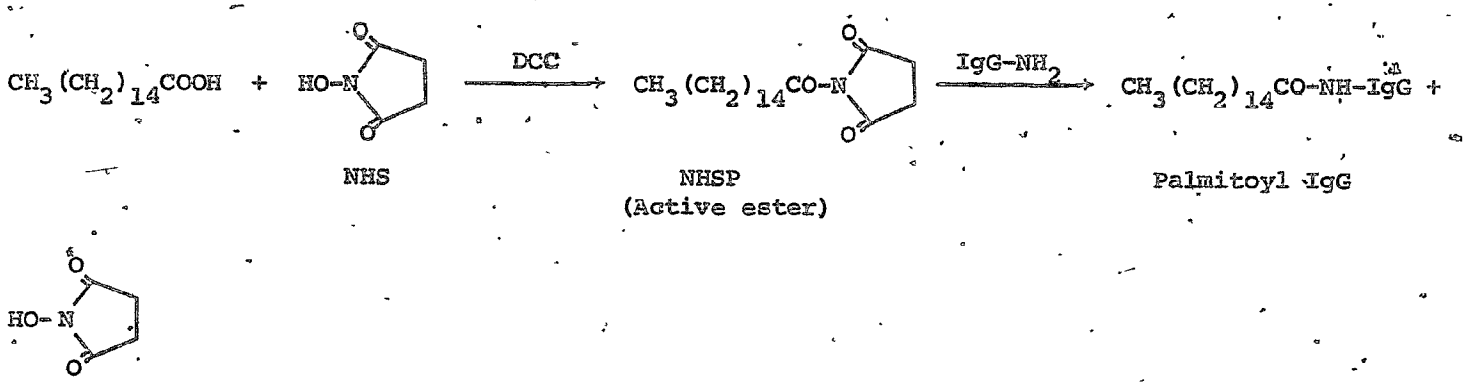


Table 4 (continued)

7. Palmitoylation Method (Huang et al. 1980, Harsch et al. 1981, Huang et al. 1982, Shen et al. 1982.)



knowledge of the stability of liposomes in the medium of cell culture. The various components of the cell culture medium may have profound effects on liposome-cell interactions. For example, calcium ions are known to cause increased binding of acidic liposomes to red blood cells. (Eytan et al. 1982). The poor stability of cholesterol free liposomes in the serum is very well known (Kirby et al. 1980a). It may sometimes be necessary to eliminate one or more of the interfering components of the cell culture medium to simplify the interpretation of interaction studies (Noteboom 1983).

There are different ways that these studies can be undertaken. Either the liposomal contents or the liposomal membrane, or more appropriately, both, could be used as markers. Lipid markers include radiolabelled phospholipid, cholesterol or more stable hexadecyl cholesterol ether (Pool et al. 1982). Schroit & Madsen (1983) have synthesized ^{125}I-phospholipids to see vesicle-cell transfer. Fluorescent lipids like N-(fluorescein isothiocyanyl)-phosphatidylethanolamine (N-FITC-PE) could also be used to serve the purpose. Water soluble fluorescent compounds like carboxyfluorescein could be used to make the aqueous contents fluorescent (Weinstein et al. 1977). In the case of immunoliposomes, antibodies, the third component could be labelled with ^{125}I. Recently,

liposomes containing colloidal gold are reported to be useful as a probe for liposome-cell interactions (Hong et al. 1983). Generally, in an interaction study, cells and liposomes are incubated together for a predetermined time. At the end of the incubation period liposomes are separated and the cells are washed to get rid of loosely bound liposomes. Subsequently, the cells are analysed for liposomal markers qualitatively (e.g. by fluorescence microscopy) or quantitatively (e.g. by counting radioactivity).

The separation of free liposomes from cell bound liposomes is an important step in vesicle-cell interactions. SUVs could be separated from cells by sedimenting the latter at low speed centrifugation. To facilitate the separation, materials like Ficoll 10%, Ficoll-paque, dextran 10%, etc, have been used in density gradient centrifugation (Jones & Osborn 1977, Fountain et al. 1980, Bragman et al. 1983). Liposomes usually float and cells pelletize in such density gradient medium. REV, MLV or other macrovesicles might still pose a problem with regard to their separation from cells.

Mechanisms of liposome cell interactions in vitro:

Whatever the method of study, liposomes are proposed to interact with cells in four major ways: 1) stable adsorption 2) endocytosis 3) fusion and 4) lipid exchange.

The definitions as given by Pagano & Weinstein (1978) and a brief description of each of these mechanisms follow:

1. Stable adsorption is the association of intact liposomes with the cell surface without their internalization. The term "stable" emphasizes that the adsorbed liposomes are not removed by washing of the cells. However, they could be detached by treatments such as trypsinization of the cells. (Pagano and Takeichi 1977, Chander et al. 1983). The adsorption of liposomes to cells depends on the transition temperature of phospholipids in liposomes as well as the temperature of study. "Solid" liposomes adsorb more than "fluid" liposomes. Interestingly, the relationship between temperature and adsorption to cells, as observed by Pagano and Takeichi (1977), is an inverse one. The forces involved in the adsorption process could be nonspecific (e.g. electrostatic, hydrophobic) or specific (e.g. surface receptors, antibodies).

2. Endocytosis is the uptake of intact liposomes into endocytotic vesicles. It is an active process requiring energy and therefore could be inhibited by cytochalasin B or other oxidative or glycolytic metabolic inhibitors. On endocytosis liposomes may fuse with lysosomes wherein they will be acted upon by various enzymes, causing the release of active ingredients into the cytoplasm. "Solid" liposomes are more prone to endocytosis than "fluid" ones. A recent investigation indicates that smaller liposomes are

endocytosed more easily than larger ones (Machy and Leserman 1983).

3. Fusion is the merging of lipid bilayer with the plasma membrane and concomitant release of liposome contents into cytoplasmic space. It can be distinguished from endocytosis on the basis of inhibitor studies. Fusion does not require energy and hence is not inhibited by metabolic inhibitors.

4. Lipid transfer is the transfer of lipid molecules between liposomes and cells without the association of the cell with the aqueous contents of the liposome. Proteins present at the cell surface or in the medium may mediate the transfer of lipid molecules. Loss of liposomal phospholipid to plasma high density lipoproteins (HDL) is an analogous example of lipid transfer.

Mechanisms outlined above are not mutually exclusive (Pagano and Weinstein 1978). For instance, adsorption is a prerequisite for endocytosis of the rest of the vesicle. More over, one or more mechanisms may be taking place simultaneously. However, certain mechanisms may be predominant depending on cell type, e.g. phagocytic or nonphagocytic, conditions of incubation and the properties of liposomes (charge, size, fluidity). Use of fusogens like polyethylene glycol, virus, lysolecithin, lectins, etc. to bring about cell-cell fusion are well known. These agents could similarly be used to induce fusion of liposome with

cells although lysolecithin was more toxic to cells (Szoka et al. 1981).

Kimelberg and Mayhew (1978) have illustrated the different interaction mechanisms diagramatically. These authors also state that transcapillary passage of liposomes and/or their contents may follow similar mechanisms. Several other reports have appeared to date on liposome cell interactions. Some have addressed the question of mechanism of interaction. Others have done such studies from another perspective, mainly to see the effectiveness of liposomes for drug delivery to cells or organisms in in vitro culture conditions. Both increased and decreased delivery of liposome contents has been observed (Weinstein et al. 1978, Allen et al. 1981, Norrie et al. 1982, Onaga and Baillie 1982, Patel et al. 1982, Todd et al. 1982, Deliconstantinos et al. 1983, Jansons and Panzner 1983).

DISTRIBUTION AND INTERACTIONS OF LIPOSOMES IN VIVO

Liposome behavior within the biological milieu is governed by a number of factors. Route of administration is one such important factor. Other factors are comprised of characteristics related to liposomes such as size, charge, lipid composition, dose etc. Role of these factors would be discussed appropriately under different routes of administration.

1. Intravenous route (I.V.)

On intravenous administration, liposomes rapidly interact with blood components. Serum proteins such as apolipoproteins, high density lipoproteins (HDL), could cause aggregation, alteration of permeability and destabilization of liposomes (Scherphof et al. 1978; Allen and Cleland 1980; Guo et al. 1980; Allen 1981; Gregoriadis et al. 1983). The extent of any damage to liposomes depends on their composition (Senior and Gregoriadis 1982, Bali et al. 1983). Cholesterol incorporation renders liposomes less vulnerable to attack by plasma proteins and subsequent loss of their phospholipids to HDL (Gregoriadis and Davis 1979; Kirby et al. 1980ab; Kirby and Gregoriadis 1980).

Substitution of sphingomyelin for phosphatidylcholine has improved the stability of liposomes in serum *in vitro* as well as *in vivo* (Huang et al. 1980; Kuhn 1983). Other lipid compositions e.g. 6-aminomannose derivative of cholesterol have also shown similar results (Mauk et al. 1980). Recently, however, the view "more stable liposomes may not be effective drug carriers" has been put forth by Hunt (1982). The author states that moderately stable liposomes, especially MLV, when first destabilised by blood components actually facilitate the release of a priming dose of drug needed to achieve the desired therapeutic level. The subsequent release is slow

and provides a maintenance dose over a long period of time. Thus these MLVs may possibly function as ideal sustained release drug delivery systems.

Other components of blood, namely leukocytes, platelets, erythrocytes, have also been shown to interact with liposomes (Eytan et al. 1982, Juliano et al. 1983; Kuhn et al. 1983). Leukocytes can internalize the liposomes via phagocytosis (Kuhn et al. 1983). Interestingly, stability of liposomes in whole human blood is greater than in the serum (Lelkes and Tandeter 1982; Kuhn et al. 1983), persumably due to the protective effect of erythocytes.

After the first encounter with the components of the blood, liposomes probably would be carried to the lung. Here, larger liposomes or aggregates could be arrested by lung capillaries. Pulmonary retention could also be influenced by charge and composition of liposomes (Fidler et al. 1980). The liposomes would next be coming in contact with the reticuloendothelial system (RES). Interaction of liposomes with the RES has been a subject of study by many investigators. The organs of the RES-liver, spleen, bone marrow take up liposomes similar to other colloids or suspensions. A typical distribution profile of I.V. injected sonicated liposomes could be as follows: liver 22.4%, spleen 6.0%, lung 8.6%, kidney 9.6%, plasma 11.5% (Kimelberg and Mayhew 1978).

Extravasation of small liposomes from the blood stream is possible in organs like liver, spleen and bone marrow because these organs are lined by a type of capillary called sinusoidal or discontinuous capillaries. The gaps in the endothelial lining of sinusoidal capillaries are in the order of 0.1 μm or less and also these capillaries lack subendothelial basement membrane. These properties make them amenable to extravasation of liposomes. However, capillaries of other types like continuous (e.g. skeletal muscle and smooth muscle capillaries) and fenestrated (e.g. G.I.T.) capillaries do not allow extravasation of liposomes. In inflammed tissues, permeability of blood vessels is supposed to increase. Even so, Poste (1983) did not observe any extravasation of liposomes under inflammatory conditions. Despite this fact some authors hypothesize that in diseased organs (tumors), blood capillaries may be more permeable to allow passage of liposomes (Proffitt et al. 1983).

In the hepatic uptake of liposomes, the major role is played by the Kupffer cells (also known as fixed macrophages or mesenchymal cells) of the liver. Hepatocytes or the parenchymal cells possibly play a minor role. Time may be an important factor in deciding which cell population of the liver takes up liposomes preferentially (Freise et al. 1980). Ironically, in the presence of hepatocellular tumor, only a small uptake of MTX liposomes by the liver was

observed (Freise et al. 1979). Endocytosis has been implicated as the chief mechanism for the uptake of liposomes by Kupffer cells. Liposomes administered I.V. have been shown to be present inside the Kupffer cells associated with the lysosomal compartment by light and electron microscopy (Welden et al. 1983). Further, the subcellular fractionation studies have complemented these findings implicating endocytic uptake.

The pharmacokinetic clearance and/or disposition of liposomes depends on size (Abra and Hunt 1981, 1982) lipid composition (Fidler et al. 1980, Gregoriadis and Senior 1980, Huang et al. 1980) surface charge (Fidler et al. 1980, Koa and Lon 1980, Abraham et al. 1984) and the dose (Abra and Hunt 1981, Bosworth and Hunt 1982, Beaumier et al. 1983) of liposomes. The larger the liposomes the faster their clearance (Fidler et al. 1980). The clearance half time can range from 1 hr for SUV to ~10 min for larger vesicles. However, the clearance of free drug has been found to be even more rapid with clearance half time of 3-5 min (Yatvin and Lelkes 1982). Considering just the lipid composition of liposomes, half times for clearance could range from 0.1 hr (dilaurylphosphatidylcholine-cholesterol) to 2 hr (phosphatidylcholine) to 16.5 hr (sphingomyelin) (Huang et al. 1980; Yatvin and Lelkes 1982). Negatively charged vesicles are cleared faster than positive and neutral

vesicles. Recently, it has become evident that irrespective of their original charge all liposomes acquire a negative charge in the presence of serum. In the light of this, the differential clearance rates could result from differences in the way the blood proteins interact with liposomes or from differences in their net negative charge densities (Kimelberg and Mayhew 1978).

It is interesting to note that the blood cells, though bigger than liposomes, are not scavenged by the liver until they are functional. This fact suggests the possible existence of some recognition factors on blood cells revealing them as "self". Recognition factors could also be present in the serum. These, as yet unknown, factors may opsonize (coat) liposomes causing them to be perceived as "non-self" by Kupffer cells and/or fixed macrophages in the spleen (Kimelberg and Mayhew 1978). There are not many studies related to clearance of liposomes linked to cell recognition ligands. As regards to immunoliposomes, however, the clearance is expected to be enhanced if the Fc regions of the antibody are exposed, rather than hidden, on the surface of liposomes. Circulating monocytes and reticuloendothelial cells, through their Fc receptors, would rapidly react with the Fc region of the antibody and clear the liposomes.

RES blockade:

Several efforts have been made to reduce the RES uptake of liposomes so that their interactions with other tissues may be improved. One strategy has been to use very small liposomes like SUV that are cleared from circulation more slowly than the larger MLV, REV or LUVs. The other approach has been to block the RES. RES blockade could be accomplished by I.V. administration of substances like charcoal (Hudson and Hay 1981, Souhami et al. 1981), latex particles, methylpalmitate, dextran sulfate (Souhami et al. 1981) or empty liposomes (Abra et al. 1980). Perhaps empty liposomes are less toxic in comparison to other non-biodegradable particulate materials. The efficiency of RES blockade could be improved by using liposomes comprised of appropriate lipids, e.g. 6-aminomannose derivative of cholesterol (Wuetal 1981, Proffitt et al. 1983), glycolipids like lactosylceramide (Spanjer and Scherphof 1983), lactosylcerebroside and dimannosyl diglyceride (Szoka and Mayhew 1983). Thus a RES blockade which is reversible in 24 hrs, can be imposed easily. Despite this, the value of this method in the context of liposome mediated drug delivery, has not been impressive at all (Poste 1983). The reason is the inability of liposomes to reach tissues outside the mononuclear phagocyte system. Repeated administration of RES blockers or liposomes may paralyse the RES function

leading to toxic problems (Poste 1983). This point should be borne in mind while attempting long term therapy with liposomes.

Finally, there are certain anatomical sites which the intravenously administered liposomes cannot reach (Juliano 1981). One of them is the CNS and the other is the urinary tract. Liposomes cannot reach the CNS because they cannot cross the blood brain barrier. Similarly, they are not filtered by glomeruli and hence cannot appear in the urinary tract.

2. Intraperitoneal route (I.P.)

Convenience makes this a common route of administration in animal studies but not so in humans. Only in situations such as diseases of the peritoneal cavity or ovarian cancer, is this route of administration used in humans (Mayhew and Papahadjopoulos 1983, Ozols et al. 1984). By and large, the distribution of liposomes or their contents observed after I.P. administration, is qualitatively similar to that after I.V. administration. Quantitative differences, however, do exist. Compared to I.V. route, two to three times less uptake in RES organs like liver, spleen and higher uptake in gut, lymph glands has been seen by I.P. route (Ellens et al. 1981). Also the peritoneal cavity functions as a reservoir of vesicles for some hours (Ellens et al. 1981). The transport of liposomes from the peritoneal cavity into the general

circulation and eventually to tissues named above possibly occurs via lymphatic pathway. It could also take place through the capillary lining of the cavity. According to Poste (1983) terminal lymphatics (i.e. lympatic capillaries) offer a far less formidable barrier to the passage of liposomes than continuous and fenestrated blood capillaries. Perhaps the I.P. route is beneficial for targeting liposomes to the lymphatic system (Hisaoka et al. 1982).

In many cases the I.P. route is less toxic than the I.V. route. For example, toxicity was reduced when actinomycin D was administered in liposome form by I.P. route to ascites tumor bearing mice. However, it may not be so with liposomes containing certain other drugs. MTX entrapped liposomes administered I.P. (0.25 mg MTX per ≈ 30 g mouse) caused sudden death of all the mice in 24 hrs but no death occured on I.V. administration of the same liposomes in the same dose for 5 days (Freise et al. 1979). Tyrrel et al. (1976) reports a 100 fold increase in toxicity of intraperitoneally given MTX liposomes over free drug from unpublished data of Colley.

3. Subcutaneous and intramuscular route (S.C. and I.M.)

Liposomes administered subcutaneously may have an increased biological half-life depending on their composition. Subcutaneously administered insulin liposomes provided prolonged hypoglycemic effect compared to free

insulin (Stevenson et al. 1982). This prolongation effect was attributed to the protection of insulin from degradation, provided by liposomes. Similarly Segal et al. (1976) found delayed release of entrapped materials from large MLV injected into the rat testicles. Liposomes injected I.M. or S.C. behave similar to the intraperitoneally administered liposomes in that they exhibit initial slow clearance from the site of injection (Fountain et al. 1980b), followed by absorption into lymphatics and then into the blood stream leading to localization in RES. Liposomes injected into the foot pad also have similar fate. Subcutaneous or I.M. route could therefore possibly be used to deliver drugs to the draining regional lymph node. An example of this is the delivery of anticancer drugs or radioimaging agents to lymph node metastasis (Khato et al. 1983, Osborne et al. 1983).

4. Intraarticular route

This route of administration of liposomes has been investigated for the treatment of arthritis. Results in animals as well as humans have shown prolonged localized action of drug entrapped in liposomes (Dingle et al. 1978, Page-Thomas and Phillips 1979). Although most studies dealt with cortisol palmitate liposomes, MTX liposomes have also been studied by intraarticular injection to improve its

antiinflammatory activity (Foong and Green 1983; Green and Foong 1983).

5. Intratracheal route

Liposomes have been administered into the tracheae in aerosol form for local treatment of lung tumors and/or metastases or respiratory distress syndrome (Sachman and Tsao 1980). Liposomes, so administered, remain within the lung over an extended period of time. Little or no redistribution *via* circulation into other organs has been observed.

6. Oral route

A report of oral administration of insulin in liposomes, first appeared in 1976 (Patel and Ryman 1976). Studies reported later showed much better hypoglycemic activity possibly due to better lipid composition of liposomes (Dapergolas and Gregoriadis 1976). However, there are reports indicating the inability of liposomes to be absorbed from the gut. (Deshmukh *et al.* 1981, Schwinke *et al.* 1984).

Further, some scientists report that the liposomes are completely degraded by the detergent action of bile salts. Nevertheless, the reports indicate the possibilities for oral administration of liposome encapsulated drugs at least to protect them from premature digestion (Deshmukh *et al.* 1981). Liposomes for this purpose should be formulated so as to withstand the rigors of the

gastrointestinal environment such as acids, digestive enzymes and detergents like bile salts. According to some reports, "solid" dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC) liposomes are more stable in G.I.T. than "fluid" egg PC liposomes, possibly due to diminished hydrolysis of the former by the pancreatic phospholipases (Papergolas and Gregoriadis 1976, Rowland and Woodley 1980, 1981). More recently liposomes made of lipids, e.g. diether and dialkyl analog of PC. (Deshmukh et al. 1981), which are resistant to the action of phospholipase have been reported. The mode of absorption of orally administered liposomes and their contents could be analogous to that of chylomicrons, at least partially. Accordingly liposomes might follow lymphatic pathways of the gut with less or no absorption via the portal vein, which is sometimes beneficial. Apart from insulin, other substances like gentamycin (Morgan and Williams 1980), heparin (Ueno et al. 1982), factor VIII (Hemker et al. 1980) have also been shown to be absorbed from G.I.T. when encapsulated in liposomes. Very recently Nagata et al. (1984) have reported superior effect of oral liposomal vitamin K_1 over other dosage forms.

7. Dermal Application

The hazards of systemic administration could be completely avoided if liposomes could be applied topically. The ingenious idea of exploitation of liposomes as selective

topical drug delivery systems was conceived and investigated first in this laboratory by Mezei and Gulasekharam (1980). Studies in rabbits (Gulasekharam 1980, Mezei and Gulasekharam 1980) and guinea pigs (unpublished observations) have shown that indeed drug disposition is altered favorably when applied in liposomal form. That is to say - higher amounts of drug appeared in epidermis, dermis, the target sites and lower amounts in subcutaneous tissue. Further modification into a suitable pharmaceutical dosage form maintained this favourable disposition pattern (Mezei and Gulasekharam 1982). Dermal application of liposome entrapped drugs has great potential from the following perspectives: systemic toxicity of some topically administered drugs (e.g., corticosteroids) could be reduced or eliminated and the quality of topical therapy could be enhanced by selective dermal delivery of drugs.

However, the mechanism for the favorably altered drug disposition by liposomes, is only speculative at this stage. One possibility is that liposomes act as a depot releasing the drug slowly but in a sustained fashion (Yatvin and Lelkes 1982). Dermal use of liposomes as humectants has also been reported (Idson 1981, Rieger 1981).

8. Ophthalmic application

One of the early reports of topical application of liposome encapsulated drugs into the eye was that of Smolin

et al. (1981). According to the report, in herpes infection of the eye, the therapeutic action of iodoxuridine was greatly improved when administered in liposome encapsulated form. Corneal uptake of liposome encapsulated indoxole and penicillin G was investigated by Schaeffer and Krohn (1982). The findings suggested enhanced transcorneal flux of both the drugs by liposomisation. Megaw et al. (1981) have reported an increased binding of liposomes to the ocular lens mediated by lectin Con A.

Investigations of ocular distribution of triamcinolone acetonide and dihydrostreptomycin sulfate have been carried out in this laboratory (Singh and Mezei 1983, 1984, Singh 1982). Based on these studies, the authors recommended liposomes as suitable ophthalmic drug carriers for lipid soluble drugs but not for water soluble drugs. Stratford et al. (1983) found opposite effects on corneal and conjunctival absorption of liposomal epinephrine and inulin. Epiniphrine absorption decreased by 50% whereas inulin absorption increased 10 times by liposome entrapment. Very recently Barza et al. (1984) reported that liposome encapsulation might extend the effect of subconjuctivally injected antibiotic like gentamycin.

9. Miscellaneous routes

A recent report (Forssen and Tokes 1983) says that the intradermal injection of doxorubicin liposomes produced less dermal toxicity compared to free doxorubicin. It was

therefore implicated that the dermal toxicity of doxorubicin arising from extravasation of drug during I.V. infusion could be eliminated by liposome entrapment. Apart from intragastric route discussed earlier insulin liposomes have also been administered *via* sublingual route (Ueingarten *et al.* 1981). Results indicated enhanced effect of insulin entrapped in liposomes.

Toxicity of Liposomes

Large volumes of lecithin emulsions (e.g. Intralipid) are given intravenously without apparent toxicity. Considering this, empty liposomes which are also composed mainly of phospholipids should not be toxic in the doses administered. Despite this expectation there are some reports concerning the toxicity of liposomes. Such toxicity depends mainly on their composition and dose. Stearylamine containing liposomes were most toxic while negative liposomes with phosphatidyl serine or dicetylphosphate were more toxic than neutral liposomes (Adams *et al.* 1977). Intracerebral injection of stearylamine or dicetylphosphate containing liposomes into mice produced toxic symptoms such as epileptic seizures, cerebral necrosis, etc. (Adams *et al.* 1977). The same liposomes administered in a lesser dose, did not show any toxic symptoms. To some extent the toxicity also depends on the size of liposomes. Though of similar composition (PS:PC:CHOL), MLVs of size 0.5-3.2 μm were more toxic (LD_{50}=300 mg/kg) than SUVs of size 0.03 μm

(LD_{50}=5.5 g/kg). Mayhew and Papahadjopoulos (1983) report that they observed marked initial gross necrotic effects on normal liver when mice were injected with 5 g /kg of DPPC:CHOL liposomes. But the liver reverted back to normal state in 7 days.

Although plain liposomes may be considered relatively non-toxic chemoliposomes may exhibit increased toxicity. Such toxicity would naturally be resulting from altered tissue distribution and pharmacokinetics of entrapped drugs. Uptake by macrophages of, or sustained release from, liposomes containing cytotoxic drugs might lead to increased toxicity. This is because of the simple fact that what is sometimes good for killing cancer cells is also bad for normal cells. This aspect is particularly highlighted in the review by Kaye (1981). Sustained release of cytotoxic drugs of phase specific type (e.g. MTX, ara-c) from liposomes is good for killing cancer cells. At the same time this might adversely affect normal proliferating cells leading to increased host toxicity. Therefore, MTX and ara-c liposomes were more toxic than the "free" drug in some studies (Kaye 1981). Also, the uptake of cytotoxic liposomes by macrophages (fixed or mobile) may wipe out one of the host's vital defence mechanisms. For example, systemic administration of liposome encapsulated bleomycin and several other antitumor drugs of cycle nonspecific type,

instead of killing cancer cells, adversely enhanced metastatic spread of malignant tumor (Poste 1983). These findings highlight the fact that the liposome entrapment in some instances might make an otherwise less toxic drug into a more toxic one. Not withstanding all this, an intravenous infusion, to man, of large volumes of liposomes containing an antimitotic compound has produced no toxicity whatsoever (Coune et al. 1983).

POTENTIAL APPLICATIONS OF LIPOSOMES

As mentioned earlier, since their discovery in the early 1960s, research applications of liposomes have proliferated and diversified. The use of liposomes as drug carriers was reported first by Gregoriadis in early 1970s (Gregoriadis et al. 1974, Gregoriadis 1976). Following this they have been extensively investigated as carriers of drugs, including enzymes (Weissmann and Finkelstein 1980), chelating agents (Rahman 1980), vitamins (Alpar et al. 1981) antibiotics (Richardson 1983) alkaloids (Todorov and Deliconstantinos 1982) and corticosteroids (Shaw and Dingle 1980). Liposomes are relatively nontoxic and biodegradable because they are composed of natural constituents of body. They are versatile, allowing easy manipulation of structural characteristics like size, charge, lipid composition, etc. These beneficial properties have been partially responsible for making these lipid vesicles attractive drug carriers.

Cancer chemotherapy and enzyme therapy have been the fields of most extensive studies. These and other areas of applications will be highlighted.

Cancer chemotherapy

Among the chemotherapeutic agents anticancer drugs are highly toxic ones. The therapeutic use of these drugs is limited by their toxicity. Hence this is the area wherein improvement in the therapeutic index is sought out most desperately. The efforts made to use liposomes in cancer chemotherapy are therefore extensive. About 15 or so cytotoxic drugs have so far been encapsulated in liposomes. Among these, methotrexate (MTX), cytosine arabinoside (ara-c), doxorubicin and actinomycin are the most extensively studied ones. Investigations with liposome encapsulated anticancer agents have given rise to some interesting results. Many of these results suggest that the liposomes have a role in enhancing the efficacy of certain antitumor drugs. Papahadjopoulos et al. (1976) showed that actinomycin D entrapped in liposomes could overcome the drug resistance in Chinese hamster cell line in vitro. In vivo studies of the same drug with drug resistant tumors gave negative results (Kaye et al. 1981). An increased intracellular delivery (in vitro) of ara-CTP (active metabolite of ara-c) from liposomes was demonstrated in drug sensitive L1210 cells. The increased delivery also resulted in distinct increased cytotoxic activity in

vitro over free ara-CTP. But, ara-c and ara-CTP liposomes when tested in vivo with drug resistant mouse lymphoma failed to show any effect (Richardson and Ryman 1982).

Another interesting property of liposomes is the prolongation of plasma half-life of entrapped drugs resulting in a depot effect which could also depend on liposome size and composition (Allen and Everest 1983). This has been observed or predicted, with cytotoxic drugs such as methotrexate (Kaye et al. 1981, Richardson and Ryman 1982), actinomycin D (Kaye et al. 1981), vincristine (Firth et al. 1984), ara-c (Rustum et al. 1979) adriamycin (Forssen and Tokes 1983b) and flurodeoxyuridine (Juliano et al. 1983). However such prolongation of half-life may or may not be beneficial therapeutically depending on the type of drug or properties unique to a particular drug.

This aspect has been discussed under the toxicity of liposomes (please see page 67). Reduction in toxicity might result, in the case of cycle nonspecific drugs (e.g. actinomycin D, cyclophosphamide). The cardiac toxicity of adriamycin and general toxicity of actinomycin D was reduced as a result of liposome entrapment (Rahman et al. 1978, Olson et al. 1982, Ganapathi and Krishan 1984).

Several comparative studies on the cytotoxic effects of free and liposome encapsulated drugs have been reported.

Some workers claim that efficacy of drugs such as cytosine arabinoside (Mayhew et al. 1976), nocodazole.R (Laduron et al. 1983), carboquone (Hisaoka et al. 1982b), cis-platinum diamminodichloride (Sur et al. 1983), and doxorubicin (Mayhew and Rustum 1983) is actually enhanced by liposomal entrapment. Many of these investigations were carried out using ascites tumors or I.V. tumors (eg. L1210 leukemia). Ascites tumor bears little resemblence to tumors in a real life situation. Lately, studies involving solid tumors also began to appear. Some of the recent reports of investigations of liposomal cytotoxic drugs in solid and metastatic tumors are: methotrexate liposomes to treat solid rodent (Kosloski et al. 1978) and murine tumor (Weinstein et al. 1980), doxorubicin liposomes to treat mouse lung tumor (Abra et al. 1983), actinomycin D liposomes to treat murine renal adenocarcinoma (Kedar et al. 1980), doxorubicin liposomes to treat mouse primary and metastatic liver tumors (Mayhew and Rustum 1983). Recently, Frossen and Tokes (1983) have shown improved therapeutic benefit of doxorubicin liposomes against Lewis lung carcinoma. Such improved effect was attributed to the significant reduction in immunosuppressive activity of doxorubicin in addition to reduced cardiac toxicity and depot effect.

The effect of liposome encapsulated antitumor drugs could also be influenced by lipid composition of liposomes as evident from recent investigations (Kirby and Gregoriadis 1983).

Use of hyperthermia resulted in selective release of liposome entrapped cytotoxic agents to tumors (Weinstein et al. 1979,1980). Other than this, attempts to target liposomes to tumors have largely been unsuccessful. The non-selectivity of anticancer liposomes prompted investigators to make them targetable by use of cytophilic ligands such as antibodies, lectins, etc. (Leserman et al. 1983; Monsigny et al. 1983). Such efforts emerged only recently and although many in vitro studies of antibody targeted liposomes have been reported, most of these dealt with cells such as RBCs or spleen cells (Weinstein et al. 1978, Harsch et al. 1981, Herrman and Mescher 1981, Huang et al. 1981, Machy et al. 1982ab, Rogers 1982b, Bragman et al. 1983, Machy and Leserman 1983, Heath et al. 1984).

Very few studies dealing with cancer cells have so far been reported. Binding of antigen bearing liposomes to murine myeloma tumor cells was shown by Leserman et al. (1979,1980b). However, binding did not result in increased delivery of vesicle contents into cells. There are other reports supporting this observation (Weinstein et al. 1978, Todd et al. 1980). Heath et al. (1983) and

Matthay et al. (1984) recently reported an increase in specific toxicity of monoclonal antibody targeted methotrexate-γ-aspartate liposomes.

The first and so far the only investigation of in vivo anticancer evaluation of monoclonal antibody targeted liposomes containing an antitumor drug was reported recently by Hashimoto et al. (1983). The authors demonstrated an augmented antitumor effect of actinomycin D liposomes bearing tumor specific IgM subunits against mouse mammary tumor cells in vitro as well as in vivo (I.P. and S.C. tumor). However, many more studies are needed before the relevance of immunospecific targeting of liposomes for therapy could become clear.

A comprehensive coverage of evaluations of liposome encapsulated anticancer drugs is dealt with in review articles by several authors (Yatvin and Lelkes 1982, Kaye 1981, Mayhew and Papahadjopoulos 1983, Weinstein and Leserman 1984).

Immunomodulators

Refractory macrophages can be activated to selectively kill metastatic cancer cells by the use of immunomodulators called macrophage activating factors (MAF) (eg. muramyl dipeptide (MDP), lymphokines). MDP is rapidly excreted following parenteral administration. Encapsulation of MDP in MLV liposomes provides a mechanism to achieve an efficient macrophage activity in vivo. Application of

liposomal MAF for total eradication of metastasis has been widely investigated by Fidler and co-workers (Schroit et al. 1983). Their experiments have shown convincing results for tumor free survival of animals as well as humans with lymphnode and pulmonary metastases.

Immunoadjuants

Another promising aspect of liposomes is the promotion of strong humoral and cell mediated immune response to antigens associated with liposomes without the side effects of other adjuants (Alvin and Richards 1983). Liposomes in this regard have been experimented with associated antigens such as cholera, influenza virus (virosomes), rubella, hepatitis B virus, herpes simplex virus, diphtheria toxoid.

Stimulation of the immune response is good for active immunization, but in the context of drug targeting it is detrimental. Single injection of antibody directed liposomes would not elicit perceivable antibody response but multiple injections would (van Rooijen and van Nieuwmegen 1982). However no such antiidiotypic response was observed on multiple injections of immunoliposomes in a study by Leserman et al. (1983). In the event of positive antibody response, immunoliposomes would be coated with antibodies directed against them, preventing the specific homing of liposomes. Consequently Fc portions of the second antibody may target the liposomes to B lymphocytes in general. If this results in the killing of such

lymphocytes, the interference with drug targeting might be overcome (van Rooijen and van Nieuwmegen 1982).

Enzyme therapy

Metabolic diseases or lysosomal storage diseases, e.g. Gaucher's, Pompe, Tay-Sach's disease are characterized by accumulation of substrates into the lysosomal compartment of liver cells due to enzyme deficiency. Treatment of these diseases have failed because of the inability of exogenously administered enzymes to reach the lysosomes. Enzymes administered are inactivated by proteolysis or by antibodies before they reach lysosomes. They also give rise to hypersensitive and immunological reactions. Enclosed in liposomes, the enzymes are shielded against any damage. More importantly, liposomes being lysosomotropic, would be able to carry their enzyme payload into the targets, the lysosomes (Desnick et al. 1978, Puisieux and Benita 1982, Weissman and Finkelstein 1980).

Superoxide dismutases (SOD)

These are a class of metallo enzymes that are able to protect the organism against the toxic effect of oxygen free radicals, with the aid of catalases. It has been shown that cellular incorppration of SOD is increased by liposome encapsulation. Some believe that SOD liposomes could also reduce the side effects of radiotherapy and antitumor chemotherapy (Puisieux and Benita 1982).

Treatment of heavy metal poisoning

Iron poisoning as well as iron storage diseases such as thalassaemia have been treated successfully with liposome entrapped chelating agents like EDTA and desferrioxamine (Rahman 1980). "Naked" administration of these agents has drawbacks like poor intracellular penetration, rapid excretion and side effects. Administration by entrapment in liposomes increases the uptake of these chelating agents in the liver, the site of metal storage. As a result, elimination of toxic metals takes place faster. Other studies, in which animals loaded with plutonium, mercury, gold and treated with liposomes encapsulated chelators have again demonstrated the therapeutic value of the system (Rahman et al. 1973).

Therapy of intracellular parasitic or microbial diseases

Leishmaniasis is a tropical disease caused by Leishmania donovani, a protozoan which invades the cells of the RES. Antimonial drugs (e.g. stibophen) are the drugs of choice to treat this disease. However, these antimonials are extremely toxic to the heart and kidney. Their toxicity could be reduced several fold by administration in liposome encapsulated form. The efficacy of antimonials has been increased dramatically (700 fold) by encapsulation in liposomes, presumably due to targeting of these drugs to lysosomes of the fixed macrophages in the liver (Chapman et al. 1984). However, an entirely different mechanism, namely, production of parasite toxic oxygen metabolites was

implicated in the improved efficacy of primaquine liposomes in the therapy of visceral as well as cutaneous leishmaniasis (New et al. 1983).

Malaria is another example of intracellular parasitic disease. Pirson and his co-workers (1980), (Smith et al. 1983) studied the use of primaquine liposomes in the therapy of malaria. Though they observed reduction in toxicity of primaquine when entrapped in liposomes, there was no increase in the activity of the drug.

Apart from these parasitic diseases, there are many bacterial diseases in which the causative bacteria reside in phagocytic cells. Examples include diseases such as tuberculosis, leprosy, trypanosomiasis, brucellosis, trachoma etc. Opportunity exists for improvement in the treatment of these diseases by using liposome encapsulated drugs (Richardson 1983). In his leading article Richardson (1983) quotes the study dealing with treatment of tuberculosis using liposome encapsulated antitubercular drug. In an *in vitro* study, the bactericidal effect of dihydrostreptomycin against intraphagocytic *Staph. aureus* increased when entrapped in liposomes (Bonventre and Gregoriadis 1978). In another investigation liposome entrapped amphotericin B, a lipid soluble antibiotic was shown to be less toxic but therapeutically effective against *Candida albicans* infection in mice (Juliano et al. 1983b). The therapeutic index was increased by 2 to 6 fold

over free drug. A recent article indicates that liposomal amphotericin B is toxic to fungal cells but not to mammalian cells (Mehta et al. 1984). Desiderio and Campbell (1983) have reported an enhanced intraphagocytic killing of S. typhimurium by liposome encapsulated cephalothin.

Liposomes as diagnostic tools

Gamma emitting radionuclides or radio pharmaceuticals (e.g. ^{99m}Tc, ^{131}I) can be trapped in liposomes just as drugs can. Such liposomes could possibly be used to diagnose primary neoplasms and perhaps also to destroy them if liposomes contain destructive radionuclides (β emitters). The course of γ-emitting radionuclides can be detected using a gamma camera or a rectilinear scanner. (McDougall 1980).

It is important to note here that, for the purpose of diagnosis, mere binding of radiolabelled liposomes to target cells is enough, where as selective therapy needs their internalization as well. If it is possible to further enhance the diagnostic ability of radionuclide containing liposomes, they could be done so by anchoring appropriate antibodies to their surface.

Liposomes as topical drug delivery systems

Mezei and Gulasekharam (1980, 1982) have demonstrated the feasibility of using liposomes to deliver drugs selectively to skin. This would also have an added advantage of reducing unwanted percutaneous absorption.

Ophthalmic application of drugs in liposomes has also given rise to beneficial effects. As mentioned earlier Smolin et al. (1981) have reported the superior effect of iodoxuridine liposomes in the treatment of herpes infection of eye. Singh and Mezei (1983,1984), based on their ocular distribution studies, have recommended ophthalmic liposomes for administering lipophilic drugs.

Miscellaneous applications

Liposomes have been used as models for studying intestinal drug absorption especially of β- lactam antibiotics by Kimura et al. (1980). In the field of genetic engineering, application of liposomes for intracellular delivery of nucleic acids to alter the genotype of cells has been envisaged by Papahadjopoulos (1981).

RESEARCH HYPOTHESES AND OBJECTIVES

Liposomes have been widely investigated as carriers of drugs in general. Specifically, pioneering studies from this laboratory (Mezei and Gulasekharam 1980, 1982) have explored their potential importance in topical therapy. In these studies, the dermal disposition of the drug triamcinolone acetonide (TRMA) appeared to be altered favourably when it was applied topically in liposome encapsulated form. The next approach was to optimize the factors for selective dermal delivery of drugs using liposomes. Hence the research proposal put forth was to

investigate the effect of factors such as liposome size, surface charge and lipid composition on the disposition of topically applied TRMA liposomes. However, initial experiments soon revealed that the encapsulation of TRMA in liposomes was far from satisfactory. In the light of this new finding the goal of the project was changed to improving the liposomal encapsulation of TRMA. The present hypothesis was that the chemical modification of TRMA would improve its incorporation into the liposomes.

Another part of this investigation relates to the use of liposome encapsulated antineoplastic drugs in the therapy of cancer. There have been many reports of liposomes as site specific carriers of anticancer drugs. Recently, however, it is recognized that mere encapsulation in liposomes does not suffice and that liposomes have to be provided with target seeking cytophilic ligands such as antitumor antibodies in order to accomplish the task of targeting. Another hypothesis of this research, therefore was that liposomal encapsulation of the model anticancer agent methotrexate and further modification of liposomes with antibodies would improve the anticancer activity of methotrexate.

To test the above two hypotheses the following specific objectives were set forth:

1. To improve the encapsulation of TRMA in liposomes.
2. To prepare and characterize MTX liposomes.

3. To covalently couple antibodies to liposomes and characterize them.

4. To evalute the anticancer activity of MTX liposomes by *in vitro* and *in vivo* techniques.

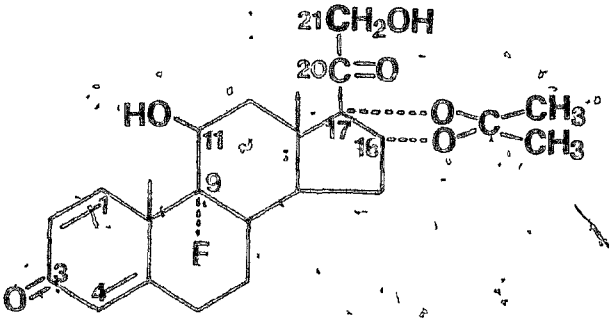
Selection of drugs

Triamcinolone acetonide and methotrexate were chosen as model drugs mainly because the previous relevant studies (Mezei and Gulasekharam 1980, 1982, Kulkarni *et al.* 1981) dealt with these drugs. Studies with the same drugs would provide a basis for comparative evaluation of different approaches.

Triamcinolone acetonide

It is an antiinflammatory drug of intermediate poteney belonging to the general class of corticosteroids (Popper and Watnick 1974). Its structure and chemical names are shown in Fig. 3 (Florey 1972). Use of TRMA in the treatment of non-infective inflammatory skin disorders such as dermatitis, psoriasis and eczema is well known (Jones and Barreuther 1981). Topical TRMA preparations are available in the form of creams, ointments and lotions with a drug concentration of 0.1% or 0.5% (Jones and Barrenther 1981).

It is noteworthy that topically administered corticosteroids could be absorbed into the systemic circulation although to a very small extent, approximately 1% of the applied dose. On entering systemic circulation



9α-fluro-11β, 16α, 17,21 -tetrahydroxypregna-1,4-diene-3, 20-dione cyclic 16,17-acetal with acetone; 9α-fluro-11β, 21-dihydroxy-16α,17α-isopropylidine-dioxy-1,4-pregnadiene-3, 20,-dione; triamcinolone 16,17-acetonide.

Figure 3. Chemical structure and alternative names for Triamcinolone Acetonide.

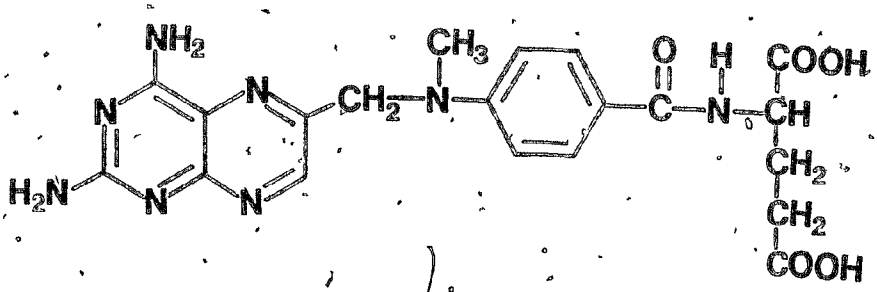
they have the potential to produce toxic effects such as suppressing of hypothalamus-pituitary-adrenal (HPA) axis and Cushing's syndrome (Hallam 1980, Morman 1981). Percutaneous absorption is more likely to occur in infants and children especially on long term application and with the use of an occlusive dressing (Gasiorowski-Mauroy 1981). A need to prevent such toxicity by reducing or eliminating the percutaneous absorption of corticosteroids is therefore evident. Hence, if liposomes could deliver TRMA selectively to skin, the significance of the achievement could not be overemphasized.

TRMA is available in ^{3}H or ^{14}C form allowing its determination in minute quantities in biological samples.

Methotrexate

It is a widely used cancer chemotherapeutic agent particularly in the treatment of neoplastic diseases such as acute lymphocytic leukemia, non-Hodgkin's lymphoma, osteosarcoma, choriocarcinoma, head and neck cancer and breast cancer (Jolivet et al. 1983, Martin and Ballentine 1983). Fig. 4 gives its chemical structure, different chemical names and other synonyms. MTX has to be stored protected from light to prevent possible photodecomposition.

MTX is an antimetabolite of folic acid. Its mode of action is by competitive and irreversible inhibition of the enzyme dihydrofolate reductase (DHFR) which in turn inhibits



N-[4- [(2,4-diamino-6-pteridinyl)-methyl]methylamine benzoyl] glutamic acid; 4-amino-10-methylfolic acid; amethopterin.

Figure 4. Chemical structure and alternative names for Methotrexate.

the formation of tetrahydrofolic acid from dihydrofolic acid. The resultant folic acid deficiency blocks nucleic acid synthesis and cell division. Thus, it is a cell cycle ("S" phase) specific drug.

High cell turnover may be eliminated in malignant tissue but other rapidly dividing normal tissue such as bone marrow, the epithelial lining of the gastrointestinal tract and the bronchioles and the cells in the hair follicles may also be severely damaged. Immunosuppression is an additional toxic side effect of MTX. Another side effect, the nephrotoxicity, probably caused by precipitation of MTX or its 7-OH metabolite, could be minimized by maintaining alkaline diuresis during treatment (Jolivet et al 1983, Anonymous 1984). Development of resistance to MTX treatment is not uncommon (Chabner et al. 1984).

Any attempt, be it liposome or otherwise, to target MTX or other cytotoxic agents preferentially to cancer cells, is a welcome measure because of the highly toxic nature of these drugs. Although it could be analysed by methods (Florey 1976, Sadee et al. 1980, Breithaupt et al. 1982, Chen et al. 1984), like HPLC, UV spectrophotometry and RIA, the availability of labelled MTX makes its quantitative analysis more sensitive and reliable.

MTX is official in USP XX, BP 1980, BPC 1979 and European Pharmacopoeia, Vol. III 1975. For human use BP 1980 recommends a dose of 5 to 100 mg at suitable intervals.

MATERIALS AND METHODS

Materials

DL-α-dipalmitoylphosphatidylcholine (DPPC), cholesterol (CHOL), stearylamine (SA), dicetyl phosphate (DCP), dipalmitoyl phosphatidylethanolamine (DPPE), egg phosphatidylethanolamine (PE), palmitoyl chloride, triamcinolone acetonide (TRMA), silica gel (chromatographic grade, 100-200 mesh), methotrexate (± amethopterin, MTX), absolute methanol, dithiothreitol (DTT), bovine serum albumin (BSA), protein standard solution were obtained from Sigma Chemical Company, St. Louis, Missouri. [6,7-^{3}H(N)]-triamcinolone acetonide (^{3}H-TRMA) of specific activity 31.3-37.0 Ci/mmol and Biofluor^{R} scintillator solution were purchased from New England Nuclear, Boston, Massachusetts. [3',5',7-^{3}H]-methotrexate sodium salt (^{3}H-MTX) of specific activity 200 mCi/mmol, ^{125}I-NaI of specific activity 15.2 mCi/μg of iodine were from Amersham, Arlington Heights, Illinois. Prepacked Sephadex G-25 M PD-10 columns, Sephadex G-50, Sephacryl S-200, Protein A Sepharose CL-4B, chromatography columns ($K_{15/90}$, Bed vol. = 90 x 1.5 cm = 154 ml; K9/15, Bed vol. = 15 x 0.9 cm = 9.5 ml), Ficoll 400, N-succinimidyl-3- (2-pyridyldithio)propionate (SPDP), Sephacryl S-400, Sepharose 4B were from Pharmacia Fine Chemicals, Pharmacia (Canada) Inc., Dorval, Quebec. Alkaline detergent solution Decon^{R} 75 concentrate, precoated silicagel 60 F 254 TLC aluminium sheets

manufactured by Merck were purchased from BDH Chemicals, Toronto, Ontario. RPMI 1640 powder medium, fetal bovine serum (FBS) were from Flow Laboratories Inc., Mississauga, Ontario. Polycarbonate filters (8 µm and 12 µm) were procured from Nucleopore Corporation, Pleasanton, California. Immersible CX-10^{R} ultrafiltration units were bought from Millipore, Bedford, Massachusetts. Polypropylene Eppendorf^{R} micro test tubes (400 µl) were from Brinkman Instruments Inc., Westbury, New York. Naphthalene, pyridine, calcium chloride dihydrate and all the solvents were from Fisher Scientific Co.

Equipments and Instruments

Equipment and instruments used were the following: Beckman Liquid Scintillation Counter LS 3133T - Beckman Instruments Inc., Fullerton, California. Gamma counter, Model 1085 - Nuclear-Chicago Corporation, Des Plaines, Illinois. Coulter counter^{R}, model Z_F - Coulter Electronics Inc., Hileah, Florida. Refrigerated centrifuges, model PR-6 and model B-20, centrifuge, universal model UV, clinical centrifuge, model CL - International Equipment Company, Needham Heights, Massachusetts. Universal Spectrophotometer PMQII and Standard Universal and GFL microscopes - Carl Zeiss, Oberkochen, West Germany. Olympus Inverted microscope - Tokyo. Mettler Type H207 and Micro Gram-atic Balances, Top loading PL300 balance - E. Mettler, Zurich, Switzerland.

LKB 700 UltroRac^{R} Fraction Collector - LKB-Produkter AB, Bromma, Sweden. Metabolic Shaking Incubator - Precision Scientific Company, Chicago. Wrist Action Shaker^{R}, Model 75 - Burrell Corporation, Pittsburgh, Pennsylvania. Buchler Portable Rotary Evaporator - Buchler Instruments Inc., Fort Lee, New Jersey. Bransonic 220 Ultrasonic Cleaner - Branson Cleaning Equipment Company, Shelton, Connecticut. Sonic dismembrator - Artek System Corporation, distributed by Fisher. BioGard Hood - the Baker Company Inc., Sanford, Maine. Incubator for tissue culture - National, a Heinicke Co. Laboratory Counter - Clay Adams, Parsippany, NJ. Beckman 152 Microfuge. Fisher Accumet^{R} pH meter model 210. Super-mixer - Lab-line Instruments, Inc., Melrose Park, ILL. Eppendorf standard fixed-volume pipettes. Pipetman continuously adjustable digital micrometer pipettes - Mandel Scientific Co. Ltd., Montreal, PQ. Swin-Lok Membrane Holders - Nucleopore, Pleasanton, Ca. USP dissolution rate testing assembly consisting of water bath and Thermomix 1480 temperature regulator (B.Braun, Melsungen AG, West Germany). Dissolution stirrer and stirrer drive model 53 (Hanson Research Corp., Northbridge, California), Masterflex^{R} Peristalysis pump, model 7016.20 and its speed controller (Cole Parmer Instrument Co., Chicago, ILL.) and Spectronic 21 Bausch and Lomb Spectrophotometer with flow through cell.

Animals:

C57BL/6J mice were ordered from Jackson Laboratory, Bar Harbour, Maine.

Antibodies:

Anti-BSA, anti-EL4 and anti-M21 polyclonal antibodies were obtained from Dr. Ghose, Department of Pathology. These antibodies were raised in rabbits by injecting either BSA, EL4 cells or M21 cells. The anti-EL4 sera, inactivated at 56°C for 30 min was absorbed extensively with washed homogenates of liver, lung, kidney and spleen from normal adult C57BL/6J mice. After heat inactivation, the anti-M21 sera was absorbed only with human red cells. IgG antibodies were obtained from these serums by fractionation with 33% saturated ammonium sulfate.

Tumors:

The EL4 lymphoma is a murine tumor originally obtained from the Chester Beatty Research Institute (London, England) in 1969 and has been maintained in Dr. Ghose's laboratory by serial I.P. passage in C57BL/6J mice. The human melanoma line M21, obtained from the Scripp's Research Institute, La Jolla, Ca. in 1979, is maintained in Dr. Ghose's laboratory, by serial transplantation of melanoma cells S.C. in the flank of nude mice of BALB/c background. The human kidney carcinoma line caki-1, obtained from the American Type Culture Collection, Rockville, MD. is maintained in Dr. Ghose's laboratory.

METHODS

STUDIES WITH TRIAMCINOLONE ACETONIDE

Attempts to improve the liposomal encapsulation of triamcinolone acetonide (TRMA)

Different methods and formulations that were tried to improve the liposomal entrapment of TRMA were as follows:

Rotary vacuum evaporation followed by mechanical shaking:

Stock solutions (5 mg/ml) of lipids and TRMA in chloroform:methanol (2:1) were used. Appropriate quantities of lipid and TRMA were placed in a suitable pear shaped flask. The solvent was evaporated in a rotary vacuum evaporator at room temperature. Alternate addition and evaporation of chloroform was done until a smooth thin film was obtained. The film was heated to 60°C for 30 minutes. To the flask was then added an appropriate quantity of aqueous swelling (dispersion) medium (in most cases aqueous 8 mM $CaCl_2$ solution) which had been prewarmed to 60°C. The flask was then immediately shaken, using a wrist action shaker, at 60°C for 30 min.

Different formulations (expressed in molar ratio) tried by the above method were:

1. DPPC:CHOL:SA (1.0:1.0:0.2)
2. DPPC:CHOL:TRMA (1.0:0.7:0.3)
3. DPPC:CHOL:TRMA:SA (0.7:0.5:0.5:0.4)
4. DPPC:CHOL:TRMA:SA (1.0:0.5:0.5:0.2)
5. DPPC:CHOL:TRMA:SA (4.0:2.0:0.5:0.6)

In all the above formulations aqueous 8 mM $CaCl_2$ solution was used as the swelling medium. The final concentration of TRMA was approximately 0.1% w/v even though initially the concentrations were sometimes higher than this.

Formulation #4 was also tried with 8 mM $CaCl_2$ solution containing 5-10% v/v alcohol as the swelling medium.

Reverse phase evaporation (REV):

DPPC 100 mg (136 micromoles), CHOL 52.66 mg (136 micromoles) and TRMA 5 mg (11.5 micromoles) were added to a 50 ml round bottom flask with a long extension neck and the solvent was removed under reduced pressure by a rotary vacuum evaporator. The residue was redissolved in a mixture of 8 ml of diethyl ether and 4.5 ml of chloroform. Water, 4.2 ml, was then added. The resulting two phase system was sonicated briefly (2-5 min) in a bath type sonicator at 0-5^{o}C. The mixture was then placed on the rotary evaporator and the organic solvent was removed under reduced pressure at room temperature. The aqueous suspension was then shaken mechanically at 50-60^{o}C for 30 minutes.

After preparing the liposomes by any of the above methods, the preparation was allowed to remain at room temperature for about an hour and then observed under the

microscope (Carl Zeiss, magnification 640X, polarized light). They were further subjected to either filtration through a polycarbonate filter (8 or 12 µm pore size) and/or centrifugation at 22000 x g.

Attempts to concentrate the liposomal preparation

By filtration through 0.45 µm millipore filter:

Liposomes were prepared using ^{3}H-TRMA by normal evaporation and shaking method. The preparation was filtered through 8 µm polycarbonate filter. The filtrate was forced though a 0.45 µm millipore filter until the volume above the filter was reduced to half the original volume. This was done using a standard filtration assembly consisting of syringe and filter holder. High force was needed to force the preparation through the filter. Radioactivity per unit volume of the preparation above the filter was measured before and after filtration.

By rotary vacuum evaporation:

A known volume of TRMA liposomal preparation containing radioactive TRMA and which was filtered through 8 µm polycarbonate filter was placed in a small pear shaped flask. The preparation was concentrated to about one-third the original volume by rotary vacuum evaporation at room temperature. Measurement of radioactivity per unit volume and microscopic observation of the preparation was done before and after concentration.

Evaluation of the supernatant of the liposomal preparation

Gel filtration chromatography of the supernatant:

Sephadex G-50 (4 g) soaked in distilled water for a day and in 8 mM $CaCl_2$ solution (containing 5% v/v alcohol) for about 2-3 hrs was packed into a column of dimensions 17 cm x 1.5 cm. The column was washed with 200-300 ml of 8 mM $CaCl_2$ solution containing 5% v/v alcohol. Void volume was determined using 2 ml of Blue dextran 2000 solution.

Cationic liposomes with TRMA were prepared by rotary vacuum evaporation followed by mechanical shaking with 8 mM $CaCl_2$ solution containing 5% v/v alcohol. The formula used was DPPC:CHOL:TRMA:SA (1.0:0.5:0.5:0.2) with 840 µCi of ^{3}H-TRMA and 5.9 mg of TRMA. The preparation was filtered through 8 µm polycarbonate filter and then centrifuged at 22000 x g for 30 minutes. Gel filtration chromatography was carried out on this supernatant.

One ml of the supernatant was applied to the column and eluted with 8 mM $CaCl_2$ solution (5% v/v alcohol). Fractions of one ml were collected. An aliquot of each fraction was added to 10 ml of Bray's solution (see appendix) and the radioactivity was determined.

Dialysis of the supernatant:

A dialysis bag was made using dialyzer tubing of length about 5 cm and inflated diameter of 1.58 cm. Supernatant, 1.5 ml, of known specific radioactivity was added to the bag. The bag was suspended in 800 ml of

magnetically stirred distilled water contained in a beaker. Dialysis was allowed to proceed at room temperature overnight. Radioactivity per unit volume of dialysate and dialysing medium was measured.

Synthesis of Triamcinolone acetonide-21-palmitate

Method of Shaw, Knight and Dingle[a] (1976):

^{3}H-TRMA (50 µCi) was placed in a 50 ml glass stoppered round bottom flask. The solvent was allowed to evaporate. Unlabelled TRMA (1.4339 g, 3.3 mmol) dissolved in pyridine (20 ml) and palmitoyl chloride[b] (2.4 ml, 7.92 mmol) were added. The mixture was stirred magnetically at room temperature for 15 hrs. The reaction mixture was then poured into 1N sulfuric acid with vigorous stirring. Crude product was separated by filtration and purified in 0.5-0.9 g batches by column chromatography on 16g silica gel (60-200 mesh) eluting with toluene:ethyl acetate:acetic acid (90:10:1), using a maximum flow rate of 150-170 ml/hr and collecting 25 ml fractions which were monitored by TLC. Fractions containing TRMA-21-palmitate were combined and recrystallized from methanol.

---

a: crude product was obtained by putting the reaction mixture in 1N H_2SO_4 instead of by vacuum evaporation as done by authors.

b: purity of palmitoyl chloride was verified by its IR spectrum.

Overall recovered yield, 0.235 g, 10.6%; conversion based on specific activity, 37.7%; mp[a] ca. 143-147°; IR (Nujol) (appendix 2): 1760 (ester C = O), 1740 (ketone C = O), 1670 (α, β - α', β' unsaturated ketone C = O), 1620 (C = C conjugated with ketone), and 890 cm^{-1} (cis CH of Δ -1,4 system); ^{1}H-NMR ($CDCl_3$): δ 0.88 (t, 3H, terminal CH_3), 0.94 (s, 3H, 18-CH_3), 1.25 (narrow m, 27H, acetonide β - CH_3 and CH_2 chain), 1.42 (s, 3H, acetonide αCH_3), 1.55 (s, 3H, 19-CH_3), 1.6-2.5 (m, 4H, $CH_2CH_2CO_2$), 4.90 (s, 2H, 21-CH_2O), 4.97 (br, 1H, 16-CHO), 6.12 (d, 1H, $J_{2,4}$ = 0.6 Hz, 4-CH), 6.44 (dd, 1H, $J_{2,4}$ = 0.6 Hz), $J_{1,2}$ = 9.5 Hz, 2-CH), and 7.22 ppm (d, 1H, $J_{1,2}$ = 9.5 Hz, 1-CH).

Anal - Calc. for $C_{24}H_{61}O_7F$: C, 71.39; H, 9.44; F, 2.82. Found: C, 71.26; H, 9.25; F, 2.80.

A portion of the reaction mixture was kept at room temperature for about a week to see whether there was any improvement in the yield.

Method using chloroform as solvent[b]:

Approximately 1 mmol of TRMA (439 mg) was dissolved in 35 ml of chloroform in a round bottom flask. Triethylamine,

---

a: Melting point was determined using Gallenkamp melting point apparatus.

b: Methylene chloride could not be used as solvent as the solubility of TRMA was less in this solvent.

0.3 ml (202 mg, 2 mmol), and palmitoyl chloride, 0.7 ml (550 mg, 2 mmol) were added. The mixture was then stirred magnetically at room temperature for abo 23 hrs, then at 42^{o} for 19 hrs. The reaction mixture was washed with 1 N H_2SO_4 (15 ml). The chloroform layer was collected and evaporated to dryness using an air jet. Dry residue was then purified by column chromatography as before. Yield 105 mg, 15.6%.

Method of Diamanti and Bianchi (1971):

Approximately 1 mmol of TRMA (435 mg) was dissolved in 4 ml of N,N-dimethylformamide in a 10 ml pear shaped flask. One ml of pyridine (160 mg, 2 mmol) and 0.7 ml (550 mg, 2 mmol) of palmitoyl chloride were added. The mixture was stirred using a magnetic stirrer for about 22 hrs. The solution was then poured into 1 N H_2SO_4 (1600 ml) with vigorous stirring. Separated solid was then filtered. This crude product was purified by column chromatography. Yield, 471 mg, 70%.

Different solvent systems were tried to separate TRMA-21-palmitate from the other components of the reaction mixture, by TLC using silica gel plates. On the basis of R_f values, the solvent system containing toluene:ethyl

acetate:glacial acetic acid (90:10:1) was chosen for column chromatography.

Preparation and comparative evaluation of TRMA and TRMA-21-palmitate liposomes

To reliably determine encapsulation efficiency, radioactive triamcinolone acetonide-21-palmitate was prepared by Diamanti and Bianchi method from [6,7^{3}H(N)]-triamcinolone acetonide. Multilamellar liposomes were prepared as described by Mezei and Gulasekharam (1980) using 13 mole% TRMA-21-palmitate and 87 mole% DPPC, a formula similar to the one recommended for cortisol-21-palmitate liposomes (Fildes and Oliver 1978). Dipalmitoyl phosphatidyl choline (160 mg) and triamcinolone acetonide-21-palmitate (22 mg, 350 µCi) in chloroform-methanol (2:1) were evaporated in a pear shaped flask using a rotary vacuum evaporator to obtain a thin film. The film was then dispersed with 22 ml of aqueous 8 mM $CaCl_2$ solution at 60^{o}C. The preparation was evaluated microscopically and then filtered through a 12 µm polycarbonate filter. The filtrate was then centrifuged at 22000 x g for 25 min. Radioactivity in each fraction was measured after each treatment. Triamcinolone acetonide liposomes were prepared in a similar manner.

STUDIES WITH METHOTREXATE

SECTION 1: REV LIPOSOMES

Preparation of MTX liposomes by reverse phase evaporation (REV) method:

Three formulation were used to prepare liposomes of different surface charges:

1. DPPC : CHOL : 0.5 : 0.5* (neutral liposomes)
2. DPPC : CHOL : SA : 0.4 : 0.5: 0.1 (positive liposomes)
3. DPPC : CHOL : DCP : 0.4 : 0.5 : 0.1 (negative liposomes)

Preparation of neutral MTX** liposomes:

DPPC 100 mg (136 µmoles) and cholesterol 52.66 mg (136 µmoles) were placed in a 50 ml round bottom flask with a long extension neck and the solvent was removed under reduced pressure by a rotary evaporator. The residue was redissolved in a mixture of 4.5 ml of chloroform and 8 ml of ether. A few drops of methanol were added to remove turbidity, if any, then MTX solution (≃ 50 mg MTX/6 ml with

---

*molar ratio

**All the operations involving MTX were conducted in dark for MTX is light sensitive. Also all the preparations containing MTX were stored in containers wrapped in aluminum foil.

or without ^{3}H-MTX) in 0.5% sodium bicarbonate (4.2 ml) was added. The resulting yellowish two-phase system was sonicated briefly (2-5 minutes) in a bath type sonicator at 0-5^{o}C. The mixture was then placed on a rotary evaporator and the organic solvent was removed under reduced pressure at room temperature (in about 30 minutes). Care was taken to prevent loss of the preparation due to frothing. The aqueous suspension was then shaken mechanically at 50-60^{o}C for 30 minutes. The preparation was allowed to stay at room temperature for about an hour and then observed under the optical microscope with a magnification of 640X.

Preparation of positively charged MTX liposomes:

These were prepared similar to neutral MTX liposomes except that lipid concentration was: DPPC 80 mg (109 µmoles), cholesterol 52.56 mg (136 µmoles) and stearylamine 7.5 mg (27 µmoles).

Preparations of negatively charged MTX liposomes:

These were prepared similarly to neutral MTX liposomes except that lipid concentration was: DPPC 80 mg (109 µmoles), CHOL 52.56 mg (136 µmoles) and dicetyl phosphate 14.9 mg (27 µmoles).

Preparation of 'empty' liposomes:

The 'empty' liposomes, containing no MTX, were prepared by using 0.5% sodium bicarbonate solution instead of the MTX solution. Presence or absence of charged lipid gave a charge or no charge to the liposome surface.

Separation of free MTX from liposome encapsulated MTX:

The liposomal preparation was centrifuged in a high speed centrifuge at 16000 rpm (22000 x g) for 25 minutes at room temperature. The pellet was then washed with 0.5% sodium bicarbonate solution by centrifugation. Finally the pellet was made up to the desired volume with 0.5% sodium bicarbonate solution. Percentage encapsulation of MTX was arrived at by comparing the amount of MTX present in the pellet to the original quantity of MTX taken for encapsulation.

Determination of MTX in liposomes:

Direct spectrophotometric assay of MTX in liposomes:

An aliquot sample (100 µl) of the neutral MTX liposomes, was diluted to 50 ml with 0.5% sodium bicarbonate solution and the absorbance was read at 370 nm using 0.5% sodium bicarbonate solution as blank. MTX has an extinction coefficient of 7×10^3 M^{-1} cm^{-1} at 370 nm. Based on this, MTX content was calculated.

Spectophotometric assay after triton-x-100 treatment:

An aliquot sample (100 µl) of the neutral MTX liposomes was diluted to 50 ml and the optical density (OD) at 370 nm was noted. Triton-X-100 (0.5 ml) was then added to 50 ml of diluted liposomal preparation (final concentration of triton-X-100 = 1% v/v) and allowed to stand at room temperature for 30 minutes. Optical density was again recorded.

Spectrophotometric assay of MTX in liposomes using 'empty' liposomes as blank:

'Empty' liposomes were prepared similar to neutral MTX liposomes. Both MTX and 'empty' liposomes were diluted to the same extent with 0.5% sodium bicarbonate solution and the OD of MTX liposomes was read at 370 nm using 'empty' liposomes as blank.

MTX determiantion in liposomes using methanol as solvent:

Preparation of standard curve: Standard solution of MTX was prepared by adding 1 ml of 0.5% sodium bicarbonate solution (to assist in dissolving MTX) to 9.07 mg of MTX and was made up to 100 ml with distilled methanol. Aliquots (1,2,3,.....7 ml) of standard solution were diluted to 25 ml with methanol and the extinction at 303 nm was measured. Calibration curve was obtained by plotting absorbance readings against concentration of MTX.

Assay of MTX liposomes:

MTX liposomes were suitably diluted with methanol and the OD was read at 303 nm using methanolic solution of empty liposomes as blank. Concentration of MTX was read off from the calibration curve.

Radioactive assay of MTX in liposomes:

Whenever ^{3}H-MTX liposomes were prepared, MTX content was quantitated by radioactive tracer technique. Aliquots of liposomes were counted in Bray's solution or Biofluor^{R} using the liquid scintillation counter.

Preliminary stability studies of neutral MTX liposomes:

MTX, 58.7 mg containing 10 μCi of ^{3}H-MTX was dissolved in 6 ml of 0.5% sodium bicarbonate solution. Neutral MTX liposomes were prepared as described earlier using 4.2 ml of this MTX solution. A 2 ml sample of liposomes was stored at 37° and another one at room temperature for one week. Free MTX was separated from liposomal MTX by centrifugation as described earlier. MTX remaining in the liposomes was determined by radioactive counting of liposome sample.

Size distribution of liposomes:

Size distribution of liposomes was determined by using a Coulter Counter equipped with a 100 micron aperture tube. The machine was precalibrated with monodisperse latex particles of standard size. Size distribution of liposomes was generated by systematically altering the aperture current, threshold and attenuation settings of the instrument.

In vitro drug release studies from positive MTX liposomes:

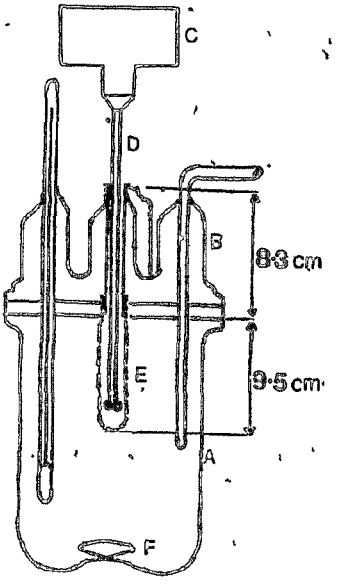
Preparation of calibration curve for MTX:

For the estimation of MTX in the dialysis medium, a calibration curve was constructed as follows: A stock solution was prepared by dissolving ~10 mg of accurately weighed MTX in 50 ml of 0.5% $NaHCO_3$ solution. The stock solution was suitably diluted to obtain MTX solutions of strength 0.2 μg/ml to 10 μg/ml. Absorbance of these

solutions were determined using Spectronic Spectrophotometer at the wavelength of 303 nm. Calibration line was obtained by plotting absorbance against drug concentration.

Drug release studies:

MTX liposomes, for drug release studies, were prepared by REV method using the formula DPPC:CHOL:SA:0.4:0.5:0.1. The release of MTX from liposomes was determined by a modification of the dynamic dialysis method of Arakawa et al. (1975) which in itself was a modification of original method of Meyer and Guttman (1968, 1970). USP dissolution test apparatus was used with modification as shown in Fig. 5. MTX liposomal preparation was diluted with 0.5% $NaHCO_3$ solution in a volume ratio of 2:1. Seven ml of this diluted liposome preparation (total MTX = 10.5-12.25 mg) was placed inside a 9.5 cm long dialysis bag (inflated diameter 1.58 cm) suspended from a 8.3 cm length tube made out of a 10 cc plastic syringe. The sac was suspended in 900 ml of 0.5% $NaHCO_3$ solution, contained in the dissolution vessel. The system was maintained at a fixed temperature by the temperature controlled water bath. The contents of the sac were stirred with a twisted glass rod rotated at ~ 125 rpm. External solution was stirred with a magnetic stirring bar. In order to determine the drug content of the external solution, the solution was continuously circulated through a flow through cell placed in a spectrophotometer which read



A, vessel; B, four-hole cover ; C, stirring motor;
D, glass stirrer; E, dialysis bag; F, stirring magnet

Figure 5. USP XVIII dissolution testing apparatus modified to study the drug release from liposomes.

the absorbance at 303 nm. The absorbance was recorded on a chart by the recording device interfaced with the spectrophotometer. Concentration of MTX was obtained by referring to the calibration curve. Experiments were done at different temperatures.

To see the effect of fetal bovine serum (FBS) on the release of MTX from liposomes, the liposomal preparation was mixed with dialysed FBS, in a volume ratio of 2:1 and was placed in the dialysis tube. The release of MTX was then determined as described earlier.

Covalent attachment of antibodies to liposomes:

Synthesis of N-[3-(2-Pyridyldithio) propionyl]dipalmitoyl phosphatidyl ethanolamine (PDP-DPPE):

PDP-DPPE was synthesized by the method of Martin et al. (1981). DPPE 34.6 mg (50 µmol) was "dissolved" in 5 ml of absolute methanol containing 7 µl (50 µmol) of triethylamine and 25 mg (80 µmol) of SPDP. The reaction was carried out under nitrogen atmosphere. Course of reaction was followed by TLC. Reaction was stopped after 5 hours. Methanol was removed by vacuum evaporation and the products were redissolved in chloroform and applied on a 6-7 g (10 ml) silica gel (100-200 mesh) column which had been activated (150°C overnight) and prewashed with 100 ml of chloroform. The column was eluted with an additional 20 ml of chloroform followed by 20 ml of each of the following, chloroform: methanol mixtures - 40:1; 30:1; 25:1; 20:1; 15:1

and finally with 60 ml of 10:1 chloroform-methanol. The maximum flow rate was about 2 ml per minute. The phosphate containing fractions eluting in 15:1 and 10:1 chloroform-methanol were combined and concentrated by rotary vacuum evaporation.

Analysis by TLC (Silica gel H; solvent system: chloroform-methanol-acetic acid 60:20:3; detecting reagent: molybdic acid reagent and/or iodine vapors) indicated phosphate positive spot of R_f value greater than that of DPPE. The product was stored at -15°C. The quantity of product obtained was too small to determine the yield.

Different solvents like methylene chloride, pyridine, dimethyl formamide, ethanol, dimethylsulfoxide were tried to dissolve DPPE so that the yield of PDP-DPPE could be improved.

Synthesis of N-[3-(2-Pyridyldithio) propionyl] phosphatidylethanolamine (PDP-PE):

PDP-PE was synthesized the same way as PDP-DPPE (Martin et al. 1981). The reaction was conducted in a screw capped Multivial (Supelco) with a Teflon^R septum under nitrogen atmosphere using egg phosphatidyl ethanolamine 37.2 mg (50 µmol*). Unlike DPPE, egg PE was

---

*Based on dioleyl form egg PE has a mol. wt. of 744 as reported in Supelco Handbook of Lipids, 6th ed. 1980, p. 10.

completely soluble in methanol. A special vial was used for better stability of egg PE during reaction. Chromatographically purified PDP-PE was weighed and the yield calculated. The product was stored in a dessiccator at -15°C.

Synthesis of N-[3-(2-Pyridyldithio)propionyl] stearlyamine(PDP-SA) (Fig. 6):

Though the principle of this synthesis is similar to the above two syntheses the procedure of synthesis and purification was developed by us. Stearylamine 260 mg (965 µmol) and SPDP 200 mg (640 µmol) were dissolved separately in 12 ml portions of absolute methanol. The solution of SPDP was added dropwise to the stearylamine solution and reaction was carried out at room temperature. Reaction was monitored by TLC. After 30 minutes of reaction, the methanol was removed by vacuum evaporation. The product was purified by column chromatography on 12 g of silica gel (100-200 mesh). Column was eluted with ethyl acetate-petroleum ether (40:60) using a maximum flow rate of 85-100 ml/hr, collecting 10 ml fractions which were monitored by TLC. Fractions containing PDP-SA were pooled and concentrated under reduced pressure. Overall recovered yield was 282 mg, i.e. 94%; mp 61°C; IR(CH_2Cl_2)(appendix 4): 3450(-NH-), 2950 [$(CH_2)_{18}$], 1690(-CO-NH-), 1525cm^{-1} (Amide II); [^{13}C]-NMR ($CDCl_3$) (appendix 5): 175, 170(cis and trans CO), 160-120 (5, pyridyl), 40-27 (several, CH_2), 23 ppm (CH_3).

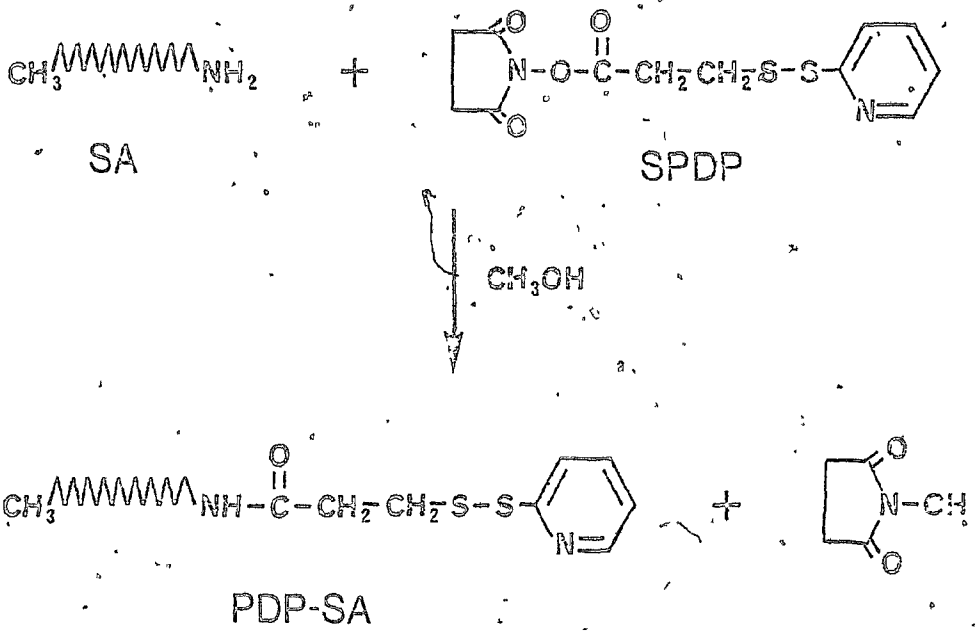


Figure 6. Synthesis of N-[3-(2-pyridyldithio)propionyl] stearylamine (PDP-SA).

Anal.-Calc. for $C_{26}H_{46}H_2S_2O$: C, 66.90; H, 9.93; N, 6.00; S, 13.74. Found C, 67.28; H, 9.89; N, 5.81; S, 13.75.

Preparation of antibody fragments:

Preparation of $F(ab')_2$ from IgG

Normal rabbit serum IgG, about 120 mg, stored precipitated in 40% saturated ammonium sulfate at 4°C was centrifuged at 1000 x g for 15 min at 4°C. Precipitate was dissolved in a minimum volume of 0.01 M phosphate buffered saline (PBS) (pH 7.2). The IgG solution was then dialysed against PBS at 4° until free from NH_4^+ ions to Nessler's reagent. This took about 2 days, changing the PBS (4 liters) four times. Protein content was determined by Biuret method.

Digestion of IgG: IgG (120 mg) in PBS (pH 7.2) was dialysed against 0.1 M sodium acetate - acetic acid buffer (pH 4) solution for about 13 hours to equilibrate the IgG solution with the buffer. Precipitate, if any, was removed by centrifugation. Protein concentration was then determined by Biuret method. Concentration of IgG was adjusted to lie between 10-20 mg/ml. For each 100 mg IgG, 2 mg of pepsin (dissolved on 0.1 M sodium acetate-acetic acid buffer) was added, which was then incubated at 37° for 18-20 hrs in a waterbath for digestion. Pepsin and other insoluble materials were removed by centrifugation (1000 rpm for 20 min). To inactivate the enzyme that was not removed

the supernatant was dialysed against PBS pH 7.2 for about 22 hrs.

Isolation of fragments: A 7.5 ml aliquot of the pepsin digest was applied to a sephacryl S-200 column ($K_{15/90}$ column, Bed volume = 90 x 1.5 cm = 154 ml) pre-equilibrated with PBS. The column was eluted with PBS collecting 2 ml fractions at 3-4 min time interval. Absorbance of each of the 100 fractions was measured at 280 nm using UV spectrophotometer. Fractions representing included volume, consisting mainly of $F(ab')_2$ and some IgG and/or Fc, were pooled.

Removal of undigested IgG and Fc by affinity chromatography: Protein A-Sepharose CL-4B, 1.5 g, was swollen in 10 ml of PBS for 1 hr at room temperature and then packed into a small chromatography column ($K_{9/15}$, Bed volume = 15 x 0.9 cm = 9.5 ml). Pooled fractions from the sephacryl S-200 column (20 ml) were filtered through this column at a flow rate of ~45 ml/hr. To remove the unbound proteins the column was washed with PBS until no more protein left the column. Fractions of 2-6 ml were collected. Absorption of each fraction was measured at 280 nm to detect the presence of protein. The bound protein (IgG and Fc) was eluted with a solution of 0.58% (0.1M) acetic acid in normal saline (pH 2.8). PBS fractions containing protein which represent $F(ab')_2$ were pooled and concentrated using immersible CS-10™ ultrafilters

(millipore) to get a $F(ab')_2$ concentration of about 10 mg/ml. $F(ab')_2$ yield was found to be 44%. The column was regenerated by washing with PBS (two column bed volumes). The column was stored at 4^{o}.

Preparation of Fab' fragments:

$F(ab')_2$ solution in PBS was dialysed against pH 5.5 buffer for about 12 hours to equilibrate with the buffer. Fab' fragments were generated from the above $F(ab')_2$ preparations (10 mg/ml) by reduction with dithiothreitol (final concentration 20 mM) for 90 min under nitrogen atmosphere at $25^{o}C$. The mixture was then placed on Sephadex G-25 column and dithiothreitol was separated from the reduced Fab' fragments with the aid of deoxygenated buffer pH 5.5. Fab' appearing in the void volume of such a column was maintained under nitrogen and used immediately for coupling to liposomes.

Preparation of liposomes for Fab' coupling:

DPPC, cholesterol and PDP-SA in the mole ratio of 0.4: 0.5: 0.1 were used to prepare empty liposomes by the reverse phase evaporation technique described earlier. Specifically, DPPC 80 mg (109 μmol), cholesterol 52.5 mg (136 μmol) and PDP-SA 12.5 mg (27 μmol) and 0.5% sodium bicarbonate solution (4.2ml) were used to prepare liposomes.

Coupling of Fab' fragments to liposomes (Fig. 7):

The liposomal preparation was diluted three times with 0.5% sodium bicarbonate solution so that the concentration

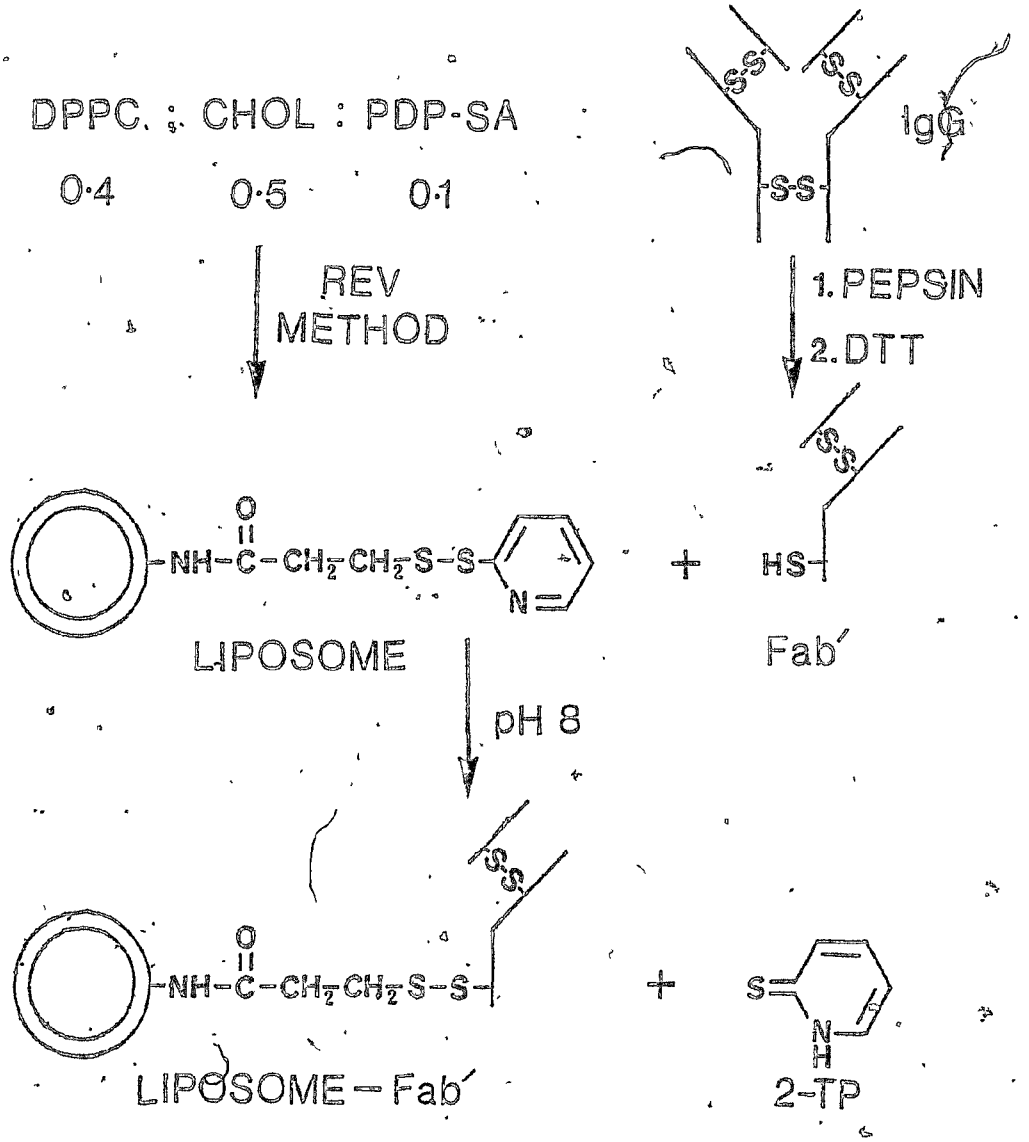


Figure 7. Schematic representation of liposome-Fab' coupling (Martin et al. 1981).

of DPPC was about 4-12 µmol/ml. An equal volume of Fab' fragments was then mixed under nitrogen in a sealed vial. The pH was adjusted to 8 with 1N sodium hydroxide. The mixture was stirred magnetically under nitrogen at 25°C for 2 hours. Reaction mixture was then centrifuged at 16000 rpm (22000 x g) for 25 min. Supernatant was set aside. The pellet was washed with 0.5% sodium bicarbonate solution by centrifugation and then made up to the desired volume with 0.5% sodium bicarbonate solution. Fab' concentration in liposomes was determined by Lowry's protein estimation method. Insoluble lipid material was removed by centrifugation before measuring the absorption at 540 nm.

Measurement of 2-Thiopyridinone (2-TP) release:

An excess DTT dissolved in 0.5% $NaHCO_3$ solution (i.e. 4 drops of 40 mg/ml DTT solution) was added to a dispersion of DPPC-Chol-PDP-SA liposomes (4:5:1, 50 µl liposomes and 3 ml 0.5% $NaHCO_3$ solution) at time 0. Absorbance was measured at 343 nm in a dual beam recording spectrophotometer (PyeUnicam SP8-100 UV spectrophotometer).

Covalent coupling of IgG to liposomes by ECDI method (Fig. 8):

Preparation of liposomes:

REV liposomes with the formula DPPC:CHOL:DPPE:0.4:0.5:0.1 were prepared as described earlier. They contained DPPC 80 mg (109 µmol), cholesterol 52.5 mg (136 µmol), DPPE 18.9 mg (27 µmol) and 0.5% $NaHCO_3$ (pH 8) solution 4.2 ml.

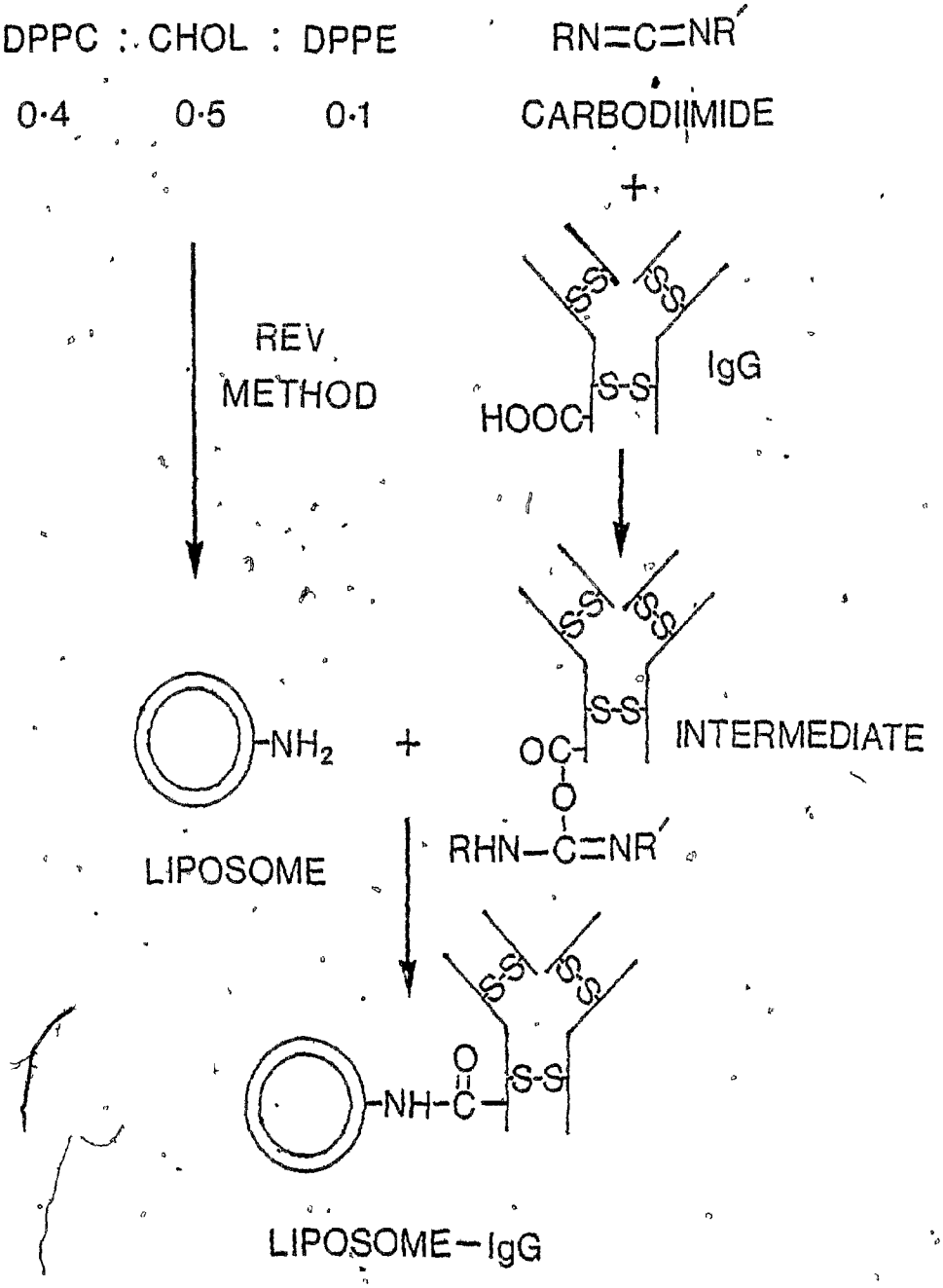


Figure 8. Schematic representation of liposome-IgG coupling by carbodiimide method.

Coupling of IgG to liposomes:

The procedure described by Endoh et al. (1981) was followed. A 20% solution (0.4 ml) (i.e. 80 mg) of 1-ethyl-3-(3-dimethylaminopropyl)carbodimide HCl was added to a mixture of liposomes (100 µl) and normal rabbit IgG solution (4 ml). The pH of the reaction mixture was found to be approximately 7.5. After standing for 2 hours at 25°C the pH was adjusted to 4.1 with 0.3 N HCl. The mixture was layered over 7 ml of Ficoll-paque and centrifuged at 3500 rpm (750 x g) for 20 min at 21°C. The liposomes floating on top were carefully withdrawn using a pasteur pipette and resuspended in .5 ml of 0.5% sodium bicarbonate solution and washed three times by centrifugation. The pellet was diluted with 100 µl of 0.5% sodium bicarbonate solution. IgG content of liposomes was determined by modified Lowry's method of protein estimation ("Methods in Enzymology" vol. 72, pp 296-297).

The nonspecific binding of IgG to liposomes was determined by the same procedure but without using ECDI and 0.3 N HCl.

Covalent coupling of IgG to liposomes by Barbet et al. (1981) method:

Preparation of liposomes:

Preparations containing large unilamellar and oligolamellar liposomes were obtained by the reverse phase evaporation method using DL-α-dipalmitoyl phosphatidyl

choline (DPPC), cholesterol, SA, and PDP-SA in a molar ratio of 4:4:1:1. Specifically, DPPC (80 mg, 109 µmol), cholesterol (42 mg, 109 µmol), SA (7.5 mg, 27.8 µmol) and PDP-SA (12.7 mg, 27.3 µmol) were dissolved in a mixture of chloroform (4.5 ml) and diethyl ether (8 ml). Next, 4.2 ml of 0.5% $NaHCO_3$ (pH 8) was added, after which, the lipid solution was sonicated at 0-5°C for 5 min in a bath-type sonicator. Organic solvents were removed from the resulting emulsion-like dispersion by a rotary vacuum evaporator. The aqueous dispersion was then shaken at 55°C for half an hour to obtain liposomes.

Preparation of PDP-IgG (Fig. 9):

Rabbit anti-BSA IgG was fractionated with 33% saturated ammonium sulfate from immune sera. Pyridyl disulfide moieties were introduced into IgG, using the heterobifunctional reagent SPDP by the method of Carlsson et al. (1978). Ten moles of SPDP was reacted with 1 mole of IgG in 0.1 M sodium phosphate buffer containing 0.15 M NaCl, pH 7.5, for 30 min. The reaction mixture was dialyzed extensively against the above buffer at 4°C in order to remove low molecular weight substances. The content of 2-pyridyl disulphide groups, as determined by the method of Carlsson et al. (1978), was 6-8.2 per mol of IgG. The modified IgG was stored at -20°C until needed.

Covalent coupling of IgG to liposomes (Fig. 10):

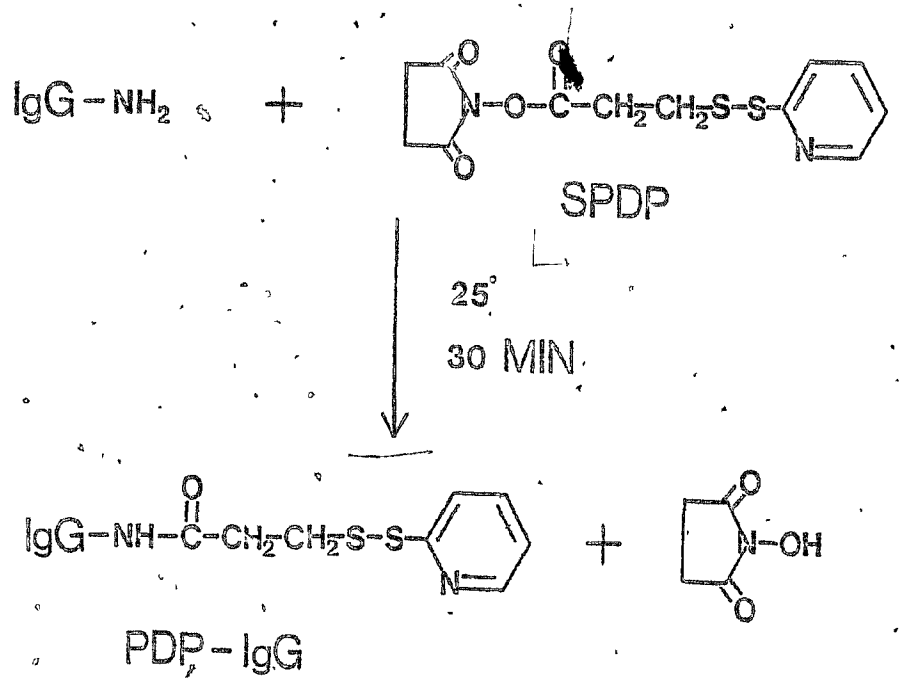


Figure 9. Preparation of PDP-IgG

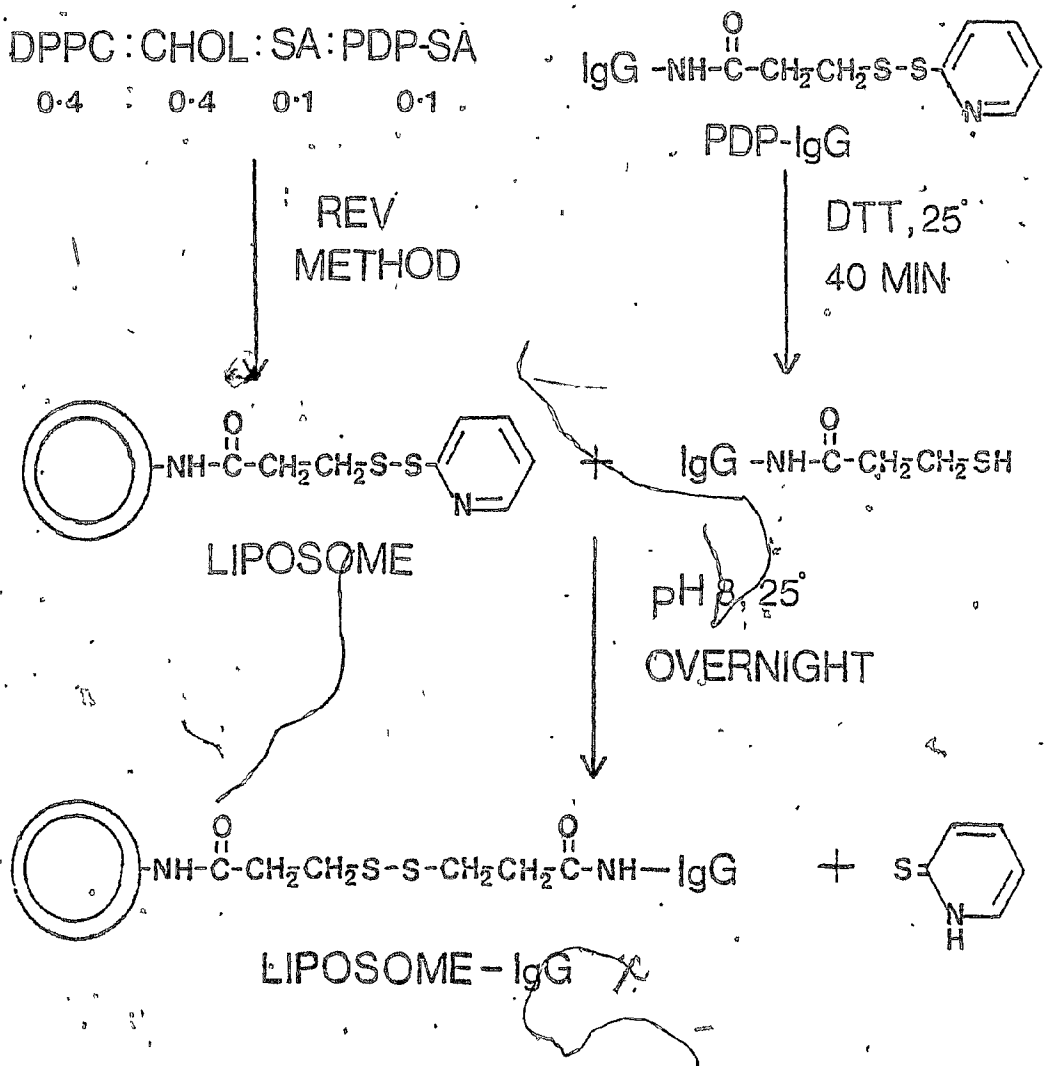


Figure 10. Schematic representation of liposome-IgG coupling (Barbet et al. 1981).

PDP-IgG was bound to liposomes by the method of Barbet et al. (1981). PDP-IgG was reduced with dithiothreitol (DTT) in 0.1 M sodium acetate buffer containing 0.15 M NaCl, pH 4.5 for 40 min. Excess DTT and pyridine-2-thione were removed by gel filtration on Sephadex G-25 M column equilibrated with 0.5% $NaHCO_3$. Thiolated IgG (8-12.6 mg) and liposomes (26 µmol of phospholipid) were stirred together overnight at room temperature and pH 8. Liposomes were separated from unbound antibody twice by centrifugation at 22000 x g for 25 min followed by washing of the pellet with 0.5% $NaHCO_3$. In order to determine non-specific adherence of antibodies to liposomes, 9 mg of PDP-IgG that had not been reduced by DTT was mixed with liposomes as above. The protein content of the liposome fraction was analysed by the method of Lowry et al. (1951).

Determination of antibody activity

Preparation of ^{125}I-BSA:

Radioiodination of bovine serum albumin (BSA) was done by the chloramine T technique (Hudson and Hay 1980, Chard 1982). Iodination procedure was carried out in a fume hood taking necessary precautions. First the following solutions were prepared: BSA, 4 mg/ml in 0.1 M PBS pH 7.5, chloramine T, 1 mg/ml in 0.1 M PBS, sodium metabisulfite, 2 mg/ml in 0.1 M PBS. A prepacked Sephadex G-25 M PD-10 column was equilibrated with 0.01 M PBS containing 2 mg/ml of BSA. BSA 8 mg in 2 ml of 0.1 M PBS was placed in a small screw capped

glass vial and 2 mCi of $Na^{125}I$ (vol. = 40 µl) was added. Iodination reaction was started by adding 120 µg of chloramine T. The reaction mixture was stirred magnetically. After 2 minutes, the reaction was stopped by adding sodium metabisulfite, 240 µg. After making up the volume to 2.5 ml with 0.1 M PBS, the whole sample was loaded on the Sephadex G-25 column and eluted with 0.01 M PBS containing BSA 2 mg/ml. The first 2.5 ml of eluate was discarded and the next 3.5 ml was collected and dialysed extensively against 0.01 M PBS. ^{125}I-BSA prepared had a specific activity of ~77.38 µCi/mg of BSA.

Antibody activity before binding to liposomes:

The antigen-binding capacity of the anti-BSA IgG was determined using ^{125}I-BSA as antigen by a method based on Farr assay (Hudson and Hay 1980). An aliquot (5 µl or so) of stock ^{125}I-BSA was diluted with cold BSA solution (4 mg/ml in 0.1 M PBS) so that 400 µg of BSA (i.e. 100 µl) had an activity of ~50000 cpm. Varying amounts of anti-BSA IgG (from ~25 to ~250 µg) were placed in each of the plastic test tubes and total volume in each tube was made up to 40 µl with 0.1 M PBS. In order to determine non-specific adherence of BSA to the tubes, only 0.1 M PBS (40 µl) was added to two of the tubes. To each tube was then added 100 µl of ^{125}I-BSA (i.e. 400 µg and 50000 cpm) and the tubes were incubated at 37°C for 2 hr in a continuous shaker. This was followed by incubation in the refrigerator

($4^{o}C$) for an additional 2 hrs with occasional shaking. At the end of the incubation period, 360 µl of 0.1 M PBS and 500 µl of saturated ammonium sulfate solution were added to each tube and mixed rapidly. After allowing the tubes to stand for 30 min, they were centrifuged at 7000 rpm for 20 min. Supernatant was discarded. Precipitate was washed twice with 50% saturated ammonium sulfate solution by centrifugation. After removing the final wash supernatant, the precipitate was counted in a gamma counter. To determine non-specific binding of ^{125}I-BSA to normal rabbit IgG (NRG), the experiment was repeated for various concentrations of NRG similar to anti-BSA IgG. Amount of BSA bound to IgG was calculated and a calibration curve was plotted. The antigen binding capacity of anti-BSA IgG, before coupling to liposomes was determined from this calibration curve.

Antibody activity after binding to liposomes:

For determining the antigen-binding capacity of the IgG linked to liposomes, an aliquot of liposome-anti-BSA IgG containing 314 µg of IgG was incubated with an excess of ^{125}I-BSA(400 µg, 55000 cpm) for 2 hr at $37^{o}C$ with continuous shaking. This was followed by incubation at $4^{o}C$ for an additional 2 hr. Liposome-antibody-antigen complex was then sedimented by centrifugation at 22000 x g for 30 min. The pellet was washed twice with 0.1 M PBS. The amount of BSA bound to liposomes was calculated by

determining the ^{125}I associated with the pellet. In control experiments the non-specific binding of ^{125}I-BSA to normal rabbit IgG-liposomes was determined. Values for specific binding to anti-BSA IgG were corrected by subtracting the amounts of radioactivity that bound non-specifically.

Leakage of drug during coupling procedure:

This was investigated by linking IgG to liposomes containing methotrexate in them. These liposomes were prepared similar to 'empty' liposomes by REV method as described already, except that a 20 mM solution of MTX in 0.5% $NaHCO_3$ was used for entrapment. Crude liposomes obtained were washed twice with 0.5% $NaHCO_3$ by centrifugation at 22000 x g for 25 min. The washed pellet, which consisted of MTX liposomes free from unentrapped MTX, was finally dispersed in a volume of 0.5% $NaHCO_3$. The leakage of entrapped MTX during antibody-liposome coupling was calculated as the difference between liposomal MTX content (determined spectrophotometrically at 303 nm in methanol) before and after attachment of thiolated normal rabbit IgG to the liposomes.

Studies of antibody mediated binding of positive MTX liposomes by melanoma cells:

Membrane Immunofluorescence assay of PDP-anti-melanoma globulin:

To see whether there was any change in the antibody

titer on thiolation, membrane immunofluorescence was performed on viable M_{21} melanoma cells. A cell suspension containing 10^7 cells/ml was prepared in 0.01 M PBS using freshly harvested cells. A series of dilutions (2 mg, 200 µg, 100 µg, 50 µg, 25 µg/ml) of thiolated and non-thiolated anti-melanoma globulin and normal rabbit globulin (negative control) were prepared in 0.01 M PBS containing 0.1% BSA. Fifty µl of cell suspension was placed in each of the several polypropylene micro test tubes of size 400 µl. Diluted antibody sample, 50 µl, was added to each tube and mixed well. Tubes were incubated at $4^{o}C$ for 30 min. After the incubation was over, cells were washed three times with cold 0.01 M PBS by centrifugation in Beckman ^{R} 152 microfuge for 2 min. Goat anti-rabbit globulin (GARG) - fluorescein isothiocyanate (FITC) conjugate (conc. 1:10), 20 µl was added to the washed cells in each tube and reincubated at 4^{o} for 30 min. Following this cells were again washed three times with 0.01M PBS by centrifugation as before. Cells were then suspended in a drop of glycerol (i.e. 90% glycerol in tris buffer). A droplet of the cell suspension was mounted on glass slide for observation by fluorescence microscopy.

Preparation of ^{3}H-MTX liposomes and antibody conjugation:

^{3}H-MTX containing liposomes were prepared by REV method using the formulation DPPC:CHOL:SA:PDP-SA:0.4:0.4:0.1:0.1. The solution used for entrapment had a MTX conc.

of 250 µg/ml. and a radioactivity of 6.736 µCi/ml. Anti-melanoma globulin and NRG were conjugated to liposomes by Barbet et al. (1981) method. Final liposome preparation consisted of 58 µg/ml of MTX, 2 mg/ml of antibody and 1.545 µCi/ml of radioactivity.

Antibody mediated binding of positive MTX liposomes by melanoma cells:

Antibody mediated binding of MTX liposomes by melanoma cells was assessed qualitatively and quantitatively by immunofluorescence and radiotracer method respectively.

Immunofluorescence technique:

Membrane immunofluorescence staining technique used for assessing the titer of the antibody was adapted here. A series of dilutions (containing antibody conc. 2 mg, 200 µg, 100 µg, 50 µg, 25 µg/ml) of MTX liposomes bearing anti-melanoma globulin, NRG or no antibody were prepared in 0.01 M PBS containing 0.1% BSA. Melanoma cells were treated with these samples following by GARG-FITC conjugate and processed as described under membrane immunofluorescence assay.

Radiotracer method:

Single cell suspension of melanoma M_{21} cells of concentration ~0.5 x 10^6 cells/ml was prepared in RPMI 1640 medium containing 10% FBS and antibiotics. These cells were plated in tissue culture dishes (60 x 15 mm) at a concentration of ~3 x 10^6 cells per dish and allowed to

grow in the incubator at 37°C. At 72 hr, floating cells in each dish were discarded and cells adhering to the dish were washed gently three times with 3 ml of cold 0.01 M PBS. Cells were then incubated with 50 µl of ^{3}H-MTX liposomes with or without conjugated antibody in 1.5 ml of RPMI 1640 medium containing no FBS, for various times at different temperatures. The final concentration of various components in the incubation mixture were as follows: MTX, 1.9-2.0 µg/ml (4.20-4.45µM); antibody, 54-69 µg/ml; radioactivity ~168860 dpm/1.5 ml. At the end of the incubation period, unbound liposomes were discarded by decantation and adhering cells were washed gently, five times with 2-3 ml of 0.01 M PBS. The cells were then detached with 1.5 ml of EDTA solution. Detached cells (1.5 ml) along with the EDTA washings of the plate (1.5 ml) were layered gently on 10 ml of 10% Ficol (with 5 mM EDTA) placed in a centrifuge tube. Tubes were centrifuged in IEC clinical centrifuge at maximum speed for 10 min. Cell pellet was washed with 6 ml of 0.01 M PBS by centrifugation for 10 min. Cell pellet was then dissolved in 1 ml of 0.5% buffered sodium dodecyl sulfate solution (see appendix). To determine the cell associated radioactivity, an aliquot (0.5 ml) was added to 15 ml of Biofluor^{R} and counted in the liquid scintillation counter. Protein estimation was done by the method of Lowry et al. (1951) using 100 µl aliquots of lysate.

In vitro studies against Caki cells:

Human renal carcinoma (Caki) cells were grown in 250 cm^3 polystyrene flasks in McCoy's medium supplemented with 15% fetal bovine serum and antibiotics (200 u penicillin, 200 µg streptomycin per ml). Cells were harvested from log phase growth. Cells were suspended by a 3-5 min incubation with 0.25% trypsin in Ca- and Mg-free balanced salt solution (BSS) at 37°C, followed by gentle pipetting. Cell viability was monitored by trypan blue exclusion test. Approximately 10^5 cells in 2 ml were seeded into each 35 mm plastic petri dish. After allowing the cells to grow for 24 hours, the medium was carefully removed. Two ml of fresh medium containing either liposome entrapped methotrexate, non-entrapped methotrexate or empty liposomes was added to each petri dish. In the first type of the cytostatic studies (i.e. in case of non-entrapped MTX and a control) these plates were allowed to incubate for 4 days at 37° in humidified atmosphere of 5% CO_2/95% air. On day 4, the final cell density in each of the petri plates was determined with a coulter counter. In the second type of cytostatic studies (i.e. the liposomal MTX, empty liposomes and a control) the medium in the plates was removed. The new medium containing the drug was added to each petri dish. This procedure was repeated every 24 hours for 3 consecutive days. 24 hours after the last change of medium, the final cell density was determined. Percent inhibition

of growth was calculated by comparing the growth of treated cells to that of the untreated cell population. Growth curve for Caki cells was determined by counting cell growth at different time intervals for up to 5 days.

In vitro studies against EL_4 lymphoma cells:

Growth curve for EL_4 lymphoma cells:

EL_4 lymphoma (EL_4) cells were grown in 200 cm^3 glass bottles in RPMI 1640 medium (18 ml) supplemented with 10% fetal bovine serum (FBS) and antibiotics (200 u penicillin, 200 µg streptomycin per ml). Cells were harvested from log phase growth (after approximately 4 days of growth). Cells were centrifuged at 1600 rpm (200 x g) for 6 min at 21°C to remove medium and then washed in PBS (50 ml) by centrifugation. After taking the initial count using hemocytometer, cell concentration was adjusted to 2 x 10^6 cells/ml with the medium. This was used as stock cell suspension. Cell viability was checked by trypan blue exclusion test. Cell suspensions of desired strength were prepared from stock cell suspension. Two ml of cell suspension in the medium was placed in 35 mm petri dishes at two different initial cell densities of 5×10^4 cells/ml and 1×10^5 cells/ml. The petri plates were incubated at 37°C in humidified atmosphere of 5% CO_2/95% air. Cell counts per plate was obtained using a coulter counter at settings of threshold 5, amplification 1 and aperture current 16. For counting, the cell suspension was diluted

to the 20 ml mark in the coulter cup. Counts were made of 3 or 4 replicate plates. Cells were counted every 24 hours up to 4 days. Growth curve was obtained by plotting the number of cells against time in hours.

Attempts to quantitate the number of cells in presence of liposomes:

Use of centrifugation to selectively settle cells:

Samples containing EL_4 cells and liposomes were centrifuged at 1600 rpm (200 x g) for 10 min at 21°C. Cell counts were made of the pellet and supernatant fractions using a coulter counter.

Use of Fluorescence Activated Cell Sorter (FACS):

Samples containing cells and liposomes were treated with acridine orange as in scheme I in order to stain the cells. Samples were then analysed using the cell sorter. Fluorescence distribution of cells and scatter distribution of liposome and/or cells were obtained.

Use of Triton X-100, sodium dodecyl sulfate, methanol and ether:

EL_4 cell suspension of strength approximately 100,000 cells/ml was prepared. Different dilutions (i.e. 1:50, 1:500, 1:5000) of neutral MTX liposomes in medium, were prepared as would be encountered in actual in vitro studies. To 1 ml of cell suspension 100 µl of liposomes were added and these samples were then incubated with different concentrations of triton-X-100, sodium

dodecyl sulfate, methanol and ether for 30 minutes at room temperature. Counts were taken in the coulter counter before and after incubation with these reagents. Cells alone and liposomes alone were also treated in the similar manner and counts taken.

Scheme I

Fluorescence staining of EL_4 cells using Acridine orange:

Prepare sample so that concentration is 1-2 x 10^6 cell/ml

↓

Take 0.2 ml of cells

↓

Add 0.5 ml of solution A*.

↓ incubate for 45 sec on ice

Add 1.2 ml of Acridine orange in solution B*

(Final concentration of Acridine orange with cell: 30 µg/ml).

↓

Observe under the fluorescnece microscope or analyse in FACS

Solution A*:	Solution B*:
0.01% triton-X-100	63.5 ml of 0.2 M Na_2HPO_4
0.08 N HCL pH 2.2	36.5 ml of 0.1 M citric acid pH 6.0
0.15 N NaCl	0.0372 g of sodium EDTA (1 mM)

*Acridine orange is made up in distilled water at 1 mg/ml.

Effect of MTX liposomes against EL_4 cells in vitro:
(Fig. 11)

EL_4 lymphoma cells were grown in RPMI 1640 medium with 10% FBS and antibiotics. Cells were harvested from log phase growth. For in vitro studies (cytostatic studies) cells were seeded by dilution into 35 mm culture plates, 2 ml/plate at approximately 50000 cells/ml and incubated at 37°C in humidified atmosphere of 5% CO_2/95% air. After 24 hours, cell counts were made of the number of cells per plate. Different dilutions of liposomes ('empty' or containing MTX) were made with medium and added to each plate and incubated as above. Three days later plates from culture were counted. In case of liposome treated samples, counts were made before and after treatment with triton-X-100. Each count consisted of at least three replicates. Growth inhibition was calculated by comparing the growth in treated samples to that of control.

In vitro studies against human melanoma M_{21} cells:

Growth curve for human melanoma cells:

Melanoma (M_{21}) cells were grown in 200 cc prescription bottles as monolayer cultures in RPMI 1640 medium (18 ml) supplemented with 10% FBS and antibiotics. Cells were harvested from log phase growth (i.e. after about 4 days of growth). Cells were suspended by a 5-8 min incubation with ~5 ml of EDTA solution (see appendix 1) at 37°C, following by gentle tapping of the bottle. The EDTA

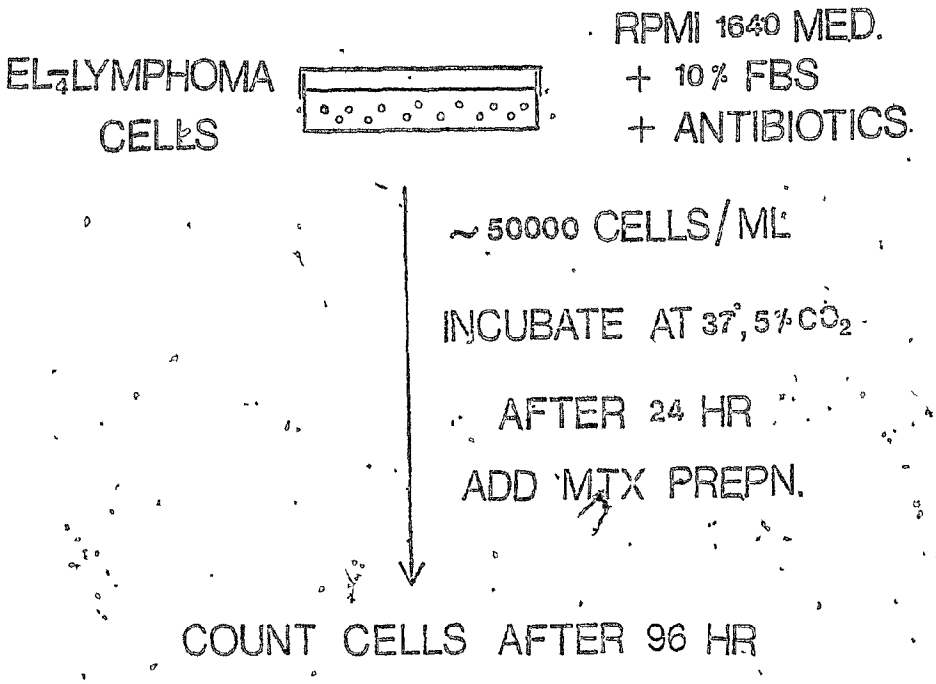


Figure 11. Anticancer evaluation of methotrexate (MTX) containing liposomes in vitro.

action was stopped by adding some spent medium (5 ml) and/or by dilution with 0.01 M PBS to a volume of 50 ml. Cells were pelleted and then washed with 50 ml of 0.01 M PBS by centrifugation. Cells were then suspended in the culture medium at a concentration of about 2×10^6 cells/ml to obtain a stock suspension. The viability of the cells was checked by trypan blue exclusion test. An aliquot of stock cell suspension was suitably diluted to obtain a cell suspension of strength 50000 cells/ml. Two ml of cell suspension (i.e. 100,000 cells) was plated in 35 x 10 mm tissue culture dish and cells were allowed to grow at 37°C. At the end of incubation, floating cells in the dish were discarded by decantation. Cells sticking to the surface of the dish were released by incubation with EDTA solution (1 ml) at 37°C and counted in the coulter counter. Cells were counted every 24 hr up to 5 days. The growth curve was constructed by plotting the number of cells against time of growth in hr.

Effect of MTX liposomes on human melanoma cells <u>in vitro</u>:

To see the effect of MTX liposomes on the growth of melanoma cells, 100,000 cells, i.e. 2 ml in each tissue culture dish were incubated at 37°C. At 24 hr, drug preparations (vol. 100 µl) were added to each dish at different concentrations and the incubation was continued. At 96 hr, incubation was stopped and the floating cells and liposomes in each plate were discarded by decantation.

Cells adhering to the dish were released by EDTA treatment and counted in the coulter counter. The inhibition of growth was calculated by comparing the cell growth in drug treated plate to that in untreated plate.

In vivo studies (Fig. 12):

EL_4 ascites tumor inhibition was tested in 12-17 weeks old, female C 57 BL/6J mice weighing about 20 g or more. Each mouse was inoculated (day 0) with 1×10^4 EL_4 lymphoma cells washed twice in PBS after aspiration from the peritoneal cavity of mouse bearing a 7 day old tumor (passaged by weekly i.p. injections of 1×10^7 cells). On days 1, 4 and 7 the various groups of mice were given I.P. injections of approximately 0.5 ml of MTX alone, 'empty' liposomes alone, physical mixture of MTX and 'empty' liposomes, or MTX liposomes. Each mouse was weighed on the days of injection; the doses of drugs were calculated on a body weight basis and the survival time was noted. Weights of mice were recorded twice weekly for 50 days and once weekly later on. During treatment mice were maintained on alkaline water to avoid possible renal toxicity. Whenever there was doubt as to the cause of death being tumor or not, a gross autopsy was done and tissues (liver, spleen, kidney, lung, femur, heart and thymus) were fixed in 10% buffered formalin phosphate ('neutral') and sent for processing and staining with hematoxylin and eosin for examination by light microscopy.

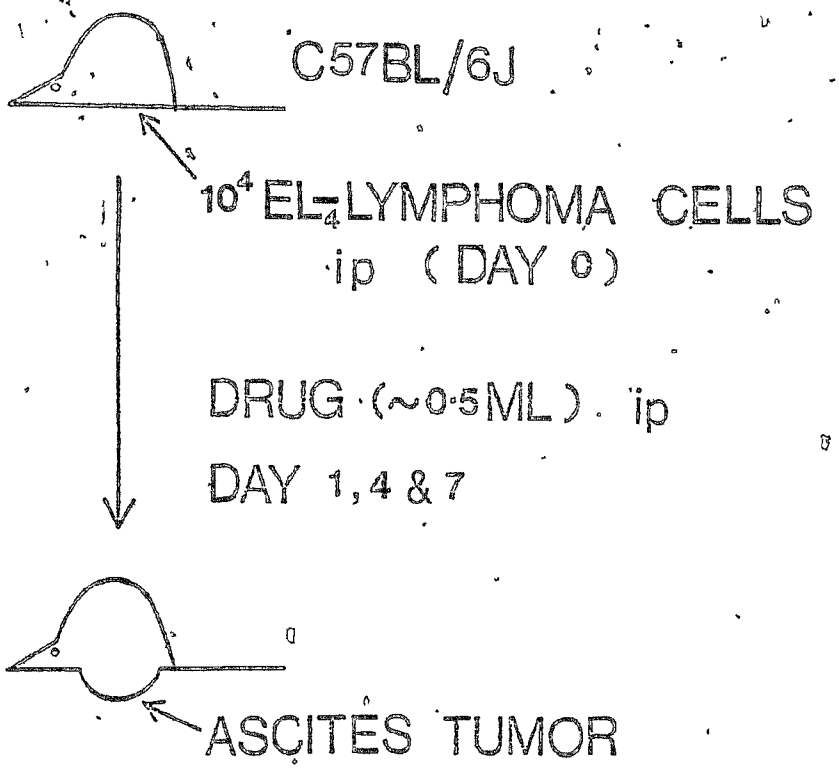


Figure 12. Anticancer evaluation of methotrexate (MTX) containing liposomes in vivo.

SECTION 2: SUV LIPOSOMES

Preparation of positively charged SUV liposomes containing MTX:

Small unilamellar liposomes with a positive surface charge were prepared using the lipid formula: DPPC:CHOL:SA, (0.5:0.4:0.1). First, the MLVs were prepared as follows. DPPC 40 mg, CHOL 16.85 mg and SA 2.94 mg were dissolved in chloroform-methanol (2:1) and solvents were evaporated, in a pear shaped flask, using a rotary vacuum evaporator to obtain a thin film. The film was then dispersed with 10 ml of ^{3}H-MTX solution (10 µCi) by shaking in a water bath at 50-55^{o}C for 30 min. The resulting MLVs were sonicated in 5 ml portions in a test tube (16 x 100 mm). The sonication was carried out using a probe-type sonicator (sonic dismembrator) at a setting of 60 in pulses lasting 20 sec in 40 sec periods for 30 min. The large liposomes and probe fragments were removed by centrifugation at 22,000 x g for 20 min. Only the upper 4 ml of the total 5 ml suspension was collected. The unencapsulated MTX was removed by exhaustive dialysis against 0.5% $NaHCO_3$.

Gel filtration chromatography to separate unencapsulated MTX:

The gel filtration chromatography through Sephadex G-25 prepacked column was tried for the separation of liposomes from unentrapped MTX. Prior to the chromatography, 2.5 ml of empty SUV were passed through the

column to avoid any nonspecific adsorption (Machy and Leserman 1983). A 1 ml sample of the liposome preparation was loaded on the column and eluted with 0.5% $NaHCO_3$. Fractions of 1 ml size were collected. An aliquot of each fraction was counted in Biofluor^R for ^3H-radioactivity. The fractions containing liposomes were pooled.

These liposomes as well as liposomes purified by dialysis were rechromatographed to determine the extent of contamination by free drug in each sample.

Covalent coupling of IgG to liposomes:

Liposomes:

For coupling antibodies, SUV liposomes were prepared with PDP-SA using the formula: DPPC: CHOL: SA: PDP-SA: 0.5: 0.3: 0.1: 0.1.

Coupling reaction:

PDP-IgG was prepared as described earlier. PDP-IgG was linked to SUV liposomes by the method of Barbet et al. (1981) essentially similar to the method described for REV liposomes. However, initially different quantities of antibody and lipid were tried for the reaction. The thiolated IgG and SUV were stirred together overnight at room temperature and pH 8.

Separation of antibody coated SUV from unbound antibody:

Attempts using Sephacryl S-400 and Sepharose 4B gels:

Sephacryl S-400 superfine (1.3 x 17.5 cm, Bed volume = 14.2 ml) and Sepharose 4B (1.3 x 17 cm, Bed volume = 13.9

ml) columns were packed. ^{3}H-MTX SUV, IgG and reaction mixture of SUV and thiolated IgG were gel filtered through these columns to see whether antibody coated liposomes could be separated from unbound antibody. Prior to the chromatography, 2 ml of empty SUV and 2 ml of fetal calf serum were passed through the column to avoid nonspecific adsorption during chromatography. In each case volume of the loaded sample was 0.5 ml. IgG fractions were followed by OD_{280}. Liposome fractions were monitored by determining the ^{3}H-radioactivity in the sample aliquots.

Ficoll-floatation centrifugation:

This technique as described by Shen et al. (1982) was used to separate the antibody coated liposomes from unbound antibody. Two ml of the liposome antibody reaction mixture was mixed with 2 ml of Ficoll solution (25% in 0.5% $NaHCO_3$) in a transparent 13.5 ml cellulose nitrate tube (16 x 76 mm). Four ml of 10% Ficoll in 0.5% $NaHCO_3$ was then overlaid, followed by 1 ml of 0.5% $NaHCO_3$. The tube was centrifuged at 3000 rpm for 30 min in an IEC low speed centrifuge. The liposomes floated to the interphase between 0.5% $NaHCO_3$ and 10% Ficoll or between 10% Ficoll and 12.5% Ficoll depending on the amount of protein associated. The liposome band was collected by puncturing the tube. Protein concentration in liposomes was estimated by BioRad method which is based on dye binding (Bradford 1976). Lowry et

al. (1951) method could not be used because of the interference by Ficoll.

Liposome binding studies with human melanoma cells:

Preparation of antibody linked liposomes:

^{3}H-MTX SUV were prepared by sonication as described earlier using DPPC 80 mg, CHOL 25.3 mg, SA 5.87 mg and PDP-SA 10.15 mg. Twenty ml of ^{3}H-MTX solution (200 µCi) was used for encapsulation. The unentrapped MTX was removed by exhaustive dialysis.

The ^{3}H-MTX SUV obtained as above were reacted with thiolated antibody (~10 mg antibody for 2 ml SUV), in a volume ratio of 2:1, overnight. The antibody coated liposomes were separated from unbound antibody by Ficoll floatation centrifugation. The protein concentration and ^{3}H-radioactivity were determined. The liposome preparation used in the binding study comprised 0.45 µCi/ml of ^{3}H-radioactivity and 1.75-3.7 mg/ml of protein. The concentration of MTX in these liposomes was negligible.

Binding studies:

These studies were carried out similar to those described for REV liposomes. Melanoma cells were incubated with 50 µl of antibody coated or plain ^{3}H-MTX SUV at 37^{o}C for 2 hr. At the end of the incubation period, cells were separated by centrifugation using 10% Ficoll (with 5 mM EDTA) in 0.01 M PBS. The washed cell pellet was solubilised in 1 ml of 0.5% buffered SDS solution. To determine the

cell associated radioactivity an aliquot (0.5 ml) was added to 10 ml of Biofluor^{R} and counted in a liquid scintillation counter. The protein concentration was determined by the method of Lowry *et al.* (1951) using 100 µl aliquots of lysate.

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

STUDIES WITH TRIAMCINOLONE ACETONIDE

Attempts to improve liposomal encapsulation of TRMA

The problem that most of the TRMA was not getting encapsulated was realized when a systematic study of TRMA liposomal preparations was undertaken. Results are shown in Table 5. The lack of liposomal encapsulation of TRMA can be assessed by determining the nature and the amount of crystals in the liposomal preparation. The presence of radioactivity in the non-liposomal fraction and the presence of a considerable amount of crystals in a liposomal preparation was an indication of poor encapsulation of TRMA since most of the crystals are too large to be within the lipid vesicles.

The percent radioactivity found in the supernatant after filtration (through polycarbonate filter 8 m or 12 m) and centrifugation (at 22000 x g) also indicates the amount of TRMA which is not associated with liposomes. The higher the percentage of radioactivity remaining on the filter and present in the supernatant the lower the amount of TRMA encapsulated in the liposomes. The filtration should remove all crystals, the centrifugation separates the liposomal fraction from any drug in solution.

Table 5
Results of the study of different methods and/or formulations of liposomal preparation with TRMA

Method and/or Formulation[a]	Observation	Comments
1. DPPC: CHOL: SA 1 1 0.2	liposomes, no crystals	Hence the crystals seen in TRMA liposomes are not due to these ingredients
2. DPPC: CHOL: TRMA 1 0.7 0.3	liposomes and considerable amount of crystals	All the TRMA is not going into the liposomes.
3. DPPC: CHOL: TRMA: SA 0.7 0.5 0.5 0.4	liposomes and considerable amount of crystals	All the TRMA is not going into the liposomes.
4. DPPC: CHOL: TRMA: SA 1 0.5 0.5 0.2	liposomes and considerable amount of crystals	All the TRMA is not going into the liposomes.
After filtration of liposomal preparation through 12 µm polycarbonate filter	liposomes and very few crystals, loss of radioactivity of about 74% (of radioactivity before filtration)	Filtration through 12 µm polycarbonate filter removes majority of the crystals. Loss of radioactivity could be due to the retention of TRMA crystals by the filter and some liposomes adsorbed to the filter
After filtration through sintered glass filter. (medium pore size)	liposomes, hardly any crystals loss of radioactivity 66%	Filtration through sintered glass filter (M) effectively removes all the crystals, but there is also a possibility of more loss of liposomes

a: Unless otherwise mentioned the method of preparation of liposomes is Mechanical shaking with 8 mM aqueous $CaCl_2$ solution after rotary vacuum evaporation.

Strength is approximately 0.1% w/v TRMA.

Table 5 (continued)

Method and/or Formulation[a]	Observation	Comments
5. DPPC: CHOL: TRMA: SA 1 0.5 0.5 0.2	liposomes and considerable amount of crystals	
Swelling liquid: 8 mM $CaCl_2$ solution containing 5-10% v/v alcohol		
After storing the preparation at room temperature for about 24 hours	crystals seem to attain bigger size	
and then filtered through 12 μm polycarbonate filter	no crystals in filtrate, but 40-70% loss of radioactivity	Use of 8 mM $CaCl_2$ containing 5-10% v/v alcohol helps to remove all the crystals when filtered through 12 μm polycarbonate filter because of bigger size of crystals
centrifugation of filtrate at 16000 rpm (22,000 x g)	60-90% of radioactivity (of filtrate) is in the supernatant	Possibly due to submicroscopic liposomes and/or free dissolved TRMA
6. DPPC: CHOL: TRMA: SA 4 2 0.5 0.6	liposomes and considerable amount of crystals	Increasing the lipid content as much as 4 times also did not prevent the presence of crystals
7. DPPC: CHOL: TRMA 5.9 5.9 0.5	bigger liposomes and considerable amount of crystals	Reverse phase evaporation which gives large liposomes, which are used for encapsulating particulate matter like chromosomes, and viruses, did not eliminate crystals

First it was thought that the poor encapsulation observed was due to improper film thickness, shaking, temperature of preparation or other technical variations. Having varied and controlled each factor it was found that these factors, although important in the preparation of the liposomes, were not the causes for poor encapsulation of TRMA. Optimizing all factors related to the method of preparation or to lipid composition it was found that only a small amount of TRMA could be encapsulated (Table 5).

Injecting an ethanolic solution of lipids into a large quantity of water is a reported method of preparing liposomes (Kremer et al. 1977). Final concentration of alcohol in such a liposomal preparation was 5-10% v/v. This concentration of alcohol was tolerated or was removed by dialysis. An idea was conceived that this concentration of alcohol, if present in our TRMA liposomal preparation, may reduce crystal formation and hence the filtration step could be avoided. Hence, a 8 mM $CaCl_2$ solution containing 5-10% v/v alcohol was used as swelling liquid. Contrary to our assumption, crystals were still observed and they were much bigger compared to those obtained with the aqueous $CaCl_2$ method (Table 5). This indicated that a bigger pore size polycarbonate filter could be used to remove all crystals

and thus provide a more complete separation of crystals and lipid vesicles. The loss of liposomes due to filtration which may occur with polycarbonate filters of lower pore size, is also reduced.

The next approach was to alter the lipid to drug ratio. It was anticipated that by increasing the phospholipid quantity a higher encapsulation of TRMA would be possible. Even a four-fold increase in phospholipid quantity failed to prevent the presence of crystals in the liposomal preparation (Table 5).

It has been reported that even particulate material like chromosomes, viruses, etc. can be encapsulated in liposomes. For encapsulation of that type, a technique called "reverse phase evaporation" has been successfully used (Szoka and Papahadjopoulos 1978). The salient feature of this technique is that it produces large uni- to oligo-lamellar liposomes with high capture volume. They are big enough to encapsulate particulate matter like the ones mentioned above. It was thought that it would be possible to improve the encapsulation of TRMA if the very fine microcrystals of TRMA could be encapsulated in these big liposomes. The reverse phase evaporation technique was, therefore, tried. Unfortunately, this technique did not prove any better than those discussed earlier.

Based on the results of all these experiments listed in Table 5, it could be concluded that the TRMA, as such, can be encapsulated into liposomes only to a very limited degree.

Gel filtration chromatography and dialysis of the supernatant

Filtration of the TRMA liposomal preparation through 8 or 12 μm polycarbonate filter removes the crystals. Subsequent centrifugation at 22000 x g pellets the liposomes. Up to about 85% of the radioactivity of the filtered liposomal preparation was found in the supernatant. This could be due to TRMA in solution or to submicroscopic liposomes containing TRMA, which are not sedimentable at 22000 x g. Ultracentrifugation for one hr at 100,000 x g also gave similar results. Gel filtration chromatography and dialysis were done to determine which possibility was correct.

Gel filtration chromatography using Sephadex G-50 has been used to separate liposomes from the free unencapsulated drug (Arakawa et al. 1975). Dialysis techniques have also been used for the same purpose (Parker et al. 1981). In the former technique, liposome associated drug will emerge in void volume while free drug remains in the retarded volume (Fig. 13).

After overnight dialysis of the supernatant the initial radioactivity was greatly reduced. The dialysate

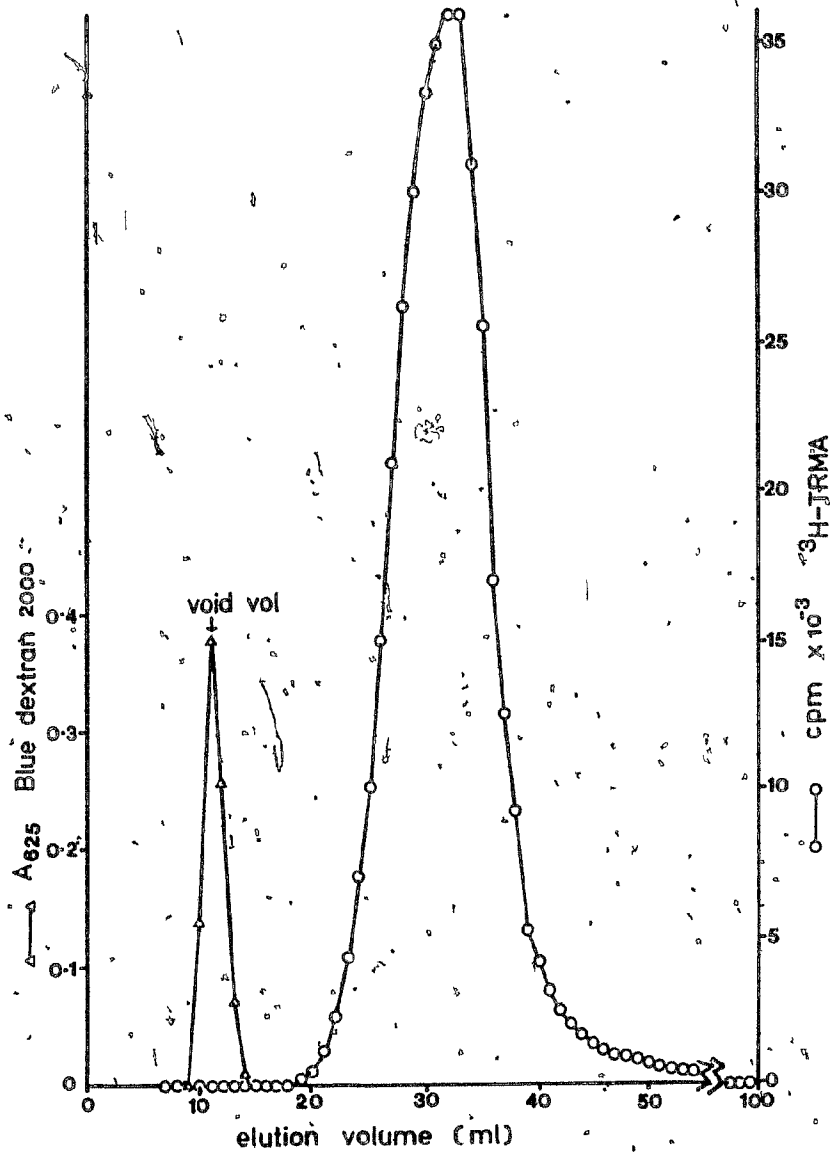


Figure 13. Sephadex G-50 elution profile of supernatant obtained by centrifugation of filtered ^{3}H-triamcinolone acetonide (TRMA) liposomal preparation.

and dialysing medium had approximately the same radioactivity per unit volume. Had there been liposomes containing TRMA in the supernatant, this equality would not be expected to be reached so easily.

The results of both these studies (i.e. gel filtration and dialysis) indicate the absence of liposome encapsulated TRMA in the supernatant. Hence the radioactivity in the supernatant can only be accounted for by the free solubilized drug.

Attempts to concentrate the liposomal preparation

The liposomal preparation referred to here, is the one obtained after filtration through 8 or 12 µm polycarbonate filter. As there was significant loss of drug in the supernatant by centrifugation, other methods, namely rotary vacuum evaporation and filtration through 0.45 µm filter were tried in order to concentrate the liposomal preparation.

Filtration

Filtration of the liposomal preparation through a filter of 0.45 µm pore size would not allow liposomes larger than 1 µm to pass through. Only the solvent and mainly the solubilized drug would pass through.

The radioactivity of the liposomal preparation collected from above the filter was almost the same before and after concentration to half the original volume. This

implies that this preparation cannot be concentrated any further by this method and possibly by ultrafiltration also.

Vacuum evaporation:

The liposomal preparation could be reduced to one-third the original volume by evaporation. Radioactivity per unit volume was three times higher than the initial value. However, the preparation which had no crystals before now contained crystals. It is probable that some of the TRMA which was in solution crystallized out because of the removal of the solvent.

Hence, these methods and also centrifugation rule out the possibility of increasing the concentration of the liposomal preparation any further.

Synthesis of TRMA-21-palmitate

One of the reasons to prepare and utilize TRMA-21-palmitate was to test the hypothesis that encapsulation efficiency can be improved by chemical modification of the drug and that the reason for the low degree of encapsulation of TRMA is due to its structure rather than inappropriate preparation techniques.

The idea for the chemical modification of TRMA to TRMA-21-palmitate was derived from a publication by Shaw et al. (1976). They had failed in their attempt to prepare a stable liposomal preparation containing the steroid, cortisol (Hydrocortisone). Chemical modification of cortisol to 21-octanoate or to 21-palmitate markedly

increased the liposomal retention of this steroid; 21-palmitate had the maximum retention. A hydrophobic "anchor" like palmitate or stearate on cortisol interacts with the lipid bilayer of the liposome and keeps the "head group" of cortisol attached to liposomes. Lack of a sufficiently long, linear hydrophobic "anchor" leads to lack of retention of that compound by the liposomes. Interaction of palmitate of cortisol-21-palmitate with the lipid bilayer was later confirmed by Fildes and Oliver (1978).

Synthesis of drug-phosphatidate compound or drug-lecithin compound (Page Thomas and Phillips 1979) is another approach used for incorporation of drug into liposomes.

It is possible that TRMA behaves similarly to cortisol, both being steroids, with respect to liposomal retention. Conversion of TRMA to TRMA-21-palmitate may induce a similar behavioral change in TRMA. Hence, it was decided to synthesize TRMA-21-palmitate, as this compound was not available commercially.

The possibility of loss of pharmacological activity on chemical modification has not been overlooked here. Cortisol palmitate had improved antiinflammatory activity compared to the absence of such an activity with cortisol acetate. Moreover, cortisol-21-palmitate was resistant to C-21 ester hydrolysis unlike smaller esters like cortisol octanoate (Shaw et al. 1978). Similar properties may be

expected of TRMA-21-palmitate. Furthermore, certain C-21 esters of TRMA have shown superior antiinflammatory effect for topical use compared to TRMA (Diamanti and Bianchi 1971).

The method of Shaw, Knight and Dingle (1976) was used in the first attempt to synthesize TRMA-21-palmitate. It was soon determined, by TLC techniques, that the synthesis was possible. The crude product (product I) obtained by this method was purified by column chromatography using chloroform:methanol (99:1) as eluant. The product (product II) obtained was expected to be TRMA-21-palmitate. Surprisingly, it had a wide melting point range, from 60 to 86°C. Palmitic acid has a melting point of 60-63°C. This led to the suspicion that the supposedly pure product was not pure but might have been contaminated with palmitic acid. The necessity arose to find out whether product II contained palmitic acid or not.

Using silica gel TLC sheet containing a fluorescent indicator, TRMA and TRMA-21-palmitate could be detected under ultraviolet light. This method could not be used for palmitic acid. After trying different reagents, chromic acid-sulfuric acid was chosen as the detecting reagent for all three. These compounds appear as dark spots on spraying the silica gel TLC plate with chromic acid-sulfuric acid and heating at 150°C for half an hour in an oven. Using the chromic and sulfuric acid mixture as the detecting reagent

and the suitable solvent system, it was possible to identify palmitic acid in product II.

Then another TLC procedure followed by column chromatographic procedure was developed for the separation of TRMA-21-palmitate from TRMA and palmitic acid. All three components had different R_f values of not more than 0.47 in the solvent system toluene:ethyl acetate:glacial acetic acid (90:10:1) (Table 6). Hence, this system was chosen for column chromatography.

Percent yield of TRMA-21-palmitate

The method developed by Shaw, Knight and Dingle gave a poor conversion as shown by specific activity. Percent recovery was very low, only 10.6% (Table 7). This could be loss due to manipulation.

Other reaction conditions were tried to improve the conversion and/or recovered yield. It was found that the Diamanti and Bianchi's method using N,N-dimethylformamide gave a much better yield (see Table 7) compared to other methods. This method was used for further synthesis of TRMA-21-palmitate, both labelled and unlabelled.

Preparation and evaluation of TRMA-21-palmitate liposomes:

Results of these studies are summarized in Table 8. These results indicate considerable improvement in liposomal encapsulation of triamcinolone acetonide as a result of palmitoylation. In the case of the triamcinolone acetonide liposomal preparation the first major loss occured after filtration through a 12 µm polycarbonate filter. This

Table 6
Development of TLC procedure to separate TRMA, TRMA-21-palmitate and palmitic acid

Solvent system	R_f value		
	TRMA	TRMA-21-palmitate	Palmitic acid
1. Chloroform: Methanol 95 5	0.55	0.87	Not possible to separate from TRMA-21-palmitate
2. Chloroform: Methanol 99 1	0.05	0.54	Not possible to separate from TRMA-21-palmitate
3. Chloroform	0.00	0.19	Not possible to separate from TRMA-21-palmitate
4. Benzene: Dioxane: Glacial Acetic Acid 90 25 1	N.D.	0.71	0.83
5. Toluene: Ethylacetate: Glacial Acetic Acid 90 10 1	0.009	0.21	0.47

N.D.: not done.

Table 7
Synthesis of TRMA-21-palmitate

Method	Percent Recovery[a]
1. solvent: pyridine: base: Pyridine	10.6[b]
2. solvent: chloroform: base: triethylamine	15.6
3. solvent: dimethylformamide: base: pyridine	70.0

a: it is possible that these values do not represent the percent conversion. Percent conversion may be same or higher as found with method 1.

b: Percent conversion calculated on basis of radioactivity was 37.7.

Table 8
Comparative data for liposomal encapsulation of triamcinolone acetonide and triamcinolone acetonide-21-palmitate

Procedure	Fraction	Concentration, mg/22 ml	
		Triamcinolone acetonide	Triamcinolone acetonide-21-palmitate
Preparation	Crude product	20	20
Filtration	Filtrate	7	19
Centrifugation of filtrate	Supernatant	6	2
	Pellet (liposomes)	1	17

filtration step is necessary with the liposomal encapsulation of a lipophilic drug. Since the drug is insoluble or very slightly soluble in the aqueous medium, only that portion is encapsulated that is in solution, or in a molecular state, intimately associated with the lipid bilayers; the remaining portion is in solid form (crystals) which, although it is probably unencapsulated, would be present in the liposomal fraction after centrifugation. The loss of 13 mg (i.e. 65%) triamcinolone acetonide by the filtration process was due mainly to unencapsulated crystals observed in the "crude" preparation. Such crystals were absent in the filtrate. No crystals were seen even in the unfiltered triamcinolone acetonide-21-palmitate liposomal fraction; consequently only 1 mg (i.e. 5%) was lost by filtration. This suggests that the palmitate form has a stronger association with the lipid layers and almost complete encapsulation is achieved.

The second major loss in the case of triamcinolone acetonide liposomes was in the supernatant after centrifugation of the filtrate. This is due mainly to the drug in solution but not associated with liposomes. Evidence for this was demonstrated by gel filtration chromatography (Fig 13). Thus the overall encapsulation of triamcinolone acetonide, determined in the final purified liposomal fraction, was only 5% while that of its palmitate derivative could be close to almost 100%, if one considers

the unavoidable loss due to the filtration and centrifugation procedures (i.e. adsorption to the filter and glassware). Another minor but inherent loss of liposomes could be due to the presence of small (<0.5 µm) liposomes which are not completely sedimented by the centrifugation force. However, for comparison purposes, both preparations were analysed by the same procedures, and consequently the same considerations should be applied.

A possible reason for increased encapsulation of the palmitate derivative could be the change in the partition coefficient from a nonfavourable one to a favourable one. For lipid soluble compounds a logarithmic partition coefficient (log P) between 1.7 and 4 is unfavourable for liposomal encapsulation (Defrise-Quertain et al. 1980). Triamcinolone acetonide has a log P of 2.53 (Hansch and Leo 1979). Palmitoylation would increase this to approximately 11 as calculated using subsituent constants (Hansch and Leo 1979).

The mechanism for increased encapsulation could be predicted to be analogous to that of cortisol-21-palmitate (Fildes and Oliver 1978). The palmitoyl chain may act as a "hydrophobic anchor" holding the steroid head group on the surface of the lipid bilayer.

One of the drawbacks in liposomal drug delivery systems is the poor encapsulation of a drug in the liposomes. That chemical modification is likely to be a

powerful approach to overcome such an obstacle is evident by this (Goundalkar and Mezei 1984) and another (Shaw et al. 1976) investigation.

STUDIES WITH METHOTREXATE

SECTION 1: REV LIPOSOMES

Formulation and preparation of MTX liposomes

After reviewing different publications related to MTX liposomes a suitable formula was designed for the preparation of MTX liposomes. DPPC, one of the most stable and uniform phospholipids, was chosen in place of the most frequently used egg or vegetable phosphatidyl choline (EPC or VPC). It is imperative that liposomes leak as little as possible when encapsulating water soluble types of drugs like MTX. Since cholesterol confers this property to liposomes, it was included in the formula. Charged lipids were used in the concentration of 10 mole% to impart desired surface charge to liposomes. Other components were included in the formulation with some specific purpose in mind as mentioned in appropriate places.

There are many different methods of preparing liposomes. A comparison of these methods in the literature reveals that reverse phase evaporation (REV) method is one of the best methods for encapsulation of water soluble types of drugs. REV method was therefore used to encapsulate MTX into the liposomes.

MTX is practically insoluble in water, alcohol, chloroform and ether. However, it is freely soluble in dilute solutions of alkali hydroxides and carbonates.

Sodium bicarbonate solution 0.5% (pH=8) was hence used to prepare MTX solution needed for liposomal encapsulation.

Use of DPPC in place of egg or vegetable phosphatidyl choline introduces certain changes in the regular REV method. Mixture of chloroform and ether is necessary to dissolve DPPC completely while ether alone is sufficient in case of EPC or VPC. Shaking the aqueous dispersion at 55°C is needed for DPPC liposomes because of the higher transition temperature of the lipid. A definite ratio of lipids, organic phase and aqueous phase, as used in the procedure is needed to ensure maximum capture volume (Szoka et al. 1978), consequently maximum encapsulation of MTX.

Separation of free MTX from liposomal MTX:

Gel filtration chromatography (using Sephadex G-50) is invariably used for quantitative separation of unencapsulated drug from liposome encapsulated drug. Centrifugation methods for this purpose may not be as quantitative as gel filtration chromatography. However, because of dilution and the time consuming nature of the latter, centrifugation was used for routine separation of free MTX.

Determination of MTX in liposomes.

MTX has an absorption maximum at 370 nm and at 303 nm. Determination of MTX in liposomes was therefore attempted by direct reading of suitable dilution of MTX liposomes at 370 nm. These direct readings gave absorption values higher

than those expected. Once we realised that these high absorption readings could be due to turbidity of liposomal samples (which we did not expect in such highly diluted samples), attempts were directed towards overcoming this problem.

Triton-X-100 has been used to cause the release of encapsulated drug from within the liposomes. With the hope of lysing the liposomes and thereby overcoming the turbidity, the liposome samples therefore were subjected to Triton-X-100 treatment. As shown by absorption readings (Table 0), the detergent treatment did not eliminate the turbidity of the liposomal preparation suggesting that Triton-X-100 may not dissolve liposomes.

To match the turbidity in MTX liposomes 'empty' liposomes of similar dilution was then tried as a blank for estimating MTX in liposomes. Though not theoretically ideal, this manipulation, provided a method to quantitatively estimate MTX in liposomes.

A more reliable procedure to determine MTX in liposomes has been recently described by Todd et al (1982). In this procedure methanol was used to dissolve MTX encapsulated in liposomes. In methanol, MTX has an absorption maximum at 303 nm where lipids have minimal extinction (in the order of 0.05 or less). This method was adopted for many of the further assays of MTX in liposomes.

Table 9
Spectrophotometric determination of methotrexate in liposomes (wave length: 370 nm)

Manipulation	Absorption readings*	Comments
1. Triton X-100 treatment		
before triton X-100	0.46	
after triton X-100	0.43	Triton X-100 ineffective in overcoming turbidity
2. Alteration of blank:		
with 0.5% $NaHCO_3$ soln as blank	0.46	
with "empty" liposomes as blank	0.21	"Empty" liposomes may possibly be used as blank

*100 µl of original liposomes diluted to 50 ml with 0.5% sodium bicarbonate solution.

A calibration line shown in Fig. 14 and Table 10 was constructed for this purpose.

Radioactive assay method

Availability of ^{3}H-MTX provided a sensitive, simple and reliable method for quantitative assays, but, because of the inconvenience of working with radioactive samples (for instance in case of coulter counting of liposome samples) and also increased cost, this method was avoided wherever possible.

MTX encapsulation efficiency

Results in Table 11 indicate that MTX encapsulation is better in positive liposomes. This could possibly be due to larger aqueous space between two like charged lipid lamellae in positive liposomes.

Results of preliminary stability studies of neutral MTX liposomes (Table 12) indicate that they are adequately stable at room temperature for the time period tested. But a slight decrease in stability could be observed at 37^{o}C.

Size distribution of liposomes

A coulter counter equipped with a 400 channel analyser and an aperture tube of 10-15 micron aperture size is recommended for size determination of liposomes (Rahman 1980) perhaps because of wide size range of liposomes. However, in the absence of such an instrument, the counter with 100 aperture tube may at least give some rough estimate of the size distribution of liposomes. One could

Table 10
Standard curve for methotrexate (MTX)
solvent: Methanol
wave length: 303 nm

MTX Conc. µg/ml	OD
3.63	0.155
7.26	0.350
10.88	0.540
14.51	0.730
18.14	0.910
21.77	1.100
25.40	1.300

Linear Regression Analysis:
slope (m) = 0.0522
y Intercept (b) = -0.0314
Correlation Coefficient = 0.9999

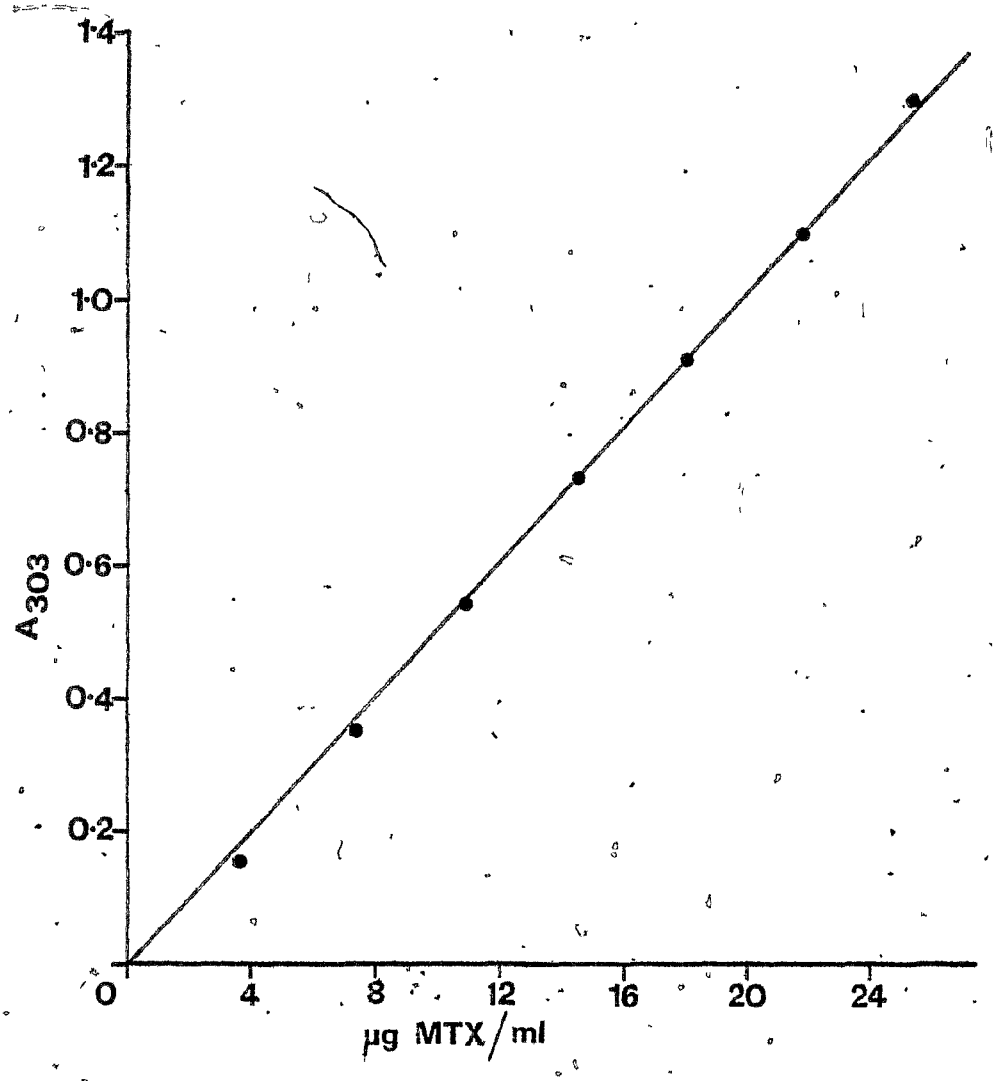


Figure 14. Calibration line for spectrophotometric determination of methotrexate (MTX) dissolved in methanol.

Table II

Some parameters of methotrexate (MTX) encapsulation in liposomes

Type of liposomes	Neutral	Positive	Negative
Percent encapsulation of MTX	9.9-10.8	18-34	N.D.
mg MTX/ml liposomes	0.8686-1.3029	3.0000-4.2000	1.0000-1.5000
mg total lipid/ml liposomes	38-53	48-70	N.D.
Final volume of liposomes (ml)	2.9-4	2-2.9	N.D.

N.D.: not done

Table 12
Results of preliminary stability studies of neutral methotrexate (MTX) containing liposomes

	at room temp.	at 37°C.
Percent MTX leaked out of liposomes after 7 days storage	8 - 18%*	28 - 34%*

*These figures may be on the high side since certain unavoidable loss in supernatant would be there with every washing.

Table 13
Size distribution of methotrexate (MTX) containing liposomes

Size µm	Percent of total Neutral liposomes	Positive liposomes
<2	67	89
2 - 5	29	11
5 - 8	4	-

expect a larger size for positive MTX liposomes because of repulsion between like charged lipid lamellae, as mentioned earlier, but they were found to be somewhat smaller in size (see Table 13) to neutral liposomes. Some of the neutral liposomes may aggregate to form larger clumps which the counter may fail to distinguish from single liposomes. This might be the reason for finding the neutral liposomes larger in size than the positively charged liposomes. On previous occasions, aggregates were indeed observed in the case of neutral liposomes when examined by optical microscopy.

In vitro drug release studies from positive MTX liposomes

Results of these studies are shown in Fig. 16 and Table 15. The concentration of the drug that is released into the dissolution medium (0.5% sodium bicarbonate) was determined by uv absorption spectrophotometry at 303 nm. Figure 15 and Table 14 represents the calibration line used for this purpose. The profiles in Fig. 16 indicate that the release of MTX from the liposomes is a slow process and is temperature dependent. However, the release at 60°C, the temperature greater than the transition temperature of DPPC, is not as great as one would expect. At, or above, the transition temperature liposomes become more "fluid" and sudden and rapid burst of their contents is normally expected. This did not happen with our liposomes. Perhaps, this is because of the presence of large percentage of cholesterol (50%) in these vesicles. The property of cholesterol to insulate against the effect of temperature on the fluidity of the membrane is well known. Fetal calf

Table 14
Calibration curve for methotrexate (MTX)
solvent:0.5% $NaHCO_3$
wave length: 303 nm

MTX conc. µg/ml	OD
0.21	0.011
0.42	0.020
0.63	0.031
0.84	0.045
1.04	0.055
2.09	0.105
4.18	0.210
6.27	0.320
8.35	0.420
10.44	0.525

Linear Regression Analysis:
Slope (m) = 0.0504
y Intercept (b) = 0.0003
Correlation coefficient = 0.9999

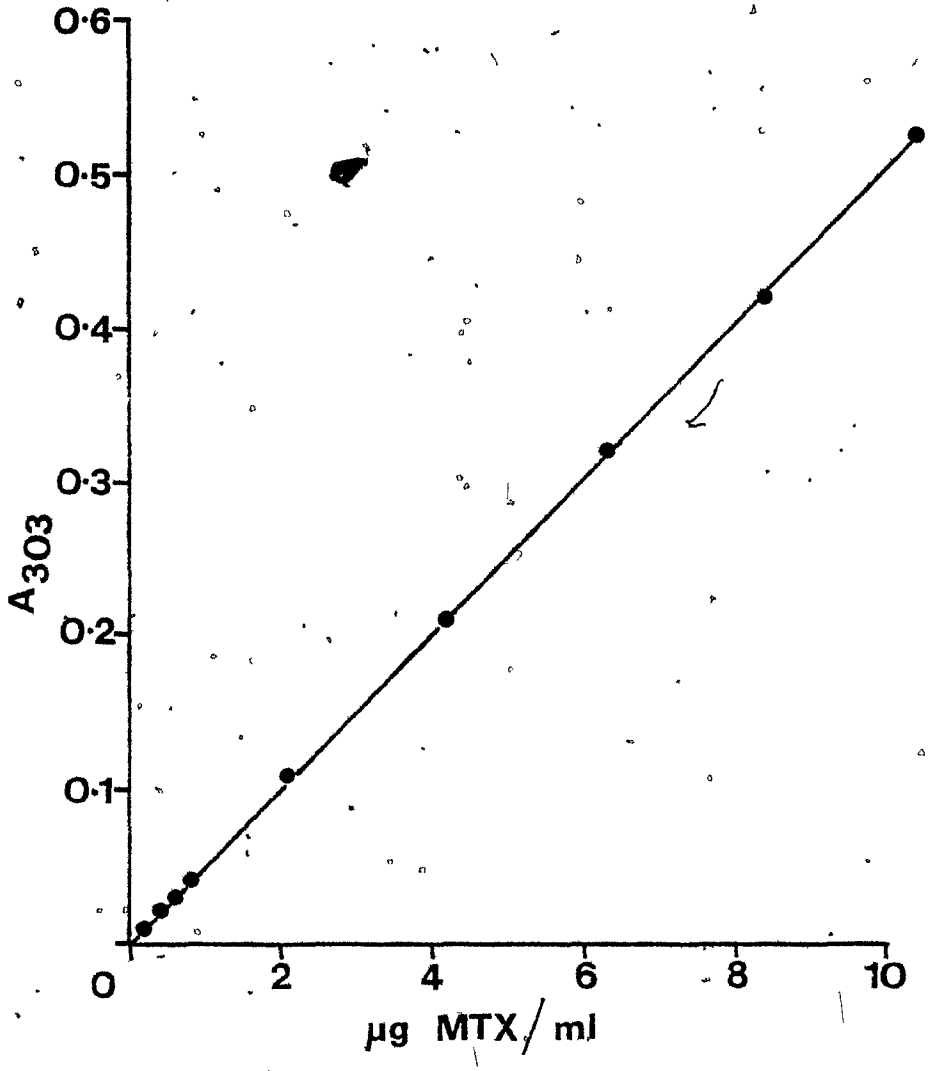


Figure 15. Calibration line for spectrophotometric determination of methotrexate (MTX) dissolved in 0.5% $NaHCO_3$.

Table 15.
Release* of methotrexate (MTX) from liposomes

Time hr	% released from free MTX soln	% MTX released from liposomes at 22°C	at 37°C	at 60°C	with FCS**
0.5	29.07	0.87	1.13	2.19	0.80
1	49.02	2.19	2.85	4.46	1.94
2	71.16	5.23	6.21	10.03	4.63
3	80.47	7.99	9.77	14.74	6.72
4	83.65	10.86	13.42	17.90	8.89
5	85.06	13.42	16.61	20.72	10.97
6	86.26	15.63	19.65	23.66	12.82
7	87.47	17.90	22.41	25.69	14.64
8	87.47	19.77	24.66	27.78	16.47

*Release is determined by dialysis technique.
**Fetal calf serum.

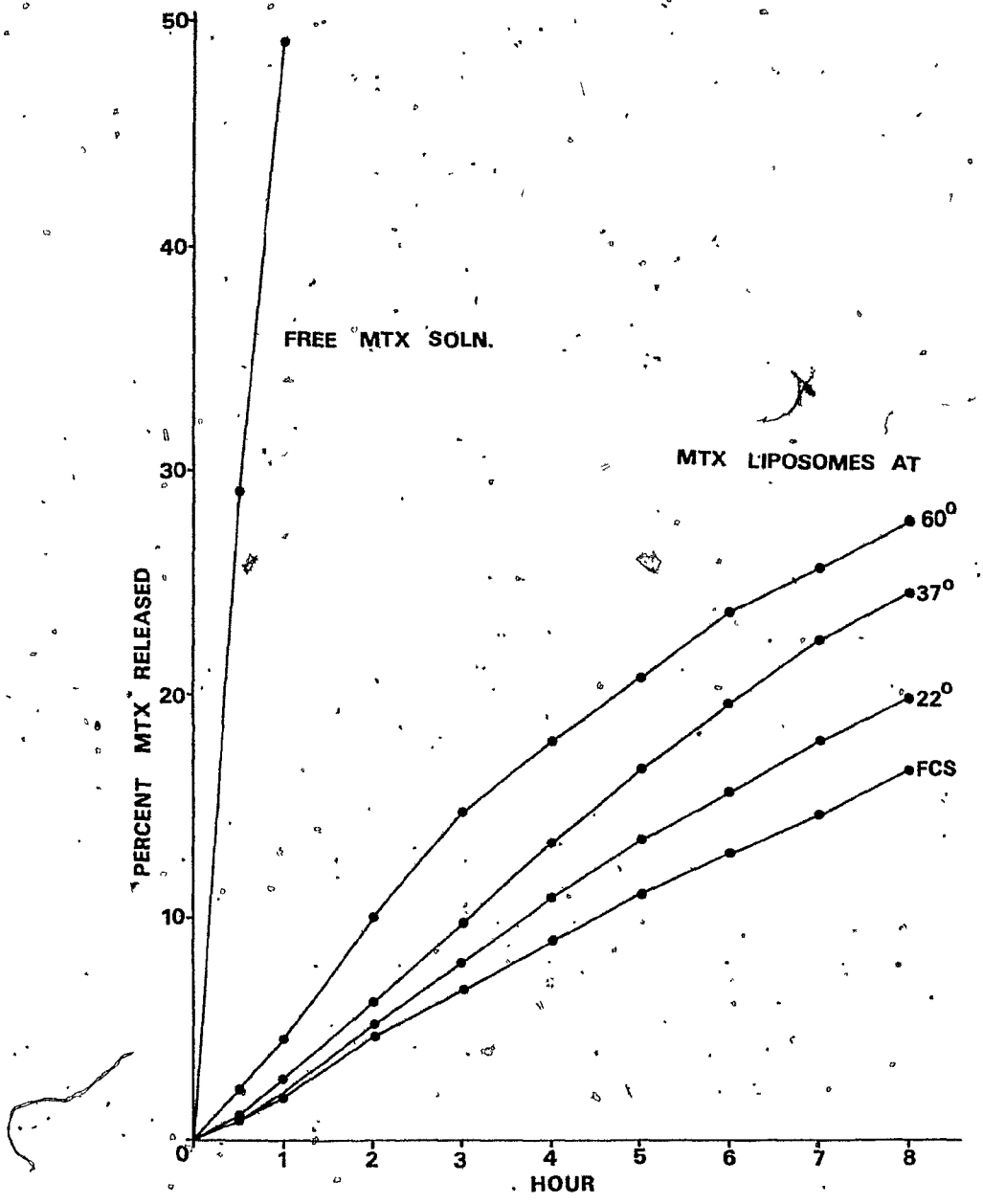


Figure 16. Release of methotrexate (MTX) as determined by dialysis technique.

serum (FCS) does not seem to have much effect on the efflux of MTX from liposomes. This is unusual because many reports have indicated that the serum proteins destabilize liposomes. Again, the seemingly stable nature of these vesicles might be the result of increased cholesterol content. Cholesterol is known to protect liposomes from attack by serum proteins. Thus the results of these studies reconfirm the protective role of cholesterol in liposomes against the effect of temperature and proteins.

Covalent attachment of antibodies to liposomes

Although this aspect is relatively new in the field of liposome research, a number of methods have been reported (see Table 3) for covalent coupling of antibodies or proteins to liposomes. Percent attachment has been very low in most of these methods. The method described by Martin et al. (1981) involves the attachment of Fab' to liposomes via disulfide bonds. Because the percentage of antibody attached and also the amount of antibodies attached per micromole of phospholipid have been comparatively higher by this method we decided to test it for our study. In this method there is a need to synthesize 3-(2-pyridyl dithio) propionyl derivative of a lipid since the latter is required to be present in the liposomes for covalently linking Fab' via disulfide bond.

Synthesis of PDP-DPPE

Egg phosphatidyl ethanolamine, which is frequently used by other investigators, is chemically less stable because of the presence of unsaturated fatty acyl moiety in the molecule. The resulting PDP-PE also becomes prone to oxidation giving rise to stability problems. Hence we planned to synthesize a more stable alternative i.e. PDP-DPPE.

The yield of PDP-DPPE obtained however, was too small to be characterized. It was soon realised that this was due to incomplete dissolution of DPPE in methanol, the solvent for the reactants. An increase in the quantity of methanol from 3 ml to 10 ml for 35 mg of DPPE was still not enough to dissolve it completely. Other solvents like methylene chloride (6 ml), ethanol (5 ml), pyridine (10 ml), dimethyl formamide (2 ml), DMSO (3 ml) and some combinations of these solvents were tried to dissolve the (35 mg) DPPE with little success. Though on heating, all the DPPE went into solution in above instances, it precipitated out on cooling to room temperature. The reaction was not tried at higher temperatures lest it may decompose SPDP.

Synthesis of PDP-PE:

Without much success in improving the yield of PDP-DPPE, we decided then to synthesize PDP-PE (from egg yolk phosphatidyl ethanolamine) though it would be expected to be less stable than PDP-DPPE.

The yield of PDP-PE was found to be 52%. TLC studies with the solvent system: Methanol-acetic acid-water: 60:20:3 revealed that R_f values of egg PE and PDP-PE were quite similar to those of DPPE and PDP-DPPE respectively (Table 16). Also silica gel F 254 (silica gel with a fluorescent indicator) TLC plates could be substituted for silica gel H plates without great difference in R_f values.

Synthesis of PDP-SA:

As a result of a "midnight brain storm" the idea to synthesize PDP-SA occurred suddenly. Stearylamine is used routinely to confer positive charge onto liposomes. It has a primary amino group to react with SPDP and a long hydrophobic stearyl group to efficiently interact with the lipid bilayer of liposomes. Because of these properties and also because PDP-SA would be more stable (by the presence of saturated fatty acyl moiety) we thought, we should try to synthesize PDP-SA and use it in place of PDP-PE for coupling Fab'. Another good point to this idea is that stearylamine is not only more stable but also cheaper compared to egg PE or DPPE.

Development of TLC procedure for separation of SA, PDP-SA, SPDP and another unidentified product of the reaction:

Initially R_f values of these compounds were determined by TLC on silica gel F 254 plates using ethyl acetate as the solvent system (Table 17). Due to the fluorescent indicator on the TLC plates, PDP-SA and SPDP

Table 16

TLC of PDP-DPPE reaction mixture on silica gel H
Solvent system: chloroform-methanol-acetic acid 60:20:3
Detecting reagent: Molybdic acid reagent
and/or iodine vapors

Component of reaction mixture	R_f
DPPE*	0.25
SPDP	0.99
Triethylamine	0.41
PDP-DPPE*	0.66

*R_f values of egg PE and PDP-PE were almost the same as those of DPPE and PDP-DPPE respectively.

Table 17
TLC of PDP-SA reaction mixture on silica gel F 254

Component of reaction mixture	R_f solvent system: Ethyl acetate	Ethyl acetate: petroleum ether 40:60
Stearylamine	0.02	0
SPDP	0.47	0.16
PDP-SA	0.48	0.22
unidentified product	0.64	0.56

could be detected under shortwave uv light. However, iodoplatinate reagent spray gave brighter, distinctly colored spots (SA-pink, PDP-SA-dark colored). Iodine vapors could also be used for visualizing these compounds on TLC. Since R_f values of these components were more than 0.4 (except stearylamine), the polarity of the solvent system was gradually decreased to get a suitable solvent system. Ethyl acetate-petroleum ether in the ratios 90:10, 80:20, 60:40, 40:60 were tried as solvent systems. The R_f value of SPBP decreased gradually. Finally ethyl acetate-petroleum ether 40:60 was chosen as the suitable solvent system for purification of PDP-SA by column chromatography based on R_f values as shown in Table 17.

The I.R. and N.M.R. spectra (appendix 4 & 5) of the solution of the synthesized product was consistent with the chemical structure of PDP-SA.

Preparation of antibody fragments and coupling to liposomes

Preparation of $F(ab')_2$ from IgG is usually accomplished by the digestion of IgG. The proteolytic enzyme, pepsin, was used to cleave the Fc portion of IgG. The elution profile of digested IgG from Sephacryl S200 is shown in Fig. 17. The undigested IgG is then removed by affinity chromatography using protein A sepharose 4B column (see elution profile in Fig. 18). Protein A (tyrosine residues) has an affinity for the Fc portion of IgG and

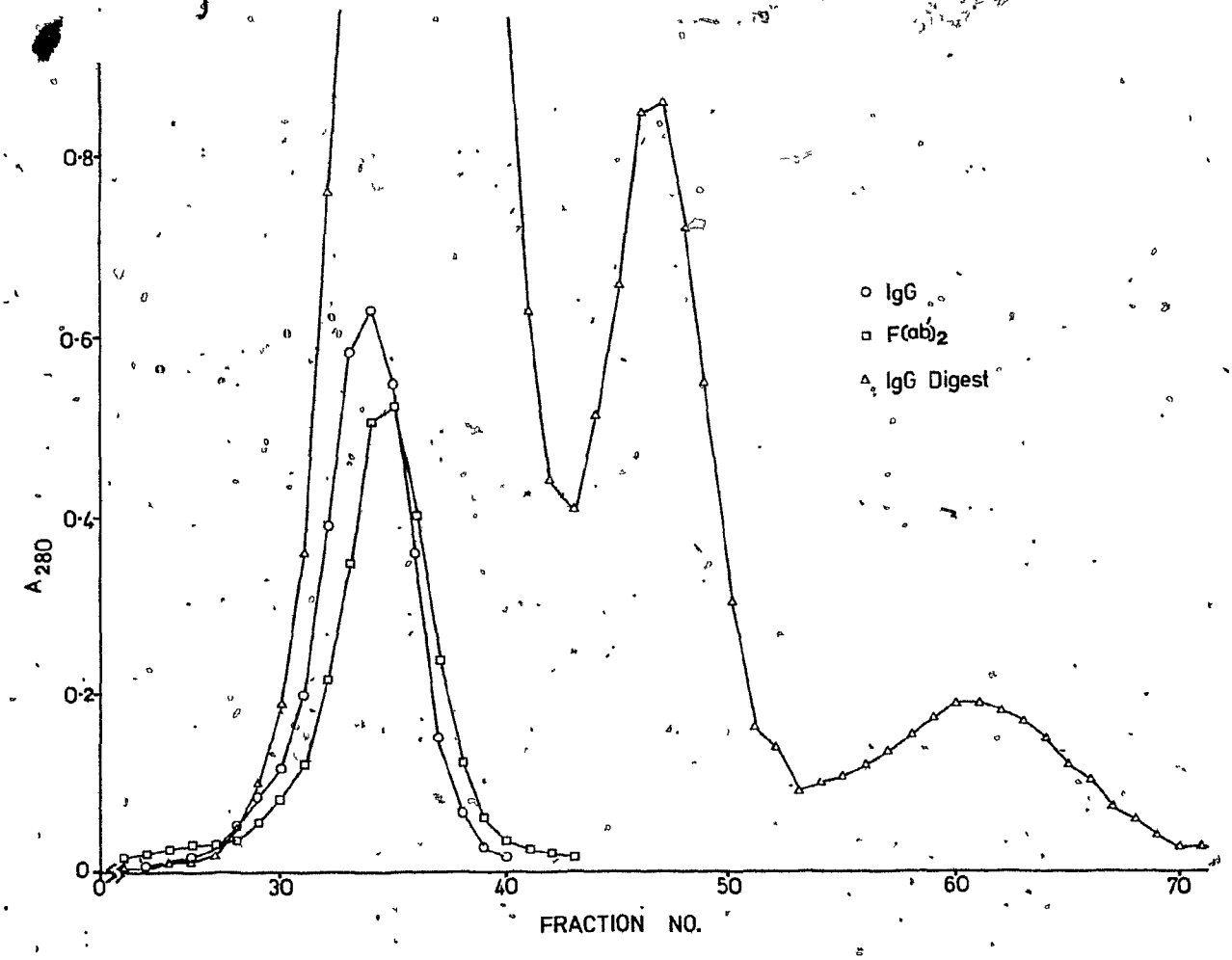


Figure 17. Elution profiles of IgG, $F(ab')_2$ and pepsin digest of IgG from Sephacryl S-200.

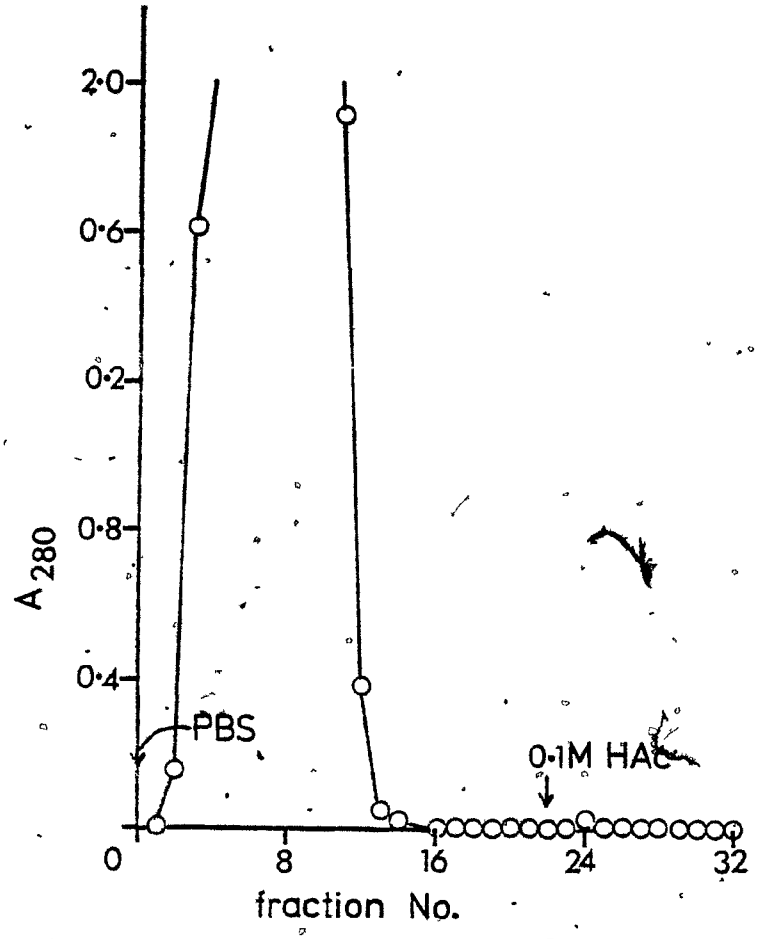


Figure 18. Isolation of $F(ab')_2$ from IgG by affinity chromatography on Protein A-Sepharose CL-4B (PBS: phosphate buffered saline, HAc: acetic acid).

thus retains IgG on the column while $F(ab')_2$ elutes out. Dithiothreitol, at pH 5.5, reduces the disulfide bond of $F(ab')_2$ producing monovalent Fab'-SH. Preparation, separation and coupling of Fab' has to be done in nitrogen atmosphere to prevent oxidation and reassociation to Fab'-SH to form Fab'-S-S-Fab' (i.e. $F(ab')_2$). Hence a closed system consisting of chromatography column connected to reaction vial was used in these experiments.

Fab'-liposome coupling takes place as a result of the disulfide exchange reaction between the thiol group on each Fab' fragment and the pyridyldithio moiety of PDP-SA molecules present in vesicle membranes. Simultaneously the chromophore 2-thiopyridinone is also released as a product of the reaction.

Results of our efforts to attach Fab' are shown in Table 18. Martin et al. (1981) reported about 14-26% coupling of Fab' with up to 600 µg of Fab' per mol phospholipid. We were unable to reproduce these comparatively high values of Fab' coupling to liposomes; our results showed only 3.2% binding. One possible reason could be that our REV liposomes were not extruded through 0.4 and 0.2 µm polycarbonate filters prior to Fab' attachment. This treatment may perhaps increase the number of available pyridyl dithio groups for interactions with Fab'-SH due to smaller and more uniform size of vesicles. Such treatment

Table 18
Covalent coupling of antibodies to liposomes using PDP-SA

	Martin et al. (1981) method	ECDI method (Endoh et al. 1981)	
	Specific binding	Specific binding	Nonspecific binding
Reaction mixture	24 mg Fab'-SH + 1 ml liposomes	420 mg IgG + 1 ml liposomes + ECDI	420 mg IgG + 1 ml liposomes
Amount bound to liposomes	770 µg Fab'/ml liposomes 29 µg Fab'/µmole DPPC	2530 µg IgG ml liposomes 97 µg IgG/µmole DPPC	300 µg IgG/ml liposomes 12 µg IgG/µmole DPPC
Percent bound	3.20	0.60	0.07

of REV MTX liposomes may possibly rupture some vesicles and release the entrapped methotrexate in which case the very purpose of choosing REV method would be dispensed with.

Other possible reason for lower attachment of Fab' could be the use of PDP-SA in place of PDP-PE. Although one could expect PDP-SA to interact similarly to PDP-PE with the lipid bilayer, the possibility of a lower fraction of total pyridyldithio moieties on the surface of liposomes in the case of the former (i.e. PDP-SA) cannot be ruled out. Fig. 19 gives an idea regarding only the total pyridyldithio moieties present in liposomes with PDP-SA. To characterize the surface pyridyldithio moieties requires the synthesis of a non-permeable reducing agent like dihydrolipoamide-dextran.

IgG coupling to liposomes by ECDI method

The lower percentage attachment and elaborate procedure involved in Fab' coupling by the Martin et al. method prompted us to try direct attachment of IgG by a simpler ECDI method. Though this sort of IgG-liposome coupling was reported as early as 1975 (Dunnick et al. 1975), a more detailed study is reported recently by Endoh et al. (1981). The mechanism of IgG coupling to liposomes via carbodiimide is illustrated in Fig. 8.

Results of this method are compared with those of the previous method in Table 18. Our values obtained here are higher compared to the values (0.13%, 20 µg IgG/µmole PC) of

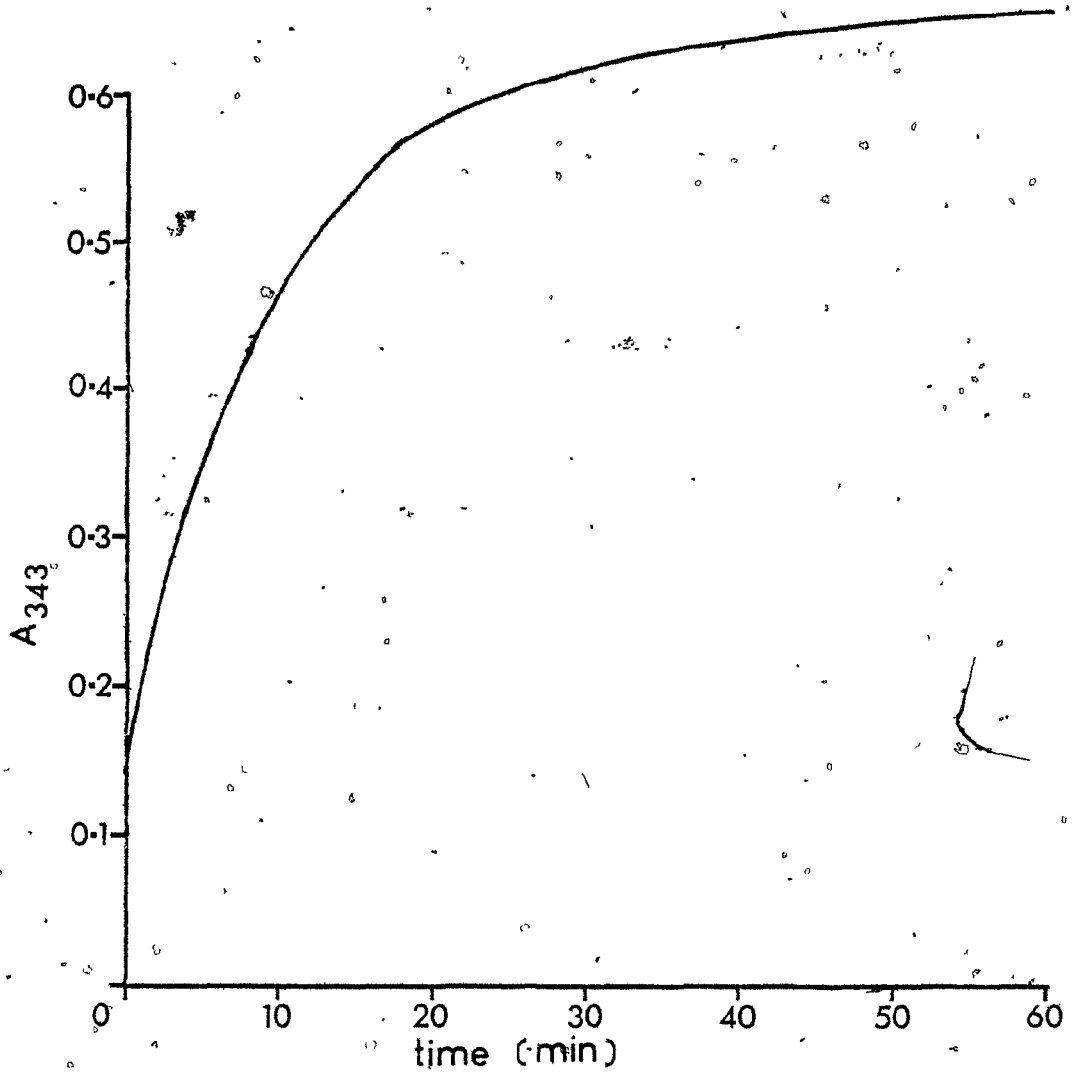


Figure 19. Release of 2-thiopyridinone from liposomes containing N-[3-(2-pyridyldithio)propionyl] stearylamine (PDP-SA) following the addition of dithiothreitol.

Endoh et al. (1981). These differences could be attributed to different reaction conditions like pH, reaction medium, etc. The amount of IgG bound per µmol of phospholipid is higher, 97 µg, compared to Fab' coupling, which was 29 µg. This could simply be due to higher amount of IgG in the reaction mixture, i.e. 420 mg IgG vs 24 mg Fab'-SH. Percentage attachment was still low, only 0.6%, and hence this method was abandoned.

IgG coupling to liposomes by the method described by Barbet et al. (1981)

Both the previous methods did not provide sufficient antibody binding efficiency. A new approach of antibody-liposome linking was therefore investigated. We utilized the method described by Barbet et al. (1981) but substituted PDP-DPPE with PDP-SA. By this method, about 24-32% of the thiolated IgG became bound to liposomes (96-123 µg IgG μmol^{-1} phospholipid) (Table 19). We thus achieved a binding efficiency comparable to that reported by Barbet et al. (1981) and Martin et al. (1981), i.e. 40% and 14-26% respectively. The non-specific adherence of antibodies to liposomes was only 0.9% (3.1 µg IgG μmol^{-1} phospholipid). This confirms that the chemical reaction between thiolated IgG and liposomes (i.e. the thiol-disulfide exchange reaction) was responsible for increased binding of IgG to liposomes.

Table 19
Covalent coupling of antibodies to liposomes using PDP-SA according to the method described by Barbet et al. (1981)

	Specific binding	Nonspecific binding
Reaction mixture	8 - 12.6 mg PDP-IgG + 1 ml liposomes (26 µmol DPPC) + DTT	9 mg PDP-IgG + 1 ml liposomes (26 µmol DPPC) No DTT
Amount bound to liposomes	2.5 - 3.2 mg IgG/ml liposomes 96-123 µg/µmol DPPC	0.08 mg IgG/ml liposomes 3.1 µg/µmol DPPC
Percent bound	24 - 32	0.9

For immunospecific liposomal targeting it is essential that the liposome-bound antibody retains its immunological reactivity. The antigen-binding capacity of anti-BSA IgG before incorporation in liposomes was found to be 0.1641 g BSA/ g anti-BSA IgG as calculated from the calibration curve in Fig. 20 (Table 20). The antigen-binding capacity of the rabbit anti-BSA IgG was reduced by only about 20% after its linkage to liposomes (Goundalkar et al. 1984a).

By virtue of the primary amino group, stearylamine can react readily with the heterobifunctional reagent SPDP to give rise to another reactive compound: PDP-SA. Stearylamine, in contrast to PE, has a well defined and simple molecular structure, i.e. it has a single hydrocarbon chain whereas PE has two fatty acyl chains of varying length and saturation. Because of its single chain, PDP-SA may better fit into the lipid bilayers of the liposomes than the two-"tailed" PDP-PE in which the length of the two chains are often unequal. Further, because its chain is saturated, PDP-SA is likely to be more stable than PDP-PE with its unsaturated fatty acyl chains. An oxidative reaction involving the PDP-PE could affect the integrity of liposomes and the stability of their linkage to antibodies. Other advantages of using PDP-SA for coupling antibodies to liposomes are the low cost of SA, the simple synthesis procedure and the almost complete quantitative conversion of SA to PDP-SA (Goundalkar et al. 1984a). Only about 20%

Table 20
Calibration curve for radioimmunoassay of anti-BSA IgG

Anti-BSA IgG µg	BSA bound to Anti-BSA IgG (a) µg	BSA bound to Normal IgG (b) µg	BSA bound specifically to anti-BSA IgG (a-b) µg
27.64	6.56	4.00	2.56
55.29	14.95	6.00	8.95
82.93	22.30	7.00	15.30
165.86	45.87	10.00	35.87
221.15	61.02	12.00	51.02
331.72	83.40	16.00	71.40
442.30	100.77	20.50	80.27

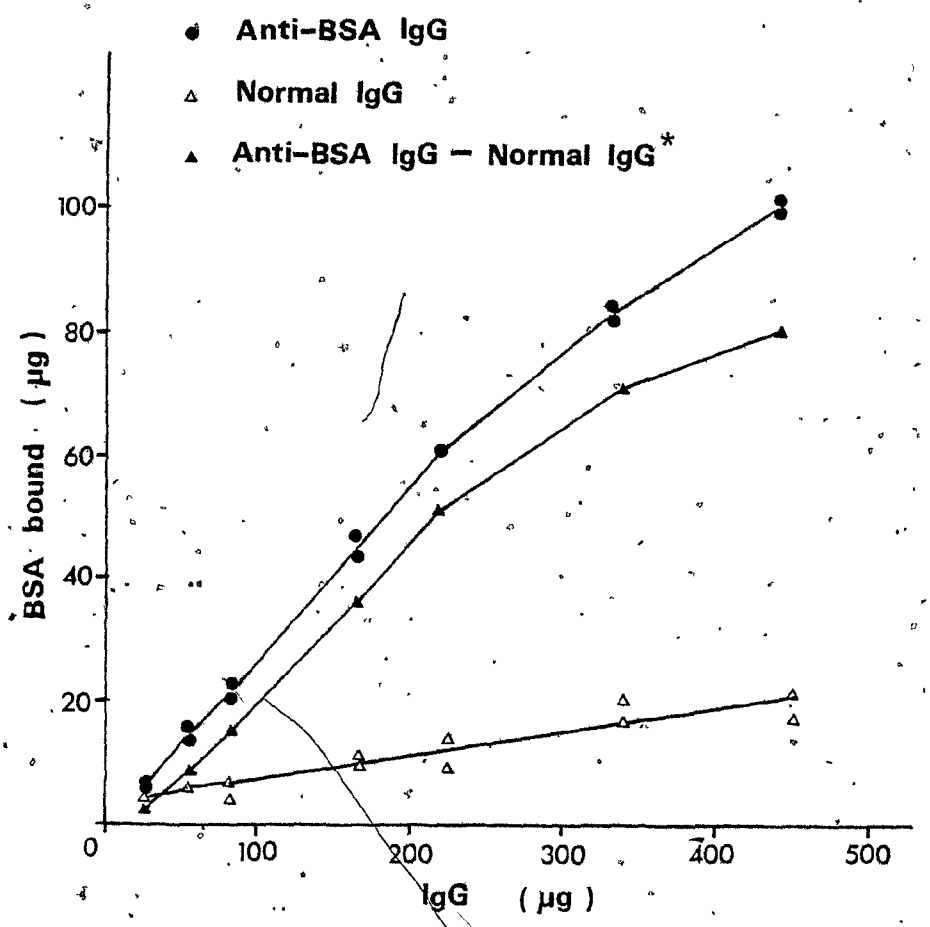


Figure 20. Calibration curve for radioimmunoassay of anti-BSA IgG.

*Indicates the difference between BSA bound to Anti-BSA IgG and Normal IgG

of entrapped MTX leaked during the overnight binding reaction.

Antibody mediated binding of positive MTX liposomes by melanoma cells

Immunofluorescence staining technique:

Antibody titer of anti-melanoma globulin, determined by the membrane immunofluorescence technique, was found to be 50 µg/ml. Thiolated globulin also had a similar titer value. It can therefore be inferred that the immunological activity is unaffected by modification of the IgG with SPDP.

By the same immunofluorescence technique we tried to assess the interaction of antibody coated liposomes with melanoma cells. Examination by fluorescence microscopy showed that at high concentration (2 mg/ml of antibody protein), both normal rabbit IgG (NRG) and anti-melanoma IgG coated liposomes were bound to cells indiscriminately. At low concentration (200 µg/ml of antibody protein), however, it appeared as though there was increased binding of liposomes to melanoma cells when the former were coated with anti-melanoma IgG (Ghose et al. 1984, Goundalkar et al. 1984b). Fig. 21 shows a photomicrographic depiction of such antibody mediated binding of liposomes to cells. Such a phenomenon, however, needs further confirmation by objective tests such as radiotracer technique.

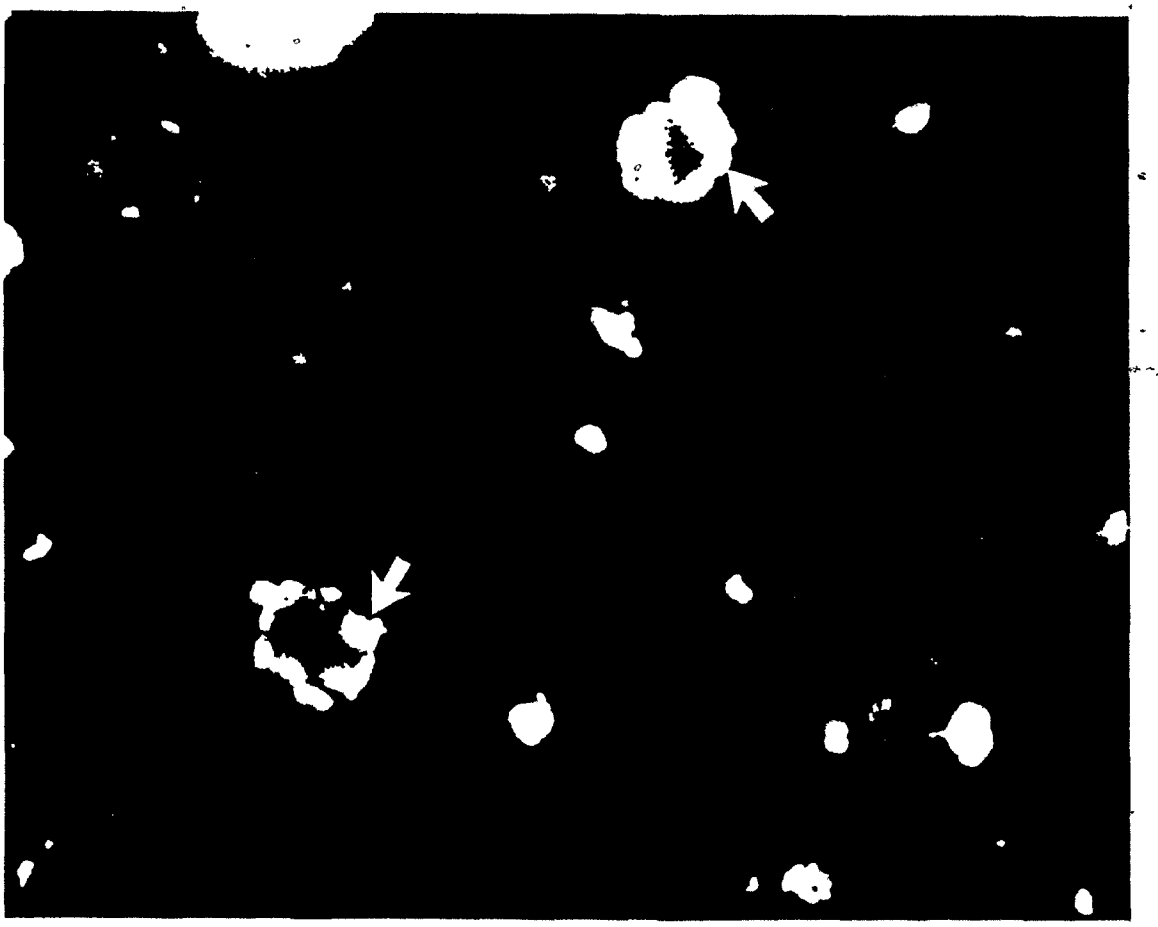


Figure 21. Fluorescence photomicrograph showing the binding of anti-melanoma globulin coated methotrexate containing liposomes to target melanoma cells.

Radiotracer technique

One of the reasons for undertaking interaction studies with melanoma cells was the fact that they are monolayer type. These cells grow attached to the surface of the plate unlike the EL_4 cells which grow in suspension. Because of their attachment to the surface of the plate, we thought that the unbound liposomes could easily be separated from the cells by decantation and washing procedure. Our initial experiments proved this to be wrong. Even after decantation and washing many liposomes though not bound to cells remained associated with cells. More washing resulted merely in increased loss of cells. Also, the characteristics of some of the cells were altered on addition of MTX liposomes resulting in agglutination. These observations led us to adopt the technique of centrifugation using 10% Ficoll for separating unbound liposomes from cells. This seemed to be a satisfactory method.

Liposome-cell interaction studies were carried out at two different temperatures for two different periods of time. Results of these experiments are presented in Table 21. Examination of the table indicates that the association of MTX with the cells increased in the following order: IgG coated MTX liposomes > MTX liposomes > free MTX solution. However, there was no significant difference in the binding of anti-melanoma globulin coated and NRG coated MTX liposomes to melanoma cells. Slightly higher association of

Table 21

Binding of antibody coated methotrexate (MTX) containing liposomes (REV) by human melanoma cells

Sample	Percent of added radioactivity associated with cells ±SD*			
	0hr**at 37°C	1hr at 37°C	2hr at 37°C	2hr at 4°
MTX soln.	N.D.	N.D.	0.0012 ±0.0003	0.005 ±0.00006
Positive MTX liposomes	3.15 ±0.22	N.D.	12.08 ±1.76	6.70 ±1.12
NRG-MTX liposomes	6.54 ±0.61	21.45 ±0.53	27.73 ±1.57	11.55 ±1.65
AMG-MTX liposomes	4.82 ±0.15	14.49 ±1.61	23.20 ±1.75	11.74 ±0.89

*standard deviation with N=3

**0 hr is the minimum time needed for mixing and centrifuging the samples; less than 15 min.

ND: not done.

liposomes bearing NRG could be attributed to the higher protein concentration (2.058 mg/ml as opposed to 1.619 mg/ml in anti-melanoma IgG-MTX liposomes) in these liposomes. This kind of result is surprising particularly in light of our previous observation by fluorescence microscopy of increased binding of antimelanoma globulin bearing liposomes to target melanoma cells. The obvious anomaly between these two findings could possibly be due to an inefficient separation of unbound liposomes from cells. Despite the presence of 10% Ficoll, some unbound liposomes because of their larger size might have settled along with the cells. Problems such as this would make it difficult to distinguish between specific and nonspecific antibody mediated binding of liposomes to cells.

In vitro studies against Caki cells

These in vitro studies were done according to the method described by Todd et al. (1982). In this method, because of the unstability of drug entrapped liposomes (for instance, due to leakage of the drug from liposomes) in presence of growth medium (Todd et al. 1982) fresh liposomes were added every 24 hours. The previous liposomes and free drug if any were removed by decantation. Cells were not disturbed in this process as they grow in monolayer fashion sticking to the bottom surface of the culture flask.

Figure 22 and Table 22 represent the growth curve for Caki cells. Results in Table 23 reveal that neutral MTX

Table 22
Growth curve for Caki cells*

Time hr	No. of cells ±SD
0	136840 ±5680
72	399480 ±5080
96	329480 ±54600
120	381200 ±19120

*Growth medium: McCoy's medium supplemented with 15% fetal bovine serum and antibiotics

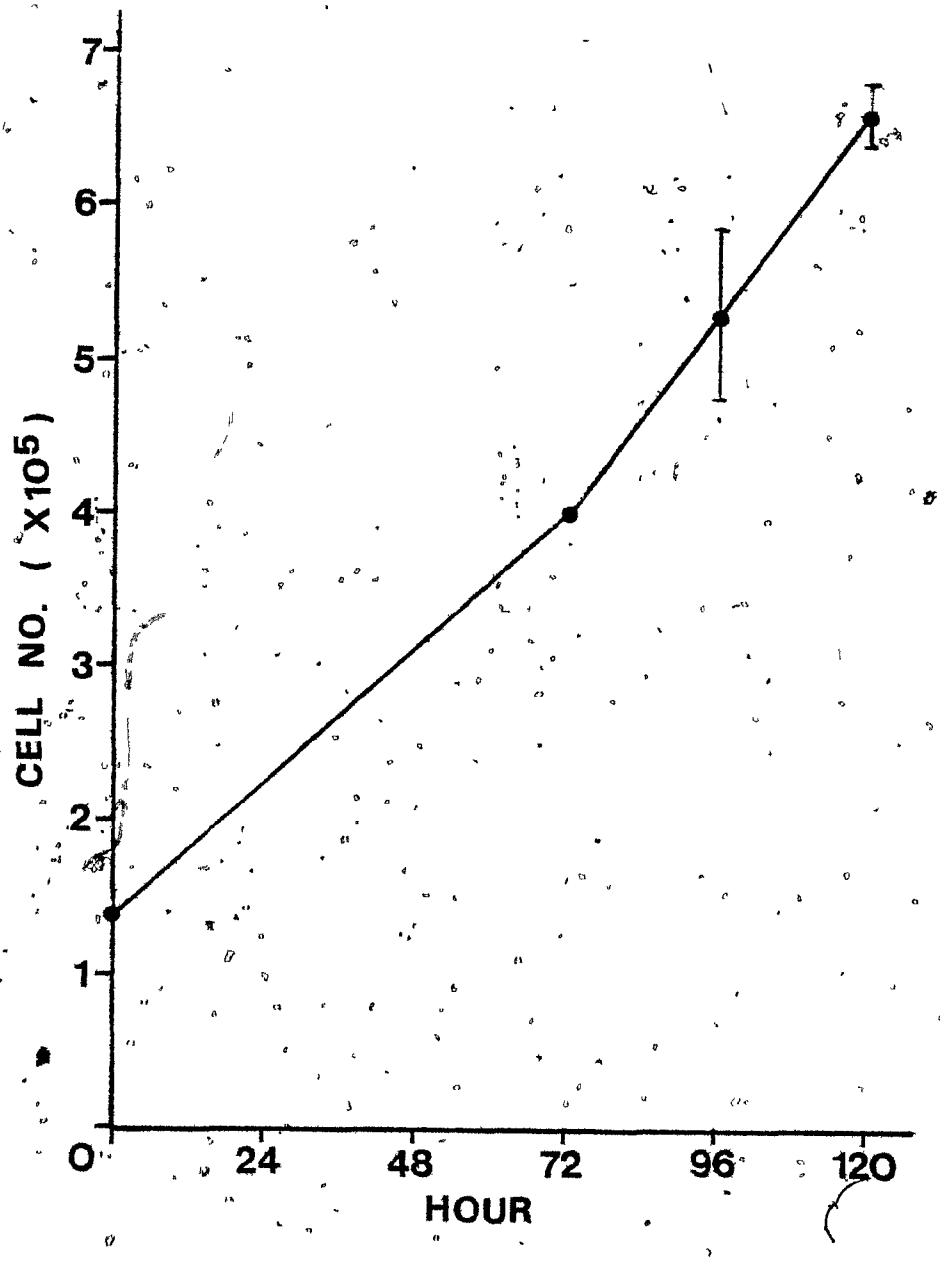


Figure 22. Growth curve for Caki cells in McCoy's medium supplemented with 15% fetal bovine serum and antibiotics.

Table 23
Effect of methotrexate (MTX) containing liposomes on Caki cells *in vitro*

Treatment	Conc. µg/ml	Percent inhibition of growth
Control	-	0
Free MTX	0.50	40
Neutral MTX liposomes	0.065	-6
	0.13	3
	0.65	8
	1.30	2
Empty liposomes	0.13	5
	1.30	2

liposomes (in the concentration range tested) are virtually ineffective against Caki cells compared to free MTX solution. Perhaps the rate of release of MTX from liposomes is too low to be effective against the cells. Furthermore, in comparison to EL_4 cells (Table 28) Caki cells are less sensitive to MTX. This is evident from the fact that with Caki cells only 40% growth inhibition is observed at a MTX concentration of 0.5 µg/ml while with EL_4 cells 86% inhibition is observed at as low a concentration as 0.1 µg/ml.

In vitro studies against EL4 lymphoma cells

EL_4 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics. Figure 23 and Table 24 represent the growth pattern for these cells.

Attempts to quantitate the number of cells in presence of liposomes:

A couple of in vitro experiments were done in which samples containing both cells and liposomes were counted using coulter counter. It was soon realized that in these experiments cell counts were being contaminated by liposomal counts. The possible theoretical contamination by liposomes in such cell counts is given in Table 25. However, this could vary unpredictably from sample to sample of liposomes, different ionic nature of liposomes and depending on time of contact between liposomes and cells. Hence a single

Table 24
Growth curves for EL_4 lymphona cells*

Time hr	No. of cells ±SD	
0	172960 ±5920	293320 ±15400
30	164360 ±1920	300040 ±9800
52	302680 ±9800	600000 ±36600
75	848000 ±28120	1640000 ±82040
105	2672000 ±84200	4020000 ±13760

*Growth medium: RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics

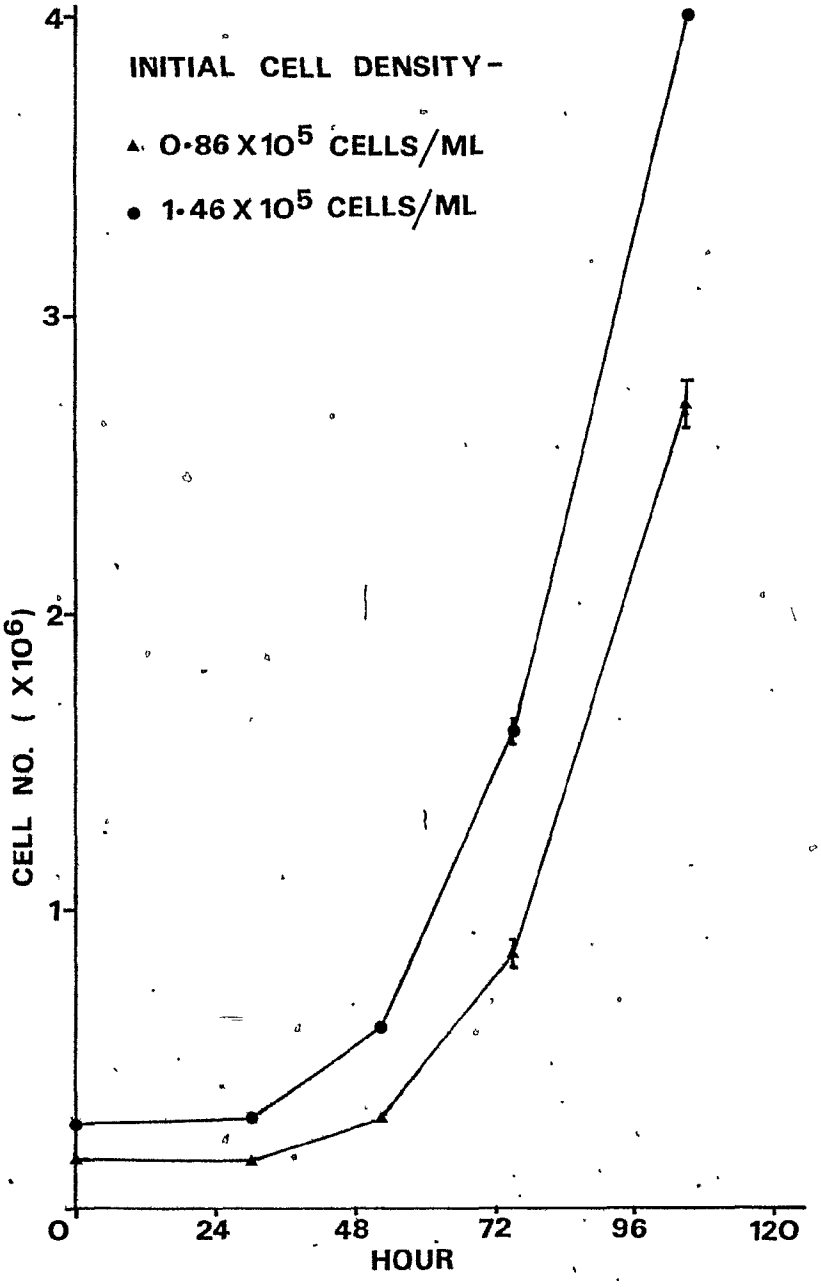


Figure 23. Growth curves for EL_4 lymphoma cells in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics.

Table 25

Expected contamination of cell counts by liposomes at a cell count of 163800 cells/2 ml (i.e. per plate)

100 µl of liposomes of dilution	Percent count contributed by liposomes at t = 24 hr	at t = 96 hr
1. 1:50	68(56)	73(10)
2. 1:500	17	1.8
3. 1:5000	2(1)	0.2 (0.09)

Note:
1. It was assumed that all the liposomes (at t=0) would be intact at t = 24 and t = 96 hr.
2. Values in parenthesis relate to 'empty' liposomes while others relate to neutral methotrexate liposomes.

suitable correction factor cannot be arrived at. Direct counting of cells using hemocytometer is not feasible because it was difficult to distinguish between cells and liposomes under an optical microscope.

Use of centrifugation to cause selective settling of cells:

To separate cells from the liposomes, centrifugation at 200 x g was tried with the hope that the cells would settle down, while liposomes would be located in the supernatant. The usual centrifugal force, 200 x g is used to settle EL_4 cells while we had used 22000 x g in the centrifugation of liposomes in the method of preparation. Unexpectably though, the results (Table 26) indicated that most of the liposomes also are settling down at this low centrifugal force. Consequently this method also proved to be futile.

Use of fluorescence activated cell sorter (FACS):

FACS, as the name suggests is an instrument used mainly for sorting of cells on the basis of fluorescence intensity of cells passing through a laser beam. For this purpose cells are to be stained with acridine orange or any other fluorescent stain. Acridine orange binds to DNA and makes cells fluorescent leaving liposomes unstained. Thus FACS can differentiate between cells and liposomes by the presence or absence of fluorescence.. FACS can also generate a scatter histogram based on light scattering by particles passing through a laser beam. Unlike a coulter counter,

Table 26
Centrifugation of neutral methotrexate (MTX) containing liposomes: 1600 rpm (200xg) for 10 min at 21°C

Dilution of liposomes	Count in Pellet	Count in Supernatant
1:50	312560 (94.5%)	18326 (5.5%)
1:500	33200 (84.5%)	6120 (15.5%)
1:5000	7280 (72%)	2840 (28%)

FACS cannot count a definite volume of sample. However, it was thought possible to obtain a ratio of cells to liposomes based on total counts due to scatter and fluorescence. Difference between scatter counts and fluorescence counts would represent liposome counts, while fluorescence counts would represent cells alone. It was thought the above ratio supplemented with a total count from coulter counter could be used to arrive at actual number of cells. However, as seen in Fig. 24, a complete scatter histogram could not be generated; a considerable number of liposomes in the lower end of size distribution range were being left out. Therefore, this resulted in an incomplete scatter count, consequently, the ratio of cells: liposomes was incorrect. Different manipulations of the instrument were tried with little success. This method was therefore abandoned.

After treating the sample with acridine orange, it was possible to clearly distinguish between liposomes and fluorescent cells by fluorescence microscopy. But again, counting of fluorescent cells using hemocytometer was not accurate enough to be adopted for our purpose.

Use of triton-X-100, sodium dodecyl sulfate (SDS, sodium lauryl sulfate), methanol and ether in the counting of cells in presence of liposomes:

Samples containing liposomes, cells and combination of both were treated with the above reagents in order to test the possibility of selectively lysing either cells or

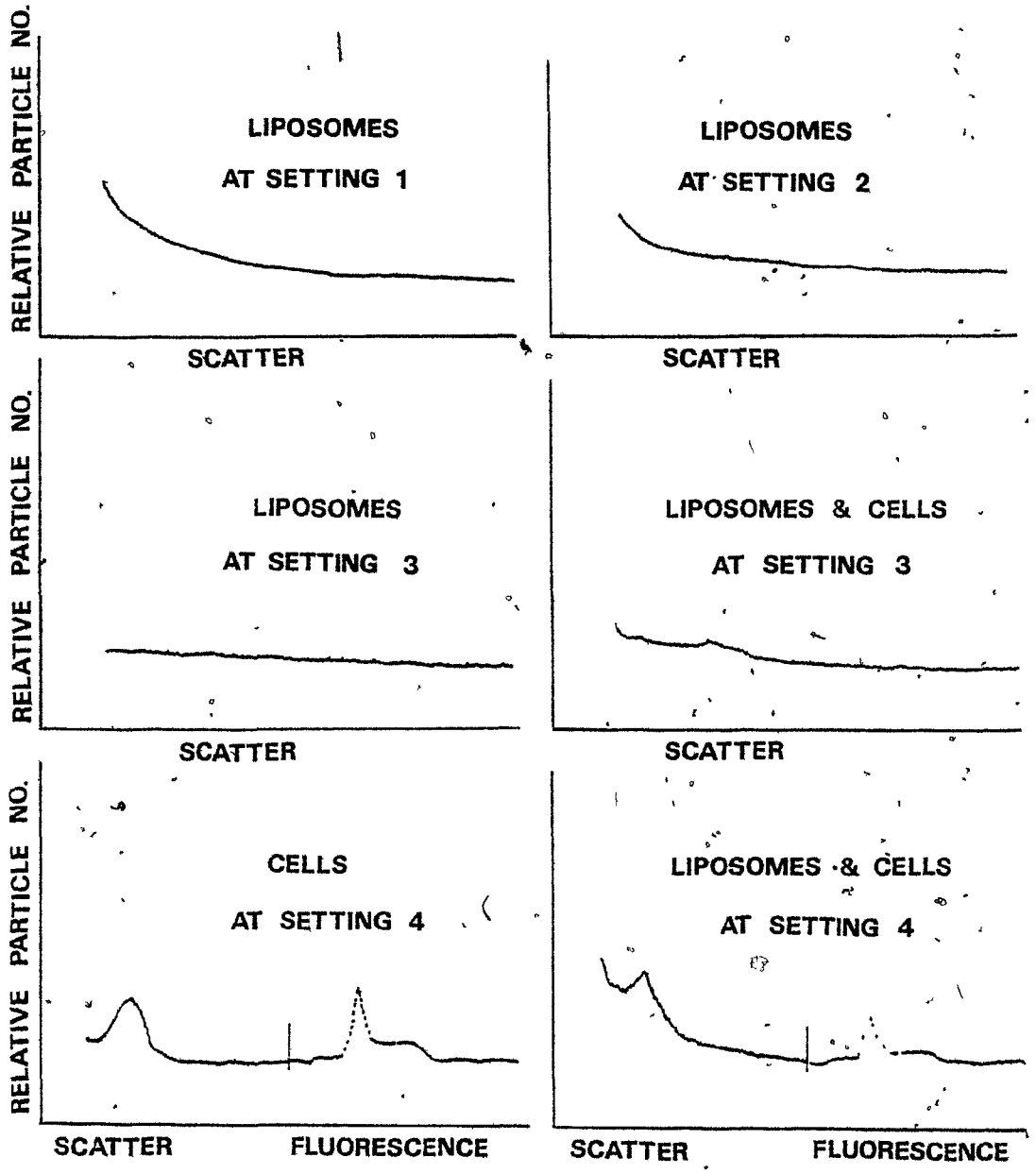


Figure 24. Scatter and Fluorescence distribution of liposomes and EL-4 lymphoma cells.

liposomes by detergent action or solvent action. Triton-X-100 has been used to cause release of entrapped substances from liposomes (Rowland and Woodley 1981). It is evident from results (Table 27) that, at a concentration of 1% triton-X-100, all cells are being lysed leaving the liposomes intact. It may seem surprising why liposomes are left intact and still triton-X-100 is used to release entrapped substances. Triton-X-100 may make liposomes leaky but without destroying their structural integrity. Structural composition may change because of interaction of triton-X-100 with the liposomal membrane. The observation of Alonso et al. (1981) is consistent with this kind of action of triton-X-100 on liposomes. Also it can be recapitulated here, that in our efforts to determine MTX in liposomes by spectrophotometry, we were unable to eliminate liposomal turbidity by triton-X-100 treatment. Perhaps this partly explains the reason for the same. The method of selectively lysing cells with triton-X-100 was then followed in all our further studies with EL_4 cells involving quantitation of cell number in presence of liposomes. Numbers in Table 27 are self explanatory regarding the effect of other reagents. SDS was nonspecific in its action on liposomes and cells. It is not clear why SDS (anionic), which is also a detergent, like triton-X-100 (neutral) may behave differently from the latter. The difference in the

Table 27

Effect of triton X-100, sodium dodecyl sulfate, methanol and ether on cells and/or liposomes.

Treatment	Percent lysis			
	cells alone	liposomes alone	Cells and liposomes together	
			actual	theoretical
Triton X-100				
0.1%	89	0	20	20
1%	99	0	25	23
10%	99	9	32	29
Sodium dodecyl sulfate				
0.1%	99	48	-	60
1%	99	61	74	70
10%	99	73	80	79
Methanol				
1%	0	0		
10%	0	0		
50%	16	2		
Ether*				
0.05%	6	5		
0.5%	7	6		
5%	7	8		

*more than 5% v/v resulted in immiscibility

chemical structure, charge, of both, may be responsible for the different action in lysing cells and lipid vesicles.

In vitro studies against EL_4 cells

Results in Table 28 indicate that liposomal MTX, irrespective of charge is less effective against EL_4 cells compared to the free MTX solution. As already mentioned, this could perhaps be due to the limited availability of free MTX as a result of slower release of MTX from liposomes. Among the three different types of liposomes, cationic liposomes seem to be more effective. Because of the electrostatic attraction, cationic liposomes may possibly interact more efficiently with cells which are usually negatively charged. This facilitated interaction may also lead to more release of free MTX from liposomes to be effective in arresting cell growth.

Results of the in vitro evaluation of targeted, i.e. anti-EL_4 globulin (AEG) coated MTX liposomes are presented in Table 29. As evident from this table, though the AEG coated liposomes were slightly more effective in inhibiting the growth of EL_4 cells than the NRG coated liposomes, such difference was not statistically significant. Also the physical mixture of AEG (i.e. specific antibody) and positively charged MTX liposomes seems to be equal in its effectiveness to that of liposomes coated covalently with AEG.

Table 28

Effect of methotrexate (MTX) containing liposomes on EL_4 lymphoma cells *in vitro*

Treatment	Conc. µg/ml	Percent inhibition of growth (± SD)
Control	-	0
Free MTX	0.01	44 (± 9)
	0.10	86 (± 2)
	1.00	86 (± 2)
Empty Neutral Liposomes	0.01*	0
	0.10*	0
Neutral MTX Liposomes	0.013	4
	0.13	4
	1.30	82
Positive MTX Liposomes	0.01	6 (± 6)
	0.10	53 (± 24)
	1.00	96 (± 6)
Negative MTX Liposomes	0.01	9
	0.10	6
	1.00	89

*Arbitrary concentration indicating the dilutions of empty liposomes similar to MTX liposomes.

Table 29
Effect of targeted methotrexate (MTX) containing liposomes on EL_4 lymphoma cells in vitro.
(Conc. 0.1 µg/ml)

Treatment	% Inhibition of growth ± SD
Control	0
Anti-EL_4-GLOB-MTX Liposomes	74.5* ±4.6
NRG-MTX Liposomes	61.1* ±10.9
Anti-EL_4-GLOB + MTX Liposomes Phy. Mix.	79.8* ±4.0

*No difference between these means as per t-test.

These results are contrary to those that we expected. Many explanations are possible for these kinds of results. One of them is the specificity of the antibody itself. These antibodies, because they are polyclonal would possess much lower specificity than the monoclonal type. Further reduction in the specificity could result from the extensive absorption procedure used in the preparation of these antibodies. The extensive absorption of the antisera with normal tissues could wash away a considerable amount of antibody activity and this would result in the reduction in the affinity of the antibody preparation. Accordingly, Uadia P. (unpublished observations) observed increased affinity in anti-melanoma antibodies obtained from unabsorbed sera compared to those obtained from absorbed sera. Secondly, even if antibodies were capable of targeting the liposomes to the EL_4 cells, if liposomes are not taken up by the cells, targeting would be futile. Recently, it is being understood that for liposomes to be taken up by cells, they have to be as small as possible (Machy and Leserman 1983, Matthay et al. 1984). The liposomes used in this study were bigger in size; their size varied from $< 2\ \mu m$ to $8\ \mu m$. This size factor could have caused hindrance for their uptake by the cells even if they were targeted. Thirdly, the amount of antibody associated with the liposomes may not be quite sufficient for the intended role

of targeting. Any combination of these factors could have given rise to results contrary to our expectations.

In vitro studies against human melanoma (M_{21}) cells:

Figure 25 and Table 30 represent the growth curve for M_{21} cells. With M_{21} cells, the effectiveness of MTX entrapped only in positively charged liposomes was tested. Results are shown in Table 31. These results are qualitatively similar to what we observed with EL_4 cells. Once again liposome entrapped MTX is less effective compared with free MTX. However, these cells show lesser sensitivity to MTX than EL_4 cells (Table 28). But, in comparison with Caki cells (Table 23), these cells show an increased sensitivity to MTX.

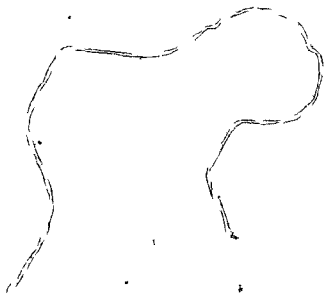
In vivo studies against murine EL_4 lymphoma:

Initial in vivo studies were conducted using both neutral and positively charged MTX liposomes. Mice bearing EL_4 ascites tumor were treated with liposomes at a MTX dose of 5 mg/kg body weight. As can be seen from Table 32, this dose caused a great amount of host toxicity resulting in early death of many of the mice. Marked decrease in weight was one of the criteria used to assign the death as due to toxicity or otherwise. Also the presence or absence of ascites tumor in the dead mice was ascertained by microscopic examination of a smear from the peritoneal cavity. Because of the great toxicity observed at a dose of 5 mg/kg, further experiments were tried by reducing the dose

Table 30.
Growth curve for human melanoma (M_{21}) cells*

Time hr	No. of cells ±SD
0	125320 ± 51200
24	121400 ± 3120
51	375040 ± 21720
75	899680 ± 27400
99	119720 ± 36080
122	1075480 ± 33680

*Growth medium: RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics.



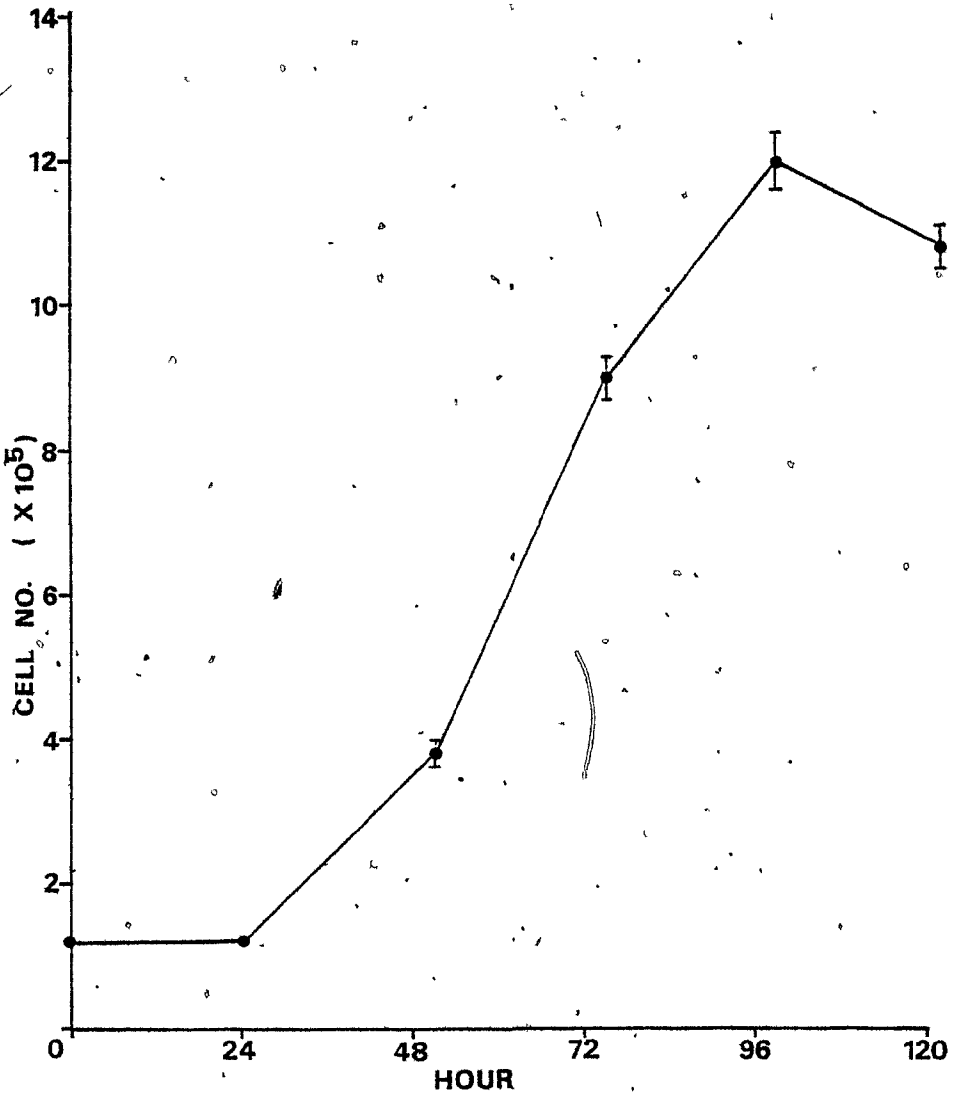


Figure 25. Growth curve for human melanoma cells in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics.

Table 31
Effect of positive methotrexate (MTX) containing liposomes on human melanoma (M_{21}) cells

Conc. of MTX	Percent inhibition of growth (± SD) Positive MTX liposomes	free MTX soln.
1 µg/ml	81.3 (±0.7)	89.9 (±0.9)
0.6 µg/ml	75.0 (±1.1)	ND*
0.3 µg/ml	72.1 (±2.9)	ND
0.2 µg/ml	17.0 (±2.3)	98.0 (±0.5)
0.1 µg/ml	0	85.4 (±0.3)
0.01 µg/ml	0	0

ND = not done

Table 32

In vivo evaluation of methotrexate (MTX) containing liposomes in the treatment of EL_4 ascites tumor (Dose of MTX = 5 mg/kg on 1, 4 and 7th days I.P.)

Treatment	Total No. of mice	Survival days	Mean survival time Days ±SD	Statistical** significance
Control (untreated)	11	14,15,15,15,17,17,17,18 18,20,28	17.6 ± 3.9	
Free MTX soln.	6	17,18,18,18,20,24	19.2 ± 2.6	N.S.
Free MTX soln. + positive empty liposomes	6	14,16,17,18,18,19	17.0 ± 1.8	N.S.
Neutral MTX liposomes	6	9*,10*,13*,20*,26,33	18.5 ± 9.6	N.S.
Positive MTX liposomes	14	4*,6*,9*,10*,10*,10*,10* 10*,14*,15*,21,22,25,33	14.2 ± 8.2	N.S.

*death due to toxicity
**student's t-test
N.S. = not significant

of MTX to 4, 3.5 and 3 mg/kg body weight. However, only positively charged MTX liposomes were tested in these and later experiments for the simple reason that these liposomes possessed the advantage of higher encapsulation and they were more effective *in vitro* than the other types of liposomes. As could be expected, a gradual reduction in the dose brought about a parallel decrease in host toxicity, as evident from the results in Table 33. The toxicity was completely eliminated at the MTX dose of 3.5 mg/kg. Further *in vivo* evaluations were therefore pursued at this dose level of MTX. It is important to note here that neither the empty liposomes, nor their physical mixture with free MTX solution were toxic. The toxicity was observed only when MTX was encapsulated in liposomes.

At the dose level of 3.5 mg/kg, the positive MTX liposomes were more effective ($p < .001$) than the free MTX solution (Table 34). An increase of about 22 percent in survival time of tumor bearing mice, was observed. However, there were no long term survivors.

With the hope that equipping the MTX loaded liposomes with tumor specific antibodies might result in improved therapeutic effectiveness, *in vivo* studies were carried out using positive MTX liposomes coated covalently with AEG. The liposomes coated with NRG served as one of the controls. Results are summarized in Table 35. Contrary to what we expected, MTX liposomes became more toxic when coated with

Table 33
Toxicity of positive methotrexate (MTX) containing liposomes*

Dose of MTX	% Death Due to Toxicity
5 mg/kg	67-75
4 mg/kg	40
3.5 mg/kg	0
3 mg/kg	0

* Empty liposomes alone or with free MTX soln. were not toxic at 5 mg/kg.

Table 34

In vivo evaluation of methotrexate (MTX) containing liposomes in the treatment of EL_4 asictes tumor
Dose of MTX = 3.5 mg/kg on day 1, 4 and 7 I.P.

Treatment	Total No. of mice	Survival days	Mean survival time Days ± SD	Statistical significance
Control (untreated)	12	16,16,17,17,17,18,18 18,18,19,20,21	17.9 ± 1.5	
Free MTX soln.	6	16,17,17,18,20,21	18.2 ± 1.9	N.S.
Positive MTX liposomes	17	17,18,19,19,19,21,21, 22,22,22,23,23,24,24, 27,28,29	22.2 ± 3.4	$p < .001$*

*t-test
N.S. = not significant

Table 35

In vivo evaluation of targeted methotrexate (MTX) containing liposomes in the treatment of EL_4 ascites tumor
Dose of MTX = 3.5 mg/kg on day 1, 4 and 7 I.P.

Treatment	Total No. of mice	Survival days	Long term survivors	Statistical** significance
1. Control (untreated)	11	17,17,18,18,18,18,19,20,20,22,25	-	
2. NRG-MTX liposomes	11	10*,10*,11*,11*,12,21,22,23,24,35+,35+[a]	2	N.S.
3. Anti-EL_4-GLOB-MTX liposomes	11	9*,11*,20*,21*,22,22,23,24,25,26,56+	1	N.S.
4. Anti-EL_4-GLOB and MTX liposomes Phy. mix.	11	14,14,23,23,24,26,27,30,31,35+,35+	2	N.S.

*death due to toxicity
**test performed: modified Wilcoxon test (Burdette and Gehan 1970)
[a] Mouse, whose survival day is denoted with a number followed by a plus sign, survived tumor free.

IgG antibodies than they were otherwise; at the dose of 3.5 mg/kg plain MTX liposomes did not cause toxicity. However, there were some long term survivors in mice treated with specific (i.e. AEG) as well as non specific (i.e. NRG) antibody coated liposomes. Long term survivors were present also in the group of mice treated with the physical mixture of AEG and MTX liposomes. Over all, statistically there was no difference in any of these treatments. On the contrary, coating the liposomes with antibodies resulted in increased host toxicity. The toxicity could not be overcome by reduction in the dose (3 mg/kg). Nor did the increase in dose (4 mg/kg) bring about any increase in the number of long term survivors (see Table 36).

In the in vitro evaluation also, no difference was observed in the effect of MTX liposomes coated with either antibody. In this respect, both in vitro as well as in vivo studies seem to indicate the same thing. In the in vivo studies too, the failure to observe superior activity of targeted MTX liposomes could be attributed to the same set of factors that were discussed under the results of in vitro experiments.

The increased toxicity of MTX entrapped in liposomes could be due to the slow clearance of MTX from the body. The clearance of the MTX could be slowed down, firstly, because of slow release of MTX from the liposomes; only the "free" form of MTX can be eliminated. Secondly, the intra

Table 36

In vivo evaluation of targeted methotrexate (MTX) containing liposomes in the treatment of EL_4 ascites tumor

Dose of MTX = 3 mg/kg on days 1, 4 and 7 I.P.

Treatment	Total No. of mice	Survival days	Mean survival time Days	± SD	Statistical** significance
1. Control (untreated)	5	14,17,17,17,18	16.6	±1.5	
2. NRG-MTX liposomes	5	9*,9*,10*,14*,22	12.87	±5.5	Not different from control (1)
3. Anti-EL_4-GLOB-MTX liposomes	5	18,20,21,23,28	22.0	±3.8	Significantly different from control (1) ($p<0.02$) and NRG-MTX liposome ($p<0.05$, t-test) but not from phy. mix.
4. Anti-EL_4-GLOB and MTX liposomes Phy. mix.	5	11,13,20,27,42+			Not different from control (1)
Dose of MTX = 4 mg/kg on days 1, 4 and 7 i.p.					
5. Control	4	17,20,22,25	21	± 3.4	
6. Anti-EL_4-GLOB-MTX liposomes	6	14*,15*,15*,19,23,	18.2	± 4.1	Not different from control as per t-test

*death due to toxicity

**modified Wilcoxon test (Burdette and Gehan 1970) was performed unless otherwise specified.

peritoneal route of administration itself could further delay the clearance of MTX. The peritoneal cavity could thus act as reservoir for toxic MTX, releasing it slowly into the lymphatic and blood circulation over a long period of time. Also, the MTX from the peritoneal reservoir, could infiltrate into the surrounding tissues, such as gastrointestinal mucosa and cause intestinal denudation which could be lethal. There is a direct correlation between the toxicity and the length of time MTX persists in the body (Chabner and Young 1973). In a trial experiment, we administered MTX liposomes, to normal mice by intravenous route at a dosage of 3.5 mg/kg on day 1, 4 and 7. None of these mice succumbed to toxic death; instead all the mice survived indefinitely like normal ones. This finding gives credence to the earlier statement that the intraperitoneal route itself could have caused increased toxicity for liposomes. Though I.P. route is usually safer than the I.V. route, this does not seem to hold true as regards to MTX liposomes, which is evident from this investigation and also from other reports (Tyrrell et al. 1976, Freise et al. 1979 and Kaye 1981).

The toxicity which vanished at a dose of 3.5 mg/kg, was restored when the liposomes were conjugated to IgG, be it specific or otherwise. In the absence of the uptake by the EL_4 cells, the antibody coated MTX liposomes could be taken up by the macrophages lining the peritoneal cavity.

This undesired uptake is conceivable because the Fc parts of IgG on liposomes can interact with Fc receptors on macrophages and trigger phagocytosis. The uptake of toxic MTX liposomes by the macrophages could be suicidal to them. The end result of all this would be to wipe out part of the immune system. Therefore the liposomes with the antibody could have been more toxic to the mice than those without antibody. Further, the toxicity could also be generated by antibody liposome conjugation process.

SECTION 2: SUV LIPOSOMES

Preparation of positively charged SUV containing MTX:

As is common practice, SUVs were prepared by sonication of MLVs. In order to do this, both bath as well as probe sonication were attempted. The bath sonication (55000 cycles/sec) was performed at 50-55^{o}C for 60 min. Visual as well as the microscopic examination of these vesicles revealed inefficient breakdown of large liposomes into SUVs. The probe sonication however, proved to be more effective in obtaining the SUVs. In the absence of PDP-SA, the liposome dispersion, became almost clear after probe sonication for 30 min. The increased clarity is an indication of reduction in size of larger MLVs into smaller SUVs. All the SUVs were hence prepared by probe sonication method.

In the presence of PDP-SA, however, the sonicated liposomes were less clear and contained a considerable amount of large liposomes. Large liposomes and probe particles could be eliminated by centrifugation at 22000 x g for 20 min. The difficulty in sonicating the PDP-SA containing liposomes could be due to the altered lipid composition itself. Despite this, it might be possible to overcome the problem by prolonging the time of sonication. Lower encapsulation efficiences (see Table 37) reflect the reduced efficiency of size reduction of PDP-SA liposomes.

Table 37
Encapsulation efficiency of ^{3}H-methotrexate (MTX) in small unilamellar vesicles (SUV)

Batch No.	SUV	SUV with PDP-SA
1	3.24%	2.2%
2	1.93%	0.67%
3	-	1.25%
mean	2.59%	1.37%
± SD	0.93%	0.77%

Separation of unentrapped drug from liposomes:

Gels like Sephadex G-25 or G-50 have often been used to separate liposomes from unentrapped drug. Compared to dialysis, it is a time saving method. Therefore, we tried this approach first. The results of Sephadex G-25 gel filtration chromatography of the sonicated ^{3}H-MTX liposomes are seen in Fig. 26. Two distinct peaks are clearly seen in this figure; one in the void volume as measured by using Blue dextran 2000 and the other in the retarded volume. The peak representing the retarded volume coincided with the peak obtained by chromatography of free MTX solution on the same column (Fig. 27). Fractions in the void volume represent liposomes. These fractions were also visually turbid indicating the presence of liposomes. The fractions containing the majority of liposomes were pooled. In order to assess the extent of separation of unencapsulated drug, the pooled liposomes were rechromatographed on Sephadex G-25 column. The elution profile in Fig. 28 was obtained as a result of this experiment. The figure gives an indication of the presence of a large quantity of free drug in the liposome sample as shown by a large peak in the retarded volume. In contrast, gel filtration of the liposomes from which the free drug was removed by exhaustive dialysis, showed only one peak at the void volume (see Fig. 29). This clearly shows the absence of any free drug in the liposomes purified by dialysis.

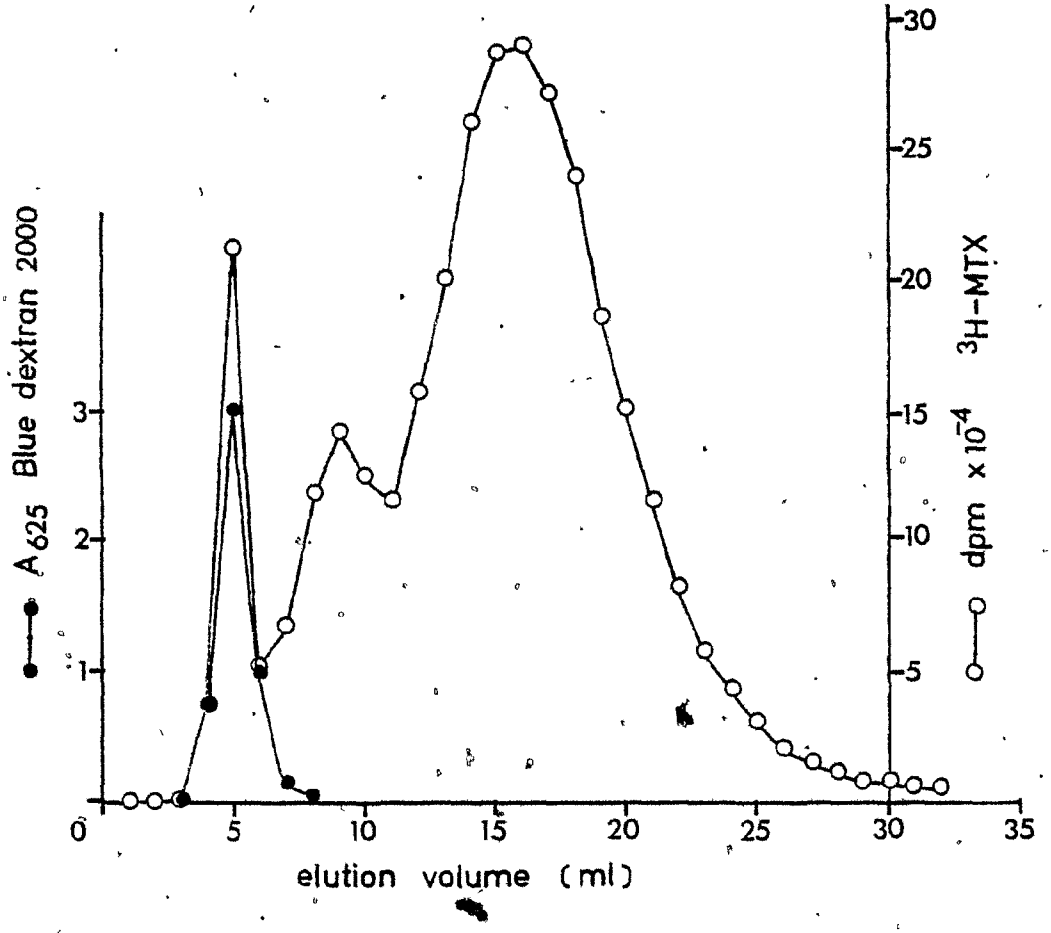


Figure 26. Elution profile of "free" and liposome (SUV) encapsulated methotrexate (MTX) from Sephadex G-25.

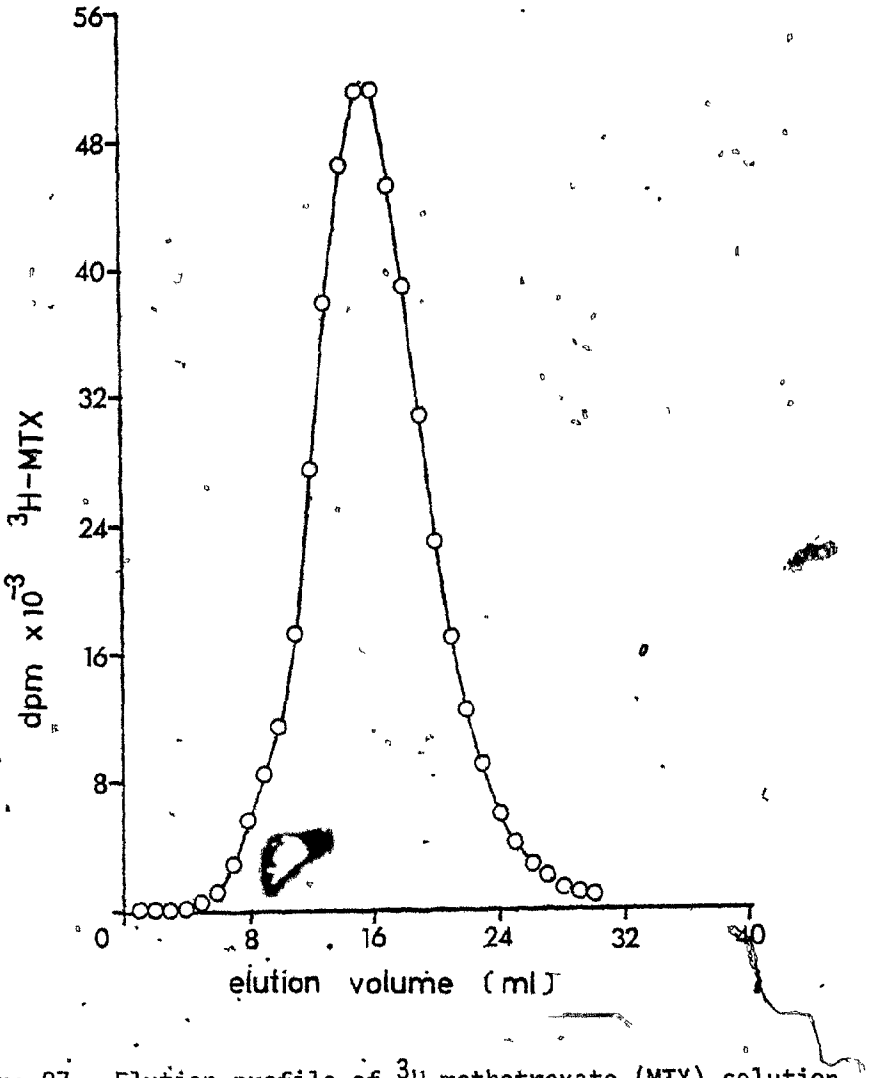


Figure 27. Elution profile of ^{3}H-methotrexate (MTX) solution from Sephadex G-25.

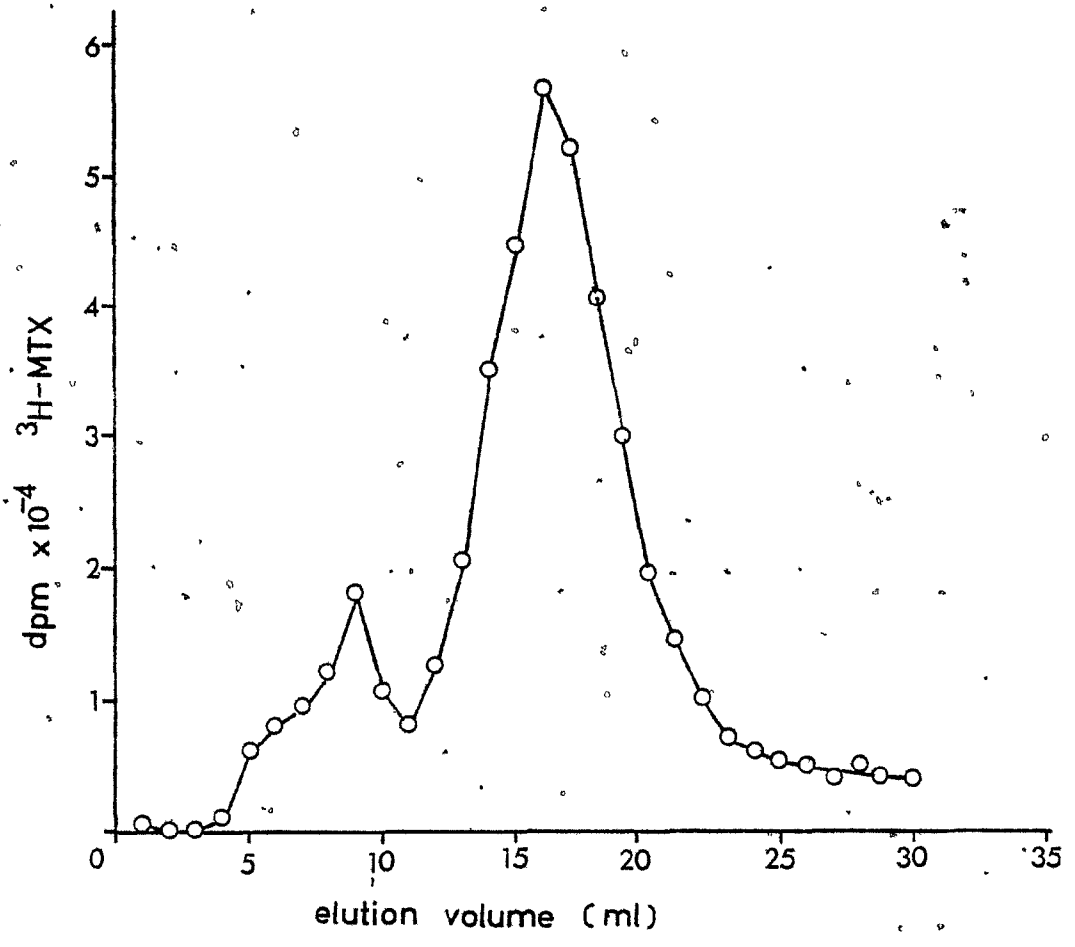


Figure 28. Rechromatography, on Sephadex G-25, of liposomal (SUV) ^{3}H-methotrexate (MTX) obtained by gel filtration.

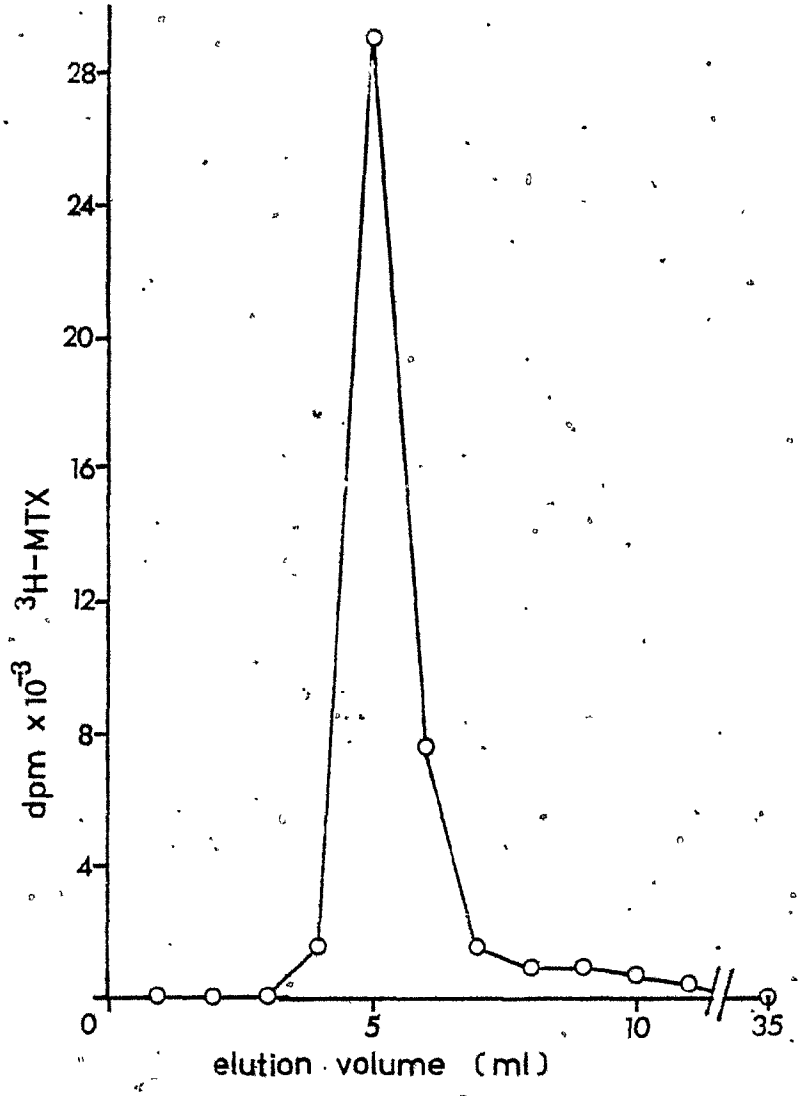


Figure 29. Sephadex G-25 elution profile of liposomal (SUV) ^{3}H-methotrexate (MTX) obtained by dialysis.

The presence of contamination as free drug in liposomes purified by gel filtration is also evident from the high encapsulation efficiency (9.1%) obtained for these SUV. This value, arrived at by comparing the area under the peaks in Fig. 26 is quite high for SUVs. On the other hand, the encapsulation efficiency (1.4%) for dialysed liposomes conforms well to the literature value for SUVs. Although gel filtration should cause no problem in removing the free drug, the method proved unsatisfactory in our case. The type of gel and height of the column could be the possible factors for such inefficiency. These results thus prove that dialysis is the best way to get rid of free drug from sonicated liposomes.

Covalent coupling of IgG to liposomes

Coupling reaction

The principle of our method of binding IgG to SUVs is the same as that described by Barbet et al. (1981). The presence of PDP-SA in liposomes and of PDP groups on IgG molecules makes the thiol disulfide exchange reaction possible. Because of this reaction, IgG binds covalently to SUV liposomes as is the case with REV liposomes.

Separation of antibody coated SUV from unbound antibody

This step of separating antibody coated liposomes from unbound antibody is quite different for SUV and REV liposomes. REV liposomes, because of their bigger size can easily be sedimented by high speed centrifugation. In

contrast, SUV's being very small in size, are difficult to sediment by this technique. Ultracentrifugation could perhaps cause settling of SUVs, but, is very difficult to redisperse the resulting pellet. Hence, unlike for REV liposomes, the technique of centrifugation for separation of unbound antibody was dispensed with for SUVs.

Many investigators have used the technique of gel filtration through appropriate gels for isolation of liposomes and antibody. Hence we tried two different gels, namely, Sephacryl S-400 superfine and Sepharose 4B for this purpose. Based on theoretical considerations (i.e. their fractionation range) both these gels should be able to resolve liposomes and IgG. As expected, liposomes and IgG are well resolved when passed through these columns (see Fig. 30,31). Unfortunately, when the liposome antibody reaction mixture was run through the columns both the columns failed to separate the liposomes from unbound IgG. A plausible explanation for this could be the aggregation of liposomes upon binding of IgG to them. Such aggregated liposomes could physically be trapped on the column. The problem of aggregation has been observed by other investigators also (Jou et al. 1984, Matthay et al. 1984). The concentration of the protein, i.e. antibody, is one of the factors responsible for aggregation. The higher the protein concentration, the higher the degree of aggregation. Aggregation has been overcome by many

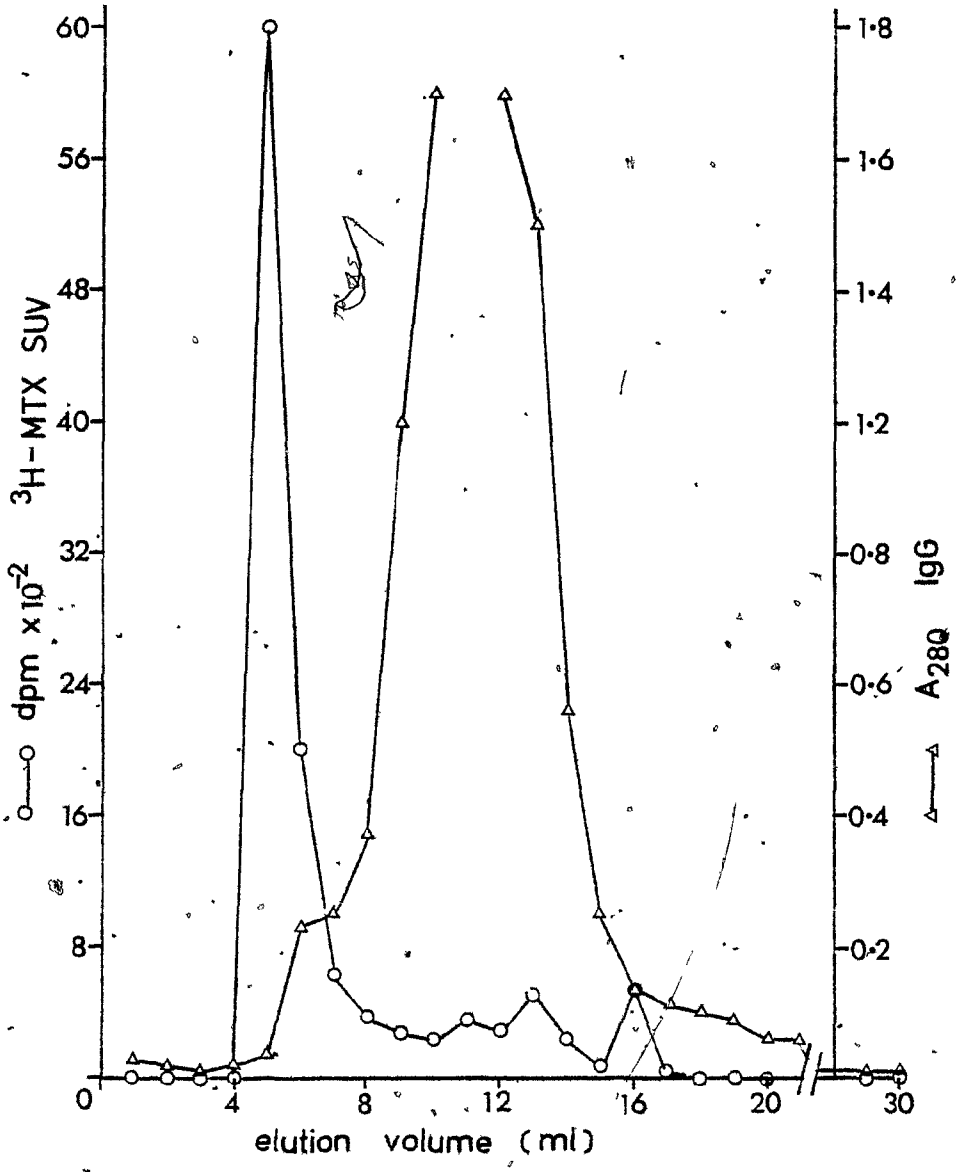


Figure 30. Elution profiles of liposomal (SUV) ^{3}H-methotrexate (MTX) and IgG from Sephacryl S-400.

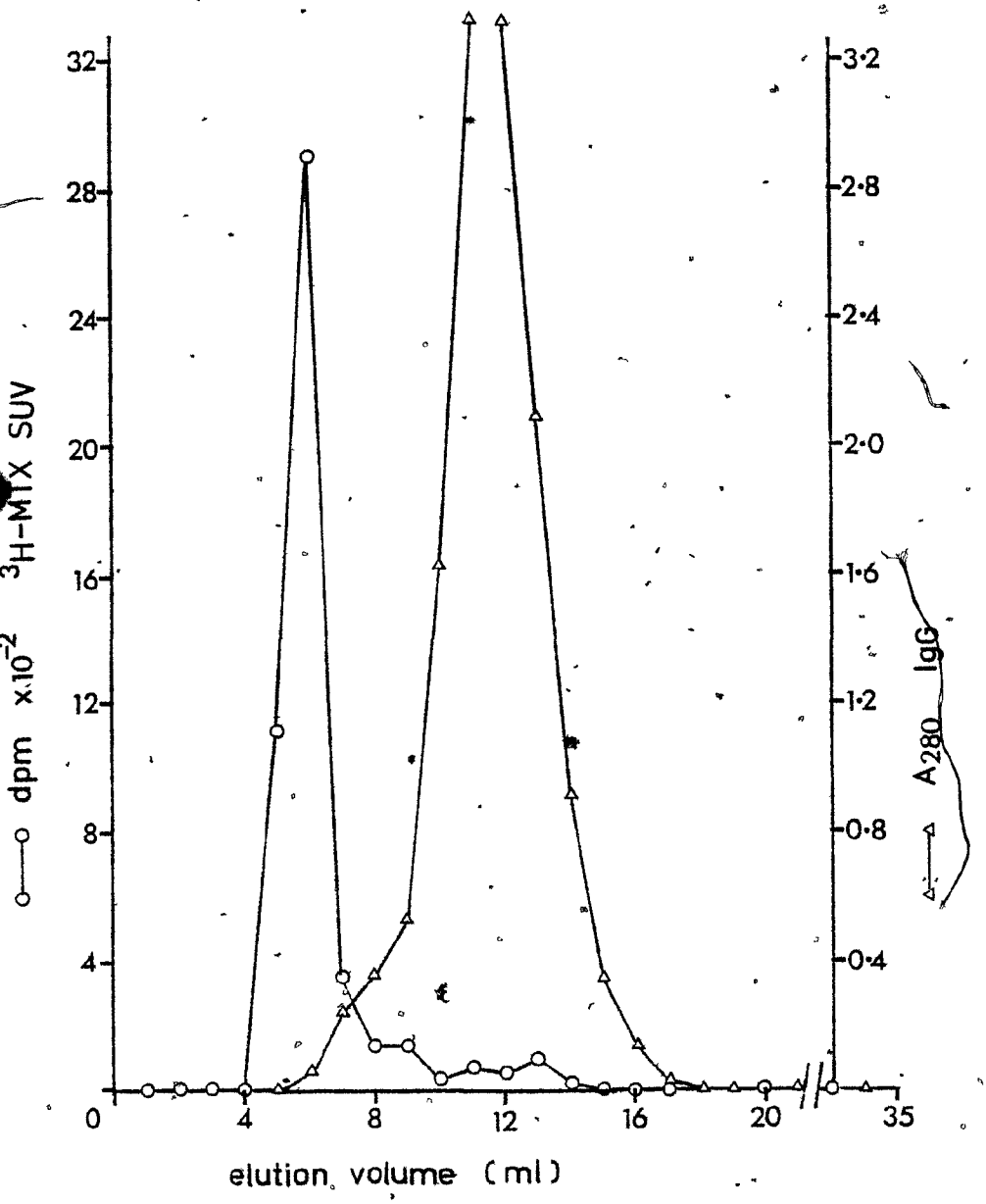


Figure 31. Elution profiles of liposomal (SUV) ^{3}H-methotrexate (MTX) and IgG from Sepharose 4B.

investigators by the use of low amounts of antibody for binding to liposomes. However, in almost all of those instances the antibody used was monoclonal. The monoclonal antibodies are supposed to possess exceptionally high specificity. In our case, the antibody used was a polyclonal one which has poor specificity compared to the monoclonal type. Therefore, it was necessary for us to have a higher concentration of IgG in our coupling reaction mixture to compensate for the lower specificity of our polyclonal antibodies.

Ficoll floatation centrifugation

This technique had been used by Shen et al. (1982) for separation of liposomes from unbound antibody. Fraley et al. (1980) had used a similar technique to isolate liposomes from unencapsulated DNA. We tested the feasibility of this approach, at first using plain liposomes and then the experiments were conducted using antibody - liposome reaction mixture. Results of these experiments are shown in Table 38. Examination of this table implies that the position of the liposomes in the Ficoll gradient depends on the concentration of protein associated with them. If the protein concentration is high (4.3 mg/ml) the liposomes settle to the bottom of the gradient. An intermediate concentration causes the liposomes to band between 10% Ficoll and 12.5% Ficoll. At low concentration, however, the liposomes float on top of 10% Ficoll below 0.5% $NaHCO_3$.

Table 38
Ficoll floatation centrifugation of antibody bound liposomes

Amount (mg) of protein (IgG) per ml of liposome in the reaction mixture	Amount of protein (IgG) associated with liposomes		Position of liposome-IgG band in the density gradient following centrifugation
	Actual obtained mg/ml	Corrected* mg/ml	
15.45 - 15.95	18.69 - 19.57	4.30 - 4.50	Bottom i.e. settles to the bottom
10.50 - 10.70	1.75 - 1.80**	2.63 - 2.70	Middle i.e. between 10% and 12.5% Ficoll
5.25 - 7.98	1.25 - 1.80	1.02 - 1.40	Top i.e. between 0% and 10% Ficoll

*Corrected to the original volume of liposomes in the reaction mixture.
**It was difficult to separate and get a more concentrated band of liposome-IgG.

This phenomenon of varying degree of floatation can easily be explained by the fact that increasing the concentration of protein with liposomes gives rise to increase in density.

The concentration of protein present in liposomes depends upon the amount of liposomes and IgG in the reaction mixture. At our ideal experimental conditions (i.e. when encapsulation efficiency of SUVs was 2.2%), 2 volumes of SUVs reacted with 1 volume of IgG solution (10 mg/ml), liposomes floated on top of the Ficoll after centrifugation. Under the same conditions, when the binding was performed using 1 volume of SUV and 1 volume of antibody, liposomes either banded between 10 and 12.5% Ficoll or settled to the bottom after centrifugation, depending on protein concentration. We were interested in liposomes which could float on 10% Ficoll, so that they can be separated from cells, in the binding studies. Therefore, to obtain such liposomes, we always used 2 volumes of liposome and 1 volume of antibody in coupling experiments.

Although the antibody bound liposomes floated on 10% Ficoll, when observed under the microscope, these liposomes showed the presence of aggregates. Attempts were made to break these aggregates by forcing the preparation through 0.4 μm polycarbonate filter. Extreme difficulty was experienced during this filtration and whatever little filtrate was obtained was devoid of liposomes. In other words, liposomes were trapped by the filter itself. A

problem such as this was also observed by Matthay et al. (1984). These authors attributed this problem to the higher protein concentration. Consistent with this notion, we had no difficulty in filtering SUV liposomes with no antibody through the 0.4 μm filter. As mentioned earlier, because we needed higher amounts of protein associated with the liposome, we could not avoid the formation of aggregates.

Liposomes binding studies with melanoma cells

These experiments were conducted with human melanoma (M_{21}) cells similar to those done with the REV liposomes. It was thought that in the former binding studies, because of the larger size of REV liposomes, some unbound liposomes might have settled along with the cells. This might have masked the increased binding of specific antibody coated liposomes to target melanoma cells. It was anticipated that the use of SUV liposomes, because of their smaller size, would overcome this problem. Another reason for conducting experiments with SUV liposomes was the recent finding that small liposomes are better than large liposomes for intracellular drug delivery (Machy and Leserman 1983).

The results of the binding studies with antibody coated SUV liposomes are presented in Table 39. These results, when compared to results obtained with REV liposomes (Table 21) suggest that liposomes bind to cells as a function of their size. The REV liposomes bound more than SUV liposomes to melanoma cells. As observed earlier, the

Table 39
Binding of antibody coated methotrexate (MTX) containing liposomes (SUV) by human melanoma cells
Time of incubation = 2 hr Temp. = 37°C

Sample	Percent of added radioactivity associated with cells ±SD
Positive MTX liposomes	3.44 ±0.20
NRG-MTX liposomes	21.44 ±0.94
AMG-MTX liposomes	5.02 ±1.12

mere presence of antibody on liposomes, further increased their binding to cells. However, unlike what was expected, these results once again fail to show any targeting, i.e. increased binding of liposomes coated with specific antibody. It is difficult to envisage a specific cause for these unexpected results. A possible reason could be the presence of aggregates in the liposomes. The aggregates might behave similar to REV liposomes and also could magnify the problem by getting enmeshed between cells.

Thus, the drawbacks associated with REV liposomes could not be overcome by SUV liposomes. Hence the cell specific targeting of antibody coated liposomes could not be observed. Further experiments, perhaps with monoclonal antibodies, might shed some light on this aspect. The high specificity of monoclonals should permit them to be used in just microgram quantities in these type of experiments. As evident from many published reports, at such small protein concentration, the aggregation of liposomes could be completely eliminated.

SUMMARY AND CONCLUSION

SUMMARY AND CONCLUSION

Studies with triamcinolone acetonide liposomes

The original objective of this study was to evaluate the effect of charge, size and lipid composition of triamcinolone acetonide (TRMA) liposomes on drug disposition. However, when attempts were made to prepare liposomes containing TRMA, it became clear that the encapsulation of TRMA was very low. Different approaches, such as alteration in lipid composition, change in swelling medium or method of preparation, etc. were tried to improve the liposomal incorporation of TRMA. Despite these attempts it was virtually impossible to get the required 0.1% w/v concentration of TRMA incorporated into the liposomes. In the light of these findings, the objective of the project was changed to improving the liposomal incorporation of TRMA by chemical modification. Previous investigations (Shaw et al. 1976) indicated that attaching a lipophilic chain to a corticosteroid (cortisol) improved the efficiency of liposomal encapsulation. On this basis we decided to prepare and test the palmitate ester of TMRA. The synthesis of this compound, triamcinolone acetonide-21-palmitate (TRMA-P) has not been previously reported. To obtain a high yield of TRMA-21- palmitate, suitable synthesis and purification procedure were developed in our laboratory. The product of the synthesis was characterized by IR, NMR

and elemental analysis. Results of the encapsulation studies with TRMA-P demonstrate a great improvement in the incorporation brought about by palmitoylation. The palmitate derivative was almost completely taken up into the liposomes proving the hypothesis that palmitoylation could enhance the liposome encapsulation of drugs (Goundalkar and Mezei 1984).

Studies with methotrexate liposomes

The main purpose of these studies was to investigate the in vivo and in vitro anticancer effect of MTX entrapped in liposomes with or without covalently linked antitumor antibodies. The reverse phase evaporation (REV) method was adopted to achieve higher encapsulation of MTX within the liposomes. The sonication method was used to prepare small unilamellar MTX liposomes which were used only in interaction studies with cells. Entrapment studies with REV liposomes indicated maximum entrapment of MTX when the liposomes had a positive surface charge. Other characterization parameters that were tested for the REV liposomes included their size and drug release pattern. These liposomes were found to be quite stable even in the presence of fetal calf serum, presumably due to the high cholesterol concentration associated with them.

To prepare targeted liposomes, antibodies were linked to them covalently. The reactive PDP-derivative of a lipid, needed for this purpose, had to be synthesized as it was not

available commercially. Our attempts to synthesize PDP-PE and PDP-DPPE by reported methods failed to provide these derivatives in sufficient quantities. However, we have synthesized a new lipid derivative with a high yield, namely PDP-SA. The chemical structure of the PDP-SA was verified by IR, NMR and elemental analysis. Using PDP-SA, we investigated two different methods of covalent binding of antibodies to liposomes. We also tested ECDI method (Endoh et al. 1981) of binding of IgG to liposomes. Our results indicated high efficiency of binding only with the method of Barbet et al. (1981). The binding procedure did not cause any substantial reduction in the activity of the antibody when tested by radioimmunoassay (Goundalkar et al. 1984a). We, therefore, followed this procedure to prepare antibody coated liposomes for further tests.

The results of our interaction studies with human melanoma cells showed increased binding of MTX liposomes to cells when they were coated with antibodies. This was observed both with REV and SUV liposomes. The examination by microscopy of the samples stained by membrane immunofluorescence revealed that larger numbers of liposomes bound to melanoma cells when liposomes were linked to anti-melanoma IgG than when they were linked to non-specific IgG (Goundalkar et al. 1984b). However, such specific binding could not be quantitated by radio tracer technique

even with SUV liposomes. The failure to quantitate the specific binding of targeted liposomes has been attributed to such factors as low specificity of antibodies, the aggregating nature of liposomes and cells, etc. More refined studies, preferably using monoclonal antibodies, would be necessary to confirm the antibody mediated targeting of liposomes to cells *in vitro*.

In vitro cytostatic studies using three different cell lines, Caki-1, EL_4 lymphoma and melanoma (M_{21}), gave qualitatively similar results. MTX was less effective in inhibiting the growth of cancer cells when encapsulated in liposomes. The diminished cytostatic effect was attributed to the slow release of MTX from liposomes and also the inability of cells to take up the REV liposomes. Among the three types of liposomes tested, positively charged MTX liposomes appear to be superior to the other two types in their growth inhibitory effect. Coating the REV MTX liposomes with antitumor antibodies did not improve their *in vitro* performance. When tested against EL_4 cells, there was no difference in the cytostatic effect of MTX liposomes coated with anti-EL_4 IgG and normal rabbit IgG.

Mouse EL_4 lymphoma (ascites tumor) tumor model was used for the *in vivo* anticancer evaluation of MTX liposomes (Ghose *et al*. 1984). The route of administration was intraperitoneal. MTX, when incorporated in REV liposomes, exhibited high host toxicity while empty

liposomes alone or mixed with the solution of MTX did not show such toxicity. The toxicity was found to be dose dependent. At, or below, the dose level of 3.5 mg/kg no toxicity was observed and further in vivo studies were conducted at this dose level using positively charged MTX liposomes. Our results have shown a statistically significant increase in survival of tumor bearing mice when treated with positively charged MTX liposomes at the 3.5 mg/kg dose. Though statistically significant, this increase was only marginal. However, for reasons unknown, the toxicity reappeared when MTX liposomes were coated with antibodies, antitumor or otherwise, and it persisted even at the dose level of 3 mg/kg. There were a few long term survivors in mice treated with 3.5 mg MTX/kg of antibody liposome conjugates. No such long term survivors were present in mice treated with unconjugated MTX liposomes. However, over all, there was no statistical difference between treatment and controls in the experiments with antibody coated liposomes.

In conclusion, our studies did not show selectivity in the action of liposome encapsulated drug in cancer therapy. However, it would be inappropriate to extrapolate these findings to other drugs in general. The action of drugs encapsulated in liposomes is governed by several factors such as route of administration, properties unique to the

drug itself, specificity of the antibodies (in the case of targeted liposomes), properties of the liposomes, etc. Our *in vitro* and *in vivo* anticancer studies were done using relatively large REV liposomes and less specific polyclonal antibodies. Use of SUV and monoclonal antibodies may provide favorable results in this regard.

REFERENCES

REFERENCES

Abra RM, Bosworth ME, Hunt CA. Liposome disposition in vivo: effects of pre-dosing with liposomes. Res Commun Chem Pathol Pharmacol 1980; 29(2):349-360.

Abra RM, Hunt CA. Liposome disposition in vivo III. Dose and vesicle size effects. Biochim Biophys Acta 1981;666:493-503.

Abra RM, Hunt CA. Liposome disposition in vivo IV: The interaction of sequential doses of liposomes having different diameters. Res Commun Chem Pathol Pharmac 1982; 36(1):17-31.

Abra RM, Hunt CA, Fu KK, Peters JH. Delivery of therapeutic doses of doxorubicin to the mouse lung using lung accumulating liposomes proves unsuccessful. Cancer Chemother Pharmacol 1983; 11:98-101.

Abraham I, Goundalkar A, Mezei M. Effect of liposomal surface charge on the pharmacokinetics of an encapsulated model compound. Biopharm Drug Dispos 1984 (in press).

Adams DH, Joyce G, Richardson VJ, Ryman BE, Wisniewski HM. Liposome toxicity in the mouse central nervous system. J Neurolog Sci 1977; 31:173-179.

Allen TM, Cleland LG. Serum induced leakage of liposome contents. Biochim Biophys Acta 1980; 597:418-426.

Allen TM. A study of phospholipid interactions between high-density lipoproteins and small unilamellar vesicles. Biochim Biophys Acta 1981; 640: 385-397.

Allen TM, McAllister L, Mansolf S, Gyorffy E. Liposome-cell interactions. A study of the interactions of liposomes containing entrapped anti-cancer drugs with the EMT6, S49 and AE$_1$ (Transport-deficient) cell lines. Biochim Biophys Acta 1981; 643:346-362.

Allen TM, Everest JM. Effect of liposome size and drug release properties on pharmacokinetics of encapsulated drug in rats. J Pharmacol Exptl Therap 1983; 226(2):539-544.

Alpar OH, Bamford JB, Walters V. The in vitro incorporation and release of hydroxocobalamin by liposomes. Int J Pharm 1981; 7:349-351.

Alving CR, Richards RL. Immunologic aspects of liposomes. In: Ostro MJ ed. Liposomes. New York: Marcel Dekker, 1983:209-288.

Anonymous. Inpharma 1984; No. 421 (28 Jan):16-17.

Arakawa E, Imai Y, Kobayashi H, Okumura K, Sezaki H. Application of drug-containing liposomes to the duration of the intramuscular absorption of water soluble drugs in rats. Chem Pharm Bull 1975; 23(10):2218-2222.

Arrowsmith M, Hadgraft J, Kellaway IW. The in vitro release of steroids from liposomes. Int J Pharm 1983; 14:191-208.

Baldwin RW. Monoclonal antibodies for drug targeting in cancer therapy. Pharm Int 1983; 4(6):137-140.

Baldwin RW, Pimm MV. Antitumor monoclonal antibodies for radioimmunodetection of tumors and drug targeting. Cancer Metastasis Rev 1983; 2:89-106.

Bali A, Dhawan S, Gupta CM. Stability of liposomes in circulation is markedly enhanced by structural modification of their phospholipid component. FEBS Lett 1983; 154(2):373-377.

Bangham AD, Standish MM, Watkins JC. Diffusion of univalent ions across the lamellae of swollen phospholipids. J Mol Biol 1965; 13:238-252.

Bangham AD, Hill MW, Miller NGA. Preparation and use of liposomes as models of biological membranes. In: Korn ED, ed. Methods in Membrane Biology. Vol. 1 New York: Plenum press, 1974:1-68.

Banker GS, Anderson NR, Kildsig DO. The future of pharmacotherapeutics; trends in drugs, dosage forms and drug delivery. Pharm Int 1983; 4(1):9-12.

Barbet J, Machy P, Leserman LD. Monoclonal antibody covalently couplied to liposomes: Specific targeting to cells. J. Suptramolec Struct Cell Biochem 1981; 16:243-258.

Barenholz Y, Gibbes D, Litman BJ, Goll J, Thompson TE, Carlson FD. A simple method for the preparation of homogeneous phospholipid vesicles. Biochemistry 1977; 16(12):2806-2810.

Barza M, Baum J, Szoka F Jr. Pharmacokientics of subconjunctival liposome encapsulated gentamycin in normal rabbit eyes. Investig Ophthalmol Vis Sci 1984; 25:486-490.

Batzri S, Korn ED. Single bilayer liposomes prepared without sonication. Biochim Biophys Acta 1973; 298:1015-1019.

Bauminger S, Wilchek M. The use of carbodiimides in the preparation of immunizing conjugates. In: Colowick SP, Kaplan NO, eds. Methods in Enzymology Vol. 70, New York: Academic press, 1980:151-159.

Beaumier PL, Hwang KJ, Slattery JT. Effect of liposome dose on the elimination of small unilamellar sphingomyelin/-cholesterol vesicles from the circulation. Res Commun Chem Pathol Pharmacol 1983; 39(2):277-289.

Blair AH, Ghose TI. Linkage of cytotoxic agents to immunoglobulins. J Immunol Methods 1983; 59:129-143.

Bonventre PF, Gregoriadis G. Killing of intraphagecytic Staphalococcus aureus by Dihydrostreptomycin entrapped within liposomes. Antimicrob Agents Chemother 1978; 13(6): 1049-1051.

Bosworth ME, Hunt CA. Liposome disposition in vivo II: Dose dependency. J Pharm Sci 1982; 71(1):100-104.

Bosworth ME, Hunt CA, Pratt D. Liposome dialysis for improved size distributions. J Pharm Sci 1982; 71(7):806-812.

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72:248-354.

Bragman KS, Heath TD, Papahadjopoulos D. Simultaneous interaction of monoclonal antibody-targeted liposomes with two receptors on K562 cells. Biochim Biophys Acta 1983; 730(2):187-195.

Breithaupt H, Kuenzlen E, Goebel G. Rapid High Pressure Liquid Chromatographic Determination of Methotrexate and its metabolites 7-Hydroxymethotrexate and 2,4-Diamino-N^{10}-methylpteroic acid in biological fluids. Anal Biochem 1982; 121:103-113.

Brunner J, Skrabal P, Hauser H. Single bilayer vesicles prepared without sonication. Physicochemical properties. Biochim Biophys Acta 1976; 455:322-331.

Burdette WJ, Gehan EA. Planning and analysis of clinical studies, Springfield, Illinois: Charles C. Thomas, 1970:72-78.

Buschl R, Ringsdorf H, Zimmermann U. Electric field induced fusion of large liposomes from natural and polymerizable lipids. FEBS Lett 1982; 150(1):38-42.

Carlsson J, Drevin H, Axen R. Protein thiolation and reversible protein-protein conjugation. N-succinimidyl 3-(2-pyridyldithio) propionate, a new heterobifunctional reagent. Biochem J 1978; 173:723-737.

Carraway KL, Koshland DE Jr. Carbodiimide modification of proteins. In: Colowick SP, Kaplan NO. eds. Methods in Enzymology Vol. 25, New York: Academic press, 1972:617-623.

Chabner BA, Young RC. Threshold methotrexate concentration for in vivo inhibition of DNA synthesis in normal and tumorous target tissues. J Clin Invest 1973; 52:1804-1811.

Chabner, Cowan, Ozols, Myers. Overcoming drug resistance in the treatment of cancer. Am Pharm 1984; NS 24(4):12-14.

Chander A, Claypool WD Jr, Strauss III JF, Fisher AB. Uptake of liposomal phosphatidylcholine by granular pneumocytes in primary culture. Am J Physiol 1983; 245(5):C397-C404.

Chapman WL Jr, Hanson WL, Alving CR, Hendricks LD. Antileishmanial activity of liposome encapsulated meglumine antimonate in the dog. Am J Vet Res 1984; 45(5):1028-1030.

Chard T. An introduction to Radioimmunoassay and related techniques. In: Work TS, Work [redacted] eds. Laboratory Techniques in Biochemistry and Molecular Biology, Vol. 6, Part 2, Amsterdam: Elsevier Biomedical Press, 1982:51-71.

Chen C, Schullery SE. Gel filtration of egg phosphatidylcholine vesicles. J Biochem Biophys Methods 1979; 1:189-192.

Chen ML, McGuire WP, Lad TE, Chiou WL. A specific HPLC assay to determine the pharmacokinetics of methotrexate in patients. Int J Clin Pharmacol Ther Toxicol 1984; 22(1):1-6.

Conner CS. Therapy with monoclonal antibodies. Drug Intell Clin Pharm 1984; 18(1):65-66.

Coune A, Sculier JP, Fruhling J et al. IV administration of a water-insoluble antimitotic compound entrapped in liposomes. Preliminary report on infusion of large volumes of liposomes. Preliminary report on infusion of large volumes of liposomes to man. Cancer Treat Reports 1983; 67(11):1031-1033.

Counsell RE, Pohland RC. Lipoproteins as potential site-specific delivery systems for diagnostic and therapeutic agents. J Med Chem 1982; 25(10):1115-1120.

Crommelin DJA, Slaats N, van Bloois L. Preparation and characterization of doxorubicin containing liposomes: I. Influence of liposome charge and pH of hydration medium on loading capacity and particle size. Int J Pharm 1983; 16:79-92.

Damen J, Dijkstra J, Regts J, Scherphof G. Effect of lipoprotein-free plasma on the interaction of human plasma high density lipoprotein with egg yolk phosphatidylcholine liposomes. Biochim Biophys Acta 1980; 620:90-99.

Dapergolas G, Gregoriadis G. Hypoglycemic effect of liposome-entrapped insulin administered intragastrically into rats. Lancet 1976; II, Part 2 (7990):824-827.

Davis SS, Illum L. Drug delivery systems. Practitioner 1983; 227:1537-1543.

Deamer D, Bangham AD. Large volume liposomes by an ether vaporization method. Biochim Biophys Acta 1976; 443:629-634.

Deamer DW. Preparation and properties of ether-injection liposomes. Ann NY Acad Sci 1978; 308:250-258.

de Duve C. Lysosomotropic agents as drugs and carriers for drugs. In: Bearn AG, ed. Better therapy with existing drugs: New uses and delivery systems. Rahway, New Jersey: Merck Sharp and Dohme International, A division of Merck and Co., Inc. 1981:119-132.

Defrise-Quertain F, Chatelain P, Ruysschaert, Delmelle M. Spin label partitioning in lipid vesicles. A model study for drug encapsulation. Biochim Biophys Acta 1980; 628:57-68.

Deliconstantinos G, Ramantanis G, Todorou DK. Interaction of ^{99m}TC-labelled liposomes with Walker tumor cells *in vitro*. Liposome mediated introduction of thaliblastine into resistant Walker tumor cells. Gen Pharmac 1983; 14(4):407-411.

Deshmukh DS, Bear WD, Brockerhoff H. Can intact liposomes be absorbed in the gut? Life Sci 1981; 28:239-242.

Desiderio JV, Campbell SG. Intraphagocytic killing of *Salmonella typhimurium* by liposome-encapsulated cephalothin. J Infect Dis 1983; 148(3):563-570.

Desnick RJ, Fiddler MB, Douglas SD, Hudson LDS. Enzyume therapy XI: Immunologic considerations for replacement therapy with unentrapped, erythrocyte- and liposome entrapped enzymes. In: Gatt S, Fraysz L, Mandel P eds. Adv Exp Med Biol Vol. 101, New York: Plenum press, 1978:753-764.

Diamanti E, Bianchi GE. New esters of triamcinolone acetonide for topical antiphlogistic use. Arzneim Forsch (Drug Res) 1971; 21(2):251-252.

Dimitriadis G. Introduction of ribonucleic acids into cells by means of liposomes. Nucleic Acids Res 1978; 5(4):1381-1386.

Dingle JT, Gordon JL, Hazleman BL *et al*. Novel treatment for joint inflammation. Nature 1978; 271:372-373.

Dunnick JK, McDougall IR, Aragon S, Goris ML, Kriss JP. Vesicle interactions with polyamino acids and antibody: *in vitro* and *in vivo* studies. J Nucl Med 1975; 16(6):483-487.

Duzgunes N, Wilschut J, Hong K et al. Physicochemical characterization of large unilamellar phospholipid vesicles prepared by reverse-phase evaporation. Biochim Biophys Acta 1983; 732(1):289-299.

Ellens H, Morsett H, Scherphof G. In vivo fate of large unilamellar sphingomyelin-cholesterol liposomes after peritoneal and intravenous injection into rats. Biochim Biophys Acta 1981; 674:10-18.

Endoh H, Suzuki Y, Hashimoto Y. Antibody coating of liposomes with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and the effect on target specificity. J Immunol Methods 1981; 44:79-85.

Enoch HG, Strittmatter P. Formation and properties of 1000 A° diameter, single bilayer phospholipid vesicles. Proc Natl Acad Sci USA 1979; 76(1):145-149.

Erlanger BF. The preparation of antigenic hapten-carrier conjugates: a survey. In: Colowick SP, Kaplan NO eds. Methods in Enzymology vol. 70, New York: Academic press, 1980:85-105.

Eytan GD, Broza R, Notsani B-E, Dachir D, Gad AE. Interactions of acidic liposomes with red blood cells. Induction of endocytosis and shedding of particles. Biochim Biophys Acta 1982; 689:464-474.

Fendler JH, Romero A. Liposomes as drug carriers. Life Sci 1977; 20:1109-1120.

Fendler JH. Optimizing Drug Entrapment in Liposomes. Chemical and Biophysical Considerations. In: Gregoriadis G, Allison AC, eds. Liposomes in Biological Systems. New York: John Wiley and Sons, 1980:87-98.

Fendler JH. Membrane Mimetic Chemistry. Chem Eng N 1984; 62(1):25-38.

Fernandez MS. Determination of surface potential in liposomes. Biochim Biophys Acta 1981; 646:23-26.

Fidler IJ; Raz A, Fogler WE, Poste G. Pulmonary localization of intravenously injected liposomes. Recent Results Cancer Res 1980; 75(2):246-251.

Fildes FJT, Oliver JE. Interaction of cortisol-21-palmitate with liposomes examined by differential scanning calorimetry. J Pharm Pharmacol 1978; 30:337-342.

Firth G, Oliver AS, McKeran RO. Studies on the use of antimitotic drugs entrapped within liposomes and of their action on a human glioma cell line. J Neurol Sci 1984; 63:153-165.

Florey K. Triamcinolone acetonide. In: Florey K ed. Analytical Profiles of Drug substances. Vol. 1; New York: Academic Press, 1972:397-421.

Florey K. Methotrexate. In: Florey K. ed. Analytical Profiles of Drug substances Vol. 5, New York: Academic press, 1976:283-305.

Foong WC, Green KL. Pharmacokinetics of liposome entrapped methotrexate injected into arthritic joints. J Pharm Pharmacol 1983; 35:84P.

Forssen EA, Tokes ZA. Use of anionic liposomes for the reduction of chronic doxorubicin-induced cardiotoxicity. Proc Natl Acad Sci USA 1981; 78(3):1873-1877.

Forssen EA, Tokes ZA. Attenuation of dermal toxicity of doxorubicin by liposome encapsulation. Cancer Treat Reports 1983a; 67(5):481-483.

Forssen EA, Tokes ZA. Improved therapeutics benefits of doxorubicin by entrapment in anionic liposomes. Cancer Res 1983b; 43:546-550.

Fountain MW, Chiovetti R Jr, Kercret H, Parrish DO, Segrest JP. Liposome-cell interactions. A rapid assay for cells in suspension culture. Biochim Biophys Acta 1980a; 596:420-425.

Fountain MW, Pedersoli WM, Ganjam VK, Ravis WR. Phosphatidylcholine-Digoxin liposome drug delivery vehicle: An evaluation of the intramuscular route of administration in dogs. Curr Ther Res 1980b; 28(4):558-561.

Fraley R, Subramani S, Berg P, Papahadjopoulos D. Introduction of liposome-encapsulated SV_{40} DNA into cells. J Biol Chem 1980; 255(21):10431-10435.

Fraley R, Papahadjopoulos D. New generation liposomes: the engineering of an efficient vehicle for intracellular delivery of nucleic acids. TIBS 1981; 6(3):77-80.

Freise J, Schmidt FW, Magerstedt P. Effect of liposome entrapped methotrexate on Ehrlich ascites tumor cells and uptake in primary liver cell tumor. J Cancer Res Clin Oncol 1979; 94:21-27.

Freise J, Muller WH, Brolsch C, Schmidt FW. In vivo distribution of liposomes between parenchymal and non parenchymal cells in rat liver. Biomedicine 1980; 32:118-123.

Frokjaer S, Hjorth EL, Worts O. Stability and storage of liposomes. In: Bundgaard H, Hansen AB, Kofod H eds. Optimization of drug delivery. Alfred Benzon Symposium 17. Munksgaard, Copenhagen 1982:384-401.

Fukuzawa K, Tokumura A, Ouchi S, Tsukatani H. Antioxidant activities of tocopherols on Fe^{2+}-ascorbate induced lipid peroxidation in lecithin liposomes. Lipids 1982; 17(7):511-513.

Ganapathi R, Krishan A. Effect of cholesterol content of liposomes on the encapsulation, efflux and toxicity of adriamycin. Biochem Pharmacol 1984; 33(4):698-700.

Gasiorowski-Mauroy ME. Topical steroids in the treatment of common dermatoses. Copmpr Ther 1981; 7(1):8-12.

Ghose T, Blair AH. Antibody linked cytotoxic agents in the treatment of cancer: current status and future prospects. J Natl Cancer Inst 1978; 61(3):657-676.

Ghose TI, Blair AH, Kulkarni PN. Preparation of antibody-linked cytotoxic agents. In: Colowick SP, Kaplan NO, eds. Methods in Enzymology Vol. 93 New York: Academic Press, 1983:280-333.

Ghose T, Blair AH, Uadia P, Kulkarni PN, Goundalkar A, Mezei M, Ferrone S. Antibodies as carriers of cancer chemotherapeutic agents. Ann NY Acad Sci 1984 (in press).

Godfrey W, Doe B, Wallace EF, Bredt B, Wofsy L. Affinity targeting of membrane vesicles to cell surfaces. Exp Cell Res 1981; 135:137-145.

Goldmacher VS. Immobilization of protein molecules on liposomes: Anchorage by artificially bound unsaturated hydrocarbon tails. Biochem Pharmacol 1983; 32(7):1207-1210.

Goll J, Carlson FD. Photon correlation spectroscopic study of the size distribution of phospholipid vesicles. Biophy J 1982; 38(1):7-13.

Goundalkar A, Mezei A. Chemical modification of triamcinolone acetonide to improve liposomal encapsulation. J Pharm Sci 1984; 73(4):834-835.

Goundalkar A, Ghose T, Mezei M. Covalent binding of antibodies to liposomes using a novel lipid derivative. J Pharm Pharmacol 1984a; 36:465-466.

Goundalkar A, Mezei M, Ghose T. Preparation of targeted liposomes and their interaction with human melanoma cells. (Abstract) Clin Biochem 1984b; 17:209.

Guey-Shuang WU, Stein RA, Mead JF. Autoxidation of phosphatidylcholine liposomes. Lipids 1982; 17(6):403-413.

Gulasekharam V. Liposomes for selective delivery of drugs in topical routes of administration. [Master's Thesis]. Halifax, Nova Scotia: Dalhousie University, 1980.

Guo LSS, Hamilton RL, Goerke J, Weinstein JN, Havel RJ. Interaction of unilamellar liposomes with serum lipoproteins and apolipoproteins. J Lipid Res 1980; 21:993-1003.

Green KL, Foong FC. The stability of methotrexate liposomes in rheumatoid synovial fluid. J Pharm Pharmacol 1983; 35:93P.

Gregoriadis G, Swain CP, Wills EJ, Tavill AS. Drug-carrier potential of liposomes in cancer chemotherapy. Lancet 1974; I, Part 2 (7870):1313-1316.

Gregoriadis G, Neerunjun ED. Homing of liposomes to target cells. Biochem Biophys Res Comm 1975; 65(2):537-544.

Gregoriadis G. The carrier potential of liposomes in biology and medicine. New Eng J Med 1976; 295(13):704-709.

Gregoriadis G. Liposomes. In: Gregoriadis G. ed. Drug Carreirs in Biology and Medicine. New York: Academic Press, 1979:287-341.

Gregoriadis G, Davis C. Stability of liposomes *in vivo* and *in vitro* is promoted by their cholesterol content and the presence of blood cells. Biochem Biophys Res Commun 1979; 89(4):1287-1293.

Gregoriadis G, Allison AC eds. Liposomes in Biological Systems. New York: John Wiley and Sons, 1980.

Gregoriadis G, Senior J. The phospholipid component of small unilamellar liposomes controls the rate of clearance of entrapped solutes from the circulation. FEBS Lett 1980; 119(1):43-46.

Gregoriadis G. Targeting of drugs: Implications in medicine. Lancet 1981; II, Part 1(8240) 1:241-247.

Gregoriadis G. Use of monoclonal antibodies and liposomes to improve drug delivery. Present status and future implications. Drugs 1982; 24(4):261-266.

Gregoriadis G, Senior J, Trouet A. eds. Targeting of drugs. New York: Plenum Press, 1982.

Gregoriadis G. Liposomes as drug carriers. Pharm Int 1983a; 4(2):33-37.

Gregoriadis G. Targeting of drugs with molecules, cells and liposomes. TIPS 1983b; 4:304-307.

Gregoriadis G, Kirby C, Senior J. Optimization of liposome behaviour *in vivo*. Biol Cell 1983; 47(1):11-18.

Gregoriadis G. ed. Liposome Technology. Vol. I-III, Florida:CRC Press, 1984.

Hallam NF. The use and abuse of topical corticosteroids in dermatology. Scott Med J 1980; 25:287-291.

Hamilton RL Jr., Goerke J, Guo LSS, Williams MC, Havel RJ. Unilamellar liposomes made with the French pressure cell: a simple preparative and semiquantitative technique. J Lip Res 1980: 21:981-992.

Hansch C, Leo A. Substituent constants for correlation analysis in chemistry and biology. New York: Wiley, 1979:19,315.

Harsch M, Walther P, Weder HG, Hengartner H. Targeting of monoclonal antibody-coated liposomes to sheep red blood cells. Biochem Biophys Res Commun 1981; 103(3):1069-1076.

Hashimoto Y, Sugawara M, Musuko T, Hojo H. Antitumor effect of actinomycin D entrapped in liposomes bearing subunits of tumor-specific monoclonal immunoglobulin M antibody. Cancer Res 1983a; 43:5328-5334.

Hashimoto Y, Sugawara M, Endoh H. Coating of liposomes with subunits of monoclonal IgM antibody and targeting of the liposomes. J Immunol Methods 1983b; 62:155-162.

Hauser H. Methods of preparation of lipid vesicles: assessment of their suitability for drug encapsulation. TIPS 1982; 3(7):274-277.

Heath TD, Fraley RT, Papahadjopoulos D. Antibody targeting of liposomes: Cell specificity obtained by conjugation of $F(ab')_2$ to vesicle surface. Science 1980; 210:539-541.

Heath TD, Macher BA, Papahadjopoulos D. Covalent attachment of immunoglobulins to liposomes via glycosphingolipids. Biochim Biophys Acta 1981; 640:66-81.

Heath TD, Montgomery JA, Piper JR, Papahadjopoulos D. Antibody targeted liposomes: Increase in specific toxicity of methotrexate-Y-aspartate. Proc Natl Acad Sci USA 1983; 80:1377-1381.

Heath TD, Fraley RT, Bentz J, Voss EW Jr., Herron JN, Papahadjopoulos D. Antibody directed liposomes. Determination of affinity constants for soluble and liposome bound antifluorescein. Biochim Biophys Acta 1984; 770:148-158.

Hemker HC, Mubler AD, Hermens WT, Zwaal RFA. Oral treatment of hemophilia A by gastrointestinal absorption of factor VIII entrapped in liposomes. Lancet 1980; I, Part 1 (8159):70-71.

Henderson NL. Recent advances in drug delivery system technology. In: Hess H-J ed. Annual reports in medicinal chemistry vol. 18, New York: Academic Press, 1983:275-284.

Herrmann SH, Mescher MF. Lymphocyte recognition of H-2 antigen in liposomes. J Supramolec Struct Cell Biochem 1981; 16:121-131.

Hisaoka M, Tsukada K, Morioka T. Studies on liposome-encapsulated carboquone. III Enhancement of lymphatic transport of carboquone by encapsulation. J Pharm Dyn 1982a; 5:27-33.

Hisaoka M, Tsukada K, Morioka T, Inomata T, Arakawa M. Studies on liposome-encapsulated carboquone. IV Enhancement of antitumor activity of carboquone against Ehrlich ascites carcinoma by encapsulation. J Pharm Dyn 1982b; 5:34-39.

Hong K, Friend DS, Glabe CG, Papahadjopoulos D. Liposomes containing colloidal gold are a useful probe of liposome-cell interactions. Biochim Biophys Acta 1983; 732:320-323.

Huang C. Studies on phosphatidylcholine vesicles. Formation and physical characteristics. Biochemistry 1969; 8(1):344-352.

Huang C, Thompson TE. Preparation of Homogeneous, Single-walled phosphatidylcholine vesicles. In: Fleischer S, Packer L, eds. Biomembranes. New York: Academic Press, 1984:485-489 (Colowick SP, Kaplan NO, eds. Methods in Enzymology, vol. 32).

Huang A, Huang L, Kennel SJ. Monoclonal antibody covalently coupled with fatty acid. J Biol Chem 1980; 255(17):8015-8018.

Huang A, Kennel SJ, Huang L. Immunoliposome labeling: A sensitive and specific method for cell surface labeling. J Immunol Methods 1981; 46(2):141-151.

Huang A, Tsao YS, Kennel SJ, Huang L. Characterization of antibody covalently coupled to liposomes. Biochim Biophys Acta 1982; 716:140-150.

Hudson L, Hay FC. Practical Immunology, 2nd ed. Oxford: Blackwell Scientific Publications, 1980:94-98.

Hunt CA, Tsang S. α-Tocopherol retards autoxidation and prolongs the shelf life of liposomes. Int J Pharm 1981; 8:101-110.

Hunt CA. Liposome disposition *in vivo* V. Liposome stability in plasma and implications for drug carrier function. Biochim Biophys Acta 1982; 719:450-463.

Hwang KJ, Luk K-FS, Beaumier PL. Hepatic uptake and degradation of unilamellar sphingomyelin/cholesterol liposomes: A kinetic study. Proc Natl Acad Sci USA 1980; 77(7):4030-4034.

Idson B. New opportunities for skin care ingredients. Drug Cosmet Ind 1981; 128(1):36-37, 90.

Illum L, Davis SS. The targeting of drugs parenterally by use of microspheres. J Parent Sci Tech 1982; 36(6):242-248.

Illum L, Jones PDE, Kreuter J, Baldwin RW, Davis SS. Adsorption of monoclonal antibodies to polyhexylcyanoacrylate nanoparticles and subsequent immunospecific binding to tumor cells *in vitro*. Int J Pharm 1983; 17:65-76.

Jansons VK, Mallett PL. Targeted liposomes: A method for preparation and analysis. Anal Biochem 1981; 111:54-59.

Jansons VK, Panzner EA. Liposomes as a means to introduce fragment A of diphtheria toxin into cells. Biochim Biophys Acta 1983; 735:433-437.

Jay M, Digenis G. Enhanced entrappment of a quaternary ammonium compound in liposomes by ion-pairing. J Pharm Sci 1982; 71(8):958-960.

Jederstrom G, Russell G. Size exclusion chromatography of liposomes on different gel media. J Pharm Sci 1981; 70(8):874-878.

Johnson HM, Brenner K, Hall HE. The use of a water soluble carbodiimide as a coupling reagent in the passive hemagglutination test. J Immunol 1966; 97(6):791-796.

Jolivet J, Cowan KH, Curt GA, Clendeninn NJ, Chabner BA. The pharmacology and clinical use of methotrexate. New Engl J Med 1983; 309(18):1094-1104.

Jones NC, Osborn MJ. Interaction of *Salmonella typhimurium* with phospholipid vesicles. Incorporation of exogenous lipids into intact cells. J Biol Chem 1977; 252(20):7398-7404.

Jones WN, Barreuther AD. Corticosteroids: When and How to use them. Drug Ther 1981; 11:135-139, 143-144.

Jou Y-H, Jarlinksi S, Mayhew E, Bankert RB. Use of heterobifunctional cross-linking reagents to prepare antibody-liposome conjugates: Development of methods to reduce liposome aggregation during conjugation. (Abstract) Fed Proc 1984; 43(7):1971.

Jou Y-H, Mazzaferro K, Mayers GL, Bankert RB. Methods for the attachment of haptens and proteins to erythrocytes. In: Colowick SP, Kaplan NO eds. Methods in Enzymology vol. 92, New York: Academic Press, 1983:257-276.

Juliano RL, Layton D. Liposomes as a drug delivery system. In: Juliano RL ed. Drug delivery systems, New York, Oxford University Press, 1980: Chap 6.

Juliano RL. Liposomes as a drug delivery system. TIPS 1981; 2(2):39-42.

Juliano RL, Hsu MJ, Peterson D, Regen SL, Singh A. Interactions of conventional or photopolymerized liposomes with platelets in vitro. Exp Cell Res 1983a; 146:422-427.

Juliano R, Lopez-Berenstein G, Mehta R, Hopfer R, Mehta K, Kasi L. Pharmacokinetic and therapeutic consequences of liposomal drug delivery: Fluorodeoxyuridine and Amphotericin B as examples. Biol Cell 1983b; 47:39-46.

Juliano RL, Hsu MJ, Regen SL, Singh M. Photopolymerized phospholipid vesicles. Stability and retention of hydrophilic and hydrophobic marker substances. Biochim Biophys Acta 1984; 770:109-114.

Kao YJ, Loo TL. Pharmacological disposition of negatively charged phospholipid vesicles in rats. J Pharm Sci 1980; 69(11):1338-1340.

Kano K, Fendler JH. Enhanced uptake of drugs in liposomes: use of labile vitamin B_{12} complexes of 6-mercaptopurine and 8-azaguanine. Life Sci 1977; 20: 1729-1734.

Kaye SB. Liposomes-Problems and promise as selective drug carriers. Cancer Treat Rev 1981; 8:27-50.

Kaye SB, Boden JA, Ryman BE. The effect of liposome (phospholipid vesicle) entrappment of actinomycin D and methotrexate on the *in vivo* treatment of sensitive and resistant solid murine tumors. Eur J Cancer 1981; 17:279-289.

Kedar A, Mayhew E, Moore RH, Williams P, Murphy GP. Effect of actinomycin D containing lipid vesicles on murine renal adenocarcinoma. J Surg Oncol 1980; 15:363-365.

Kellaway IW, Hadgraft J, Ahmed M *et al*. Liposomes-model membranes for modern medicine. Manuf Chem Aerosol News 1980a; 51(8):43-44.

Kellaway IW, Hadgraft J, Ahmed M *et al*. Liposomes-model membranes for modern medicine. Manuf Chem Aerosol News 1980b; 51(9):33-34.

Khato J, del Campo AA, Sieber SM. Carrier activity of sonicated small liposomes containing melphalan to regional lymph nodes of rats. Pharmacology 1983; 26:230-240.

Kim S, Martin GM. Preparation of cell-size unilamellar liposomes with high captured volume and defined size distribution. Biochim Biophys Acta 1981; 646:1-9.

Kim S, Turker MS, Chi EY, Sela S, Martin GM. Preparation of multivesicular liposomes. Biochim Biophys Acta 1983; 728:339-348.

Kimelberg HK, Mayhew EG. Properties and biological effects of liposomes and their uses in Pharmacology and Toxicology. In: Goldberg L, ed. CRC critical Reviews in Toxicology. Cleveland, Ohio: CRC Press, 1978:25-29.

Kimura T, Yoshikawa M, Yasuhara M, Sezaki H. The use of liposomes as a model for drug absorption: -lactam antibodies. J Pharm Pharmacol 1980; 32:394-398.

Kirby C, Gregoriadis G. The effect of the cholesterol content of small unilamellar liposomes on the fate of their lipid components *in vivo*. Life Sci 1980; 27:2223-2230.

Kirby C, Clarke J, Gregoriadis G. Effect of the cholesterol content of small unilamellar liposomes on their stability *in vivo* and *in vitro*. Biochem J 1980a; 186:591-598.

Kirby C, Clarke J, Gregoriadis G. Cholesterol content of small unilamellar liposomes controls phospholipid loss to high density lipoproteins in the presence of serum. FEBS Lett 1980b; 111(2):324-328.

Kirby C, Gregoriadis G. The effect of lipid composition of small unilamellar liposomes containing melphalan and vincristine on drug clearance after injection into mice. Biochem Pharmacol 1983; 32(4):609-615.

Kirkland JJ, Yau WW, Szoka FC. Sedimentation field flow fractionation of liposomes. Science 1982; 215:296-298.

Kosloski MJ, Rosen F, Milholland RJ, Papahadjopoulos D. Effect of lipid vesicle (liposome) encapsulation of methotrexate on its chemotherapeutic efficacy in solid rodent tumors. Cancer Res 1978; 38:2848-2853.

Kuhn SH, Gemperli B, Shephard EG et al. Interactions of liposomes with human leukocytes in while blood. Biochim Biophys Acta 1983; 762:119-127.

Kulkarni PN, Blair AH, Ghose TI. Covalent binding of methotrexate to immunoglobulins and the effect of antibody-linked drug on tumor growth, in vivo. Cancer Res 1981; 41:2700-2706.

Kurosaki Y, Kimura T, Muranishi S, Sezaki H. The use of liposomes as enzyme carriers. I. Dependence of Enzyme stability on the method of preparation. Chem Pharm Bull 1981; 29(4):1175-1178.

Knight CG, ed. Liposomes: From physical structure to therapeutic applications. New York: Elsevier Biomedical Press, 1981a.

Knight CG. Hydrophobic prodrugs in liposomes. In: Knight CG, ed. Liposomes: From physical structure to therapeutic applications. New York: Elsevier Biomedical Press, 1981b:381-389.

Kremer JMH, Esker MWJvd, Pathmamanoharan C, Wiersema PH. Vesicles of variable diameter prepared by a modified injection method. Biochemistry 1977; 16(17):3932-3935.

Kreuter J. Evaluation of nanoparticles as drug-delivery systems I: Preparation methods. Pharm Acta Helv 1983a; 58(7):196-209.

Kreuter J. Evaluation of nanoparticles as drug-delivery systems II: comparison of the body distribution of nanoparticles with the body distribution of microspheres (diameter >1 μm), liposomes and emulsions. Pharm Acta Helv 1983b; 58(8):217-226.

Kreuter J. Evaluation of nanoparticles as drug-delivery systems III: materials, stability, toxicity, possibilities of targeting and use. Pharm Acta Helv 1983C; 58(9-10):242-250.

Laduron C, Coune A, Atassi G et al. Chemotherapeutic efficacy of nocodazole encapsulated in liposomes on L1210 murine leukemia. Res Commun Chem Pathol Pharmacol 1983; 39(3):419-436.

Large P, Gregoriadis G. Phospholipid composition of small unilamellar liposomes containing melphalan influences drug action in mice bearing PC6 tumors. Biochem Pharmacol 1983; 32(7):1315-1318.

Leaver J, Alonso A, Durrani AA, Chapman D. The physical properties and photopolymerization of diacetylene containing phospholipid liposomes. Biochim Biophys Acta 1983; 732:210-218.

Lelkes PI, Tandeter HB. Studies on the methodology of the carboxyfluoprescein assay and on the mechanism of liposome stabilization by red blood cells in vitro. Biochim Biophys Acta 1982; 716:410-419.

Leserman LD, Weinstein JN, Blumenthal R, Sharrow SO, Terry WD. Binding of antigen-bearing fluorescent liposomes to the murine myeloma tumor MOPC 315. J Immunol 1979; 122(2):585-591.

Leserman LD, Barbet J, Kourilsky F. Targeting to cells fluorescent liposomes covalently coupled with monoclonal antibody or protein A. Nature 1980a; 288:602-604.

Leserman LD, Weinstein JN, Moore JJ, Terry WD. Specific interaction of myeloma tumor cells with hapten bearing liposomes containing methotrexate and carboxyfluorescein. Cancer Res 1980b; 40:4768-4774.

Leserman LD, Machy P, Barbet J. Cell specific drug transfer from liposomes bearing monoclonal antibodies. Nature 1981; 293:226-228.

Leserman LD, Machy P, Devaux C, Barbet J. Antibody bearing liposomes: Targeting in vivo. Biol Cell 1983; 47:111-116.

Levy R, Miller RA. Tumor therapy with monoclonal antibodies. Fed Proc 1983; 42(9):2650-2656.

Lichtenberg D, Markello T. Structural characteristics of phospholipid multilamellar liposomes. J Pharm Sci 1984; 73(1):122-125.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193:265-275.

Lutz D. Immunotherapy of cancer: a critical review. Int J Clin Pharmacol Ther Toxicol 1983; 21(3):118-129.

Machy P, Pierres M, Barbet J, Leserman LD. Drug transfer into lymphoblasts mediated by liposomes bound to distinct sites on H-2 encoded I-A, I-E and K molecules. J Immunol 1982a; 129(5):2098-2102.

Machy P, Barbet J, Leserman LD. Differential endocytosis of T and B lymphocyte surface molecules evaluated with antibody-bearing fluorescent liposomes containing methotrexate. Proc Natl Acad Sci USA 1982b; 79:4148-4152.

Machy P, Leserman LD. Small liposomes are better than large liposomes for specific drug delivery in vitro. Biochim Biophys Acta 1983; 730:313-320.

Magee WE, Ristow SS. Targeting to lymphoid cells of the immune net work. Pharmac Ther 1983; 21:295-323.

Margolis LB, Namiot VA, Kljukin LM. Magnetoliposomes: Another principle of cell sorting. Biochim Biophys Acta 1983; 735:193-195.

Martin FJ, Hubbell WL, Papadjopoulos D. Immunospecific targeting of liposomes to cells: A novel and efficient method for covalent attachment of Fab' fragments via disulfide bonds. Biochemistry 1981; 20(14):4229-4238.

Martin WJ, Ballentine R. Patient education on cancer chemotherapeutic agents. Am Pharm 1983; NS23(10):32-37.

Mathe G, Loc T, Bernard J. Effect sur la leucemie L1210 de la souris d'une combinaison par diazotation d'A-methopterine et de gamma-globulines de hamsters porteur de cette leucemie par heterogreffe. Comptes Rendues 1958; 246:1626-1628.

Matthay KK, heath TD, Papahadjopoulos D. Specific enhancement of drug delivery to AKR lymphoma by antibody-targeted small unilamellar vesicles. Cancer Res 1984; 44:1880-1886.

Mauk MR, Gamble RC, Baldeschwieler JD. Targeting of lipid vesicles: Specificity of carbohydrate receptor analogues for leukocytes in mice. Proc Natl Acad Sci USA 1980; 77(8):4430-4434.

Mayhew E, Papahadjopoulos D, Rustum YM et al. Inhibition of tumor cell growth in vitro and in vivo by 1- -D-Arabinofuranosylcytosine entrapped within phospholipid vesicles. Cancer Res 1976; 36:4406-4411.

Mayhew E, Papahadjopoulos D. Therapeutic applications of liposomes. In: Ostro MJ, ed. Liposomes, New York: Marcel Dekker, 1983:289-341.

Mayhew E, Rustum Y. Effect of liposome entrapped chemotherapeutic agents on mouse primary and metastatic tumors. Biol Cell 1983; 47:81-86.

McDougall IR. Liposomes as diagnostic tools. In: Gregoriadis G, Allison AC eds. Liposomes in Biological Systems, New York: John Wiley, 1980; 325-344.

Megaw JM, Takei Y, Lerman S. Lectin mediated binding of liposomes to the ocular lens. Exp Eye Res 1981; 32:395-405.

Mehta R, Lopez-Berestein G, Hopfer R, Mills K, Juliano RL. Liposomal amphotericin B is toxic to fungal cells but not to mammalian cells. Biochim Biophys Acta 1984; 770:230-234.

Meyer MC, Guttman DE. Novel method for studying protein binding. J Pharm Sci 1968; 57(9):1627-1629.

Meyer MC, Guttman DE. Dynamic dialysis as a method for studying protein binding. I: Factors affecting the kinetics of dialysis through a cellphane membrane. J Pharm Sci 1970; 59(1):33-38.

Mezei M, Gulasekharam V. Liposomes - a selective drug delivery system for the topical route of administration I. Lotion dosage form. Life Sci 1980; 26: 1473-1477.

Mezei M, Gulasekharam V. Liposomes - a selective drug delivery system for the topical route of administration: gel dosage form. J Pharm Pharmacol 1982; 34:473-474.

Milsmann MHW, Schwendener RA, Weder HG. The preparation of large single bilayer liposomes by a fast and controlled dialysis. Biochim Biophys Acta 1978; 512:147-155.

Monsigny M, Kieda C, Roche A-C. Membrane glycoproteins, glycolipids and membrane lectins as recognition signals in normal and malignant cells. Biol Cell 1983; 47:95-110.

Morgan JR, Williams KE. Preparation and properties of liposome associated gentamycin. Antimicrob Agents Chemother 1980; 17(4): 544-548.

Morii M, Abu-Zaid SS, Takeguchi N. Size and permeability of liposomes extruded through polycarbonate membranes. Int J Pharm 1983; 17(2-3):215-224.

Morman MR. Possible side effects of topical steroids. Am Fam Physician 1981; 23(1):121-125.

Morris GJ. The response of liposomes to various rates of cooling to -196°C: Effect of phospholipid: cholesterol ratio. Cryobiology 1982; 19:215-218.

Nagata M, Yotsuyanagi T, Nonomura M, Ikeda K. Coagulation recovery after warfarin-induced hypoprothrombinaemia by oral administration of liposomally-associated vitamin K_1 to rabbits. J Pharm Pharmacol 1984; 36:527-533.

New RRC, Chance ML, Heath S. Liposome therapy for experimental cutaneous and visceral leishmaniasis. Biol Cell 1983; 47:59-64.

Nicolau C, Poste G, eds. Liposomes in vivo. Biology of the cell vol. 47, France: Societe Francaise de Microscopie Electronique, 1983.

Norrie DH, Pietrowski RA, Stephen J. Screening the efficiency of intracytoplasmic delivery of materials to HeLa cells by liposomes. Anal Biochem 1982; 127:276-281.

Noteboom WD. The effects of culture conditions on the use of animal cells for drug testing. J Parenteral Sci Tech 1983; 37(6):226-231.

Nozaki Y, Lasic DD, Tanford C, Reynolds JA. Size analysis of phospholipid vesicle preparations. Science 1982; 217(4557):366-367.

Obrist R. Monoclonal antibodies as drug carriers in oncology. TIPS 1983; 4(9):375-379.

Oku N, Macdonald RC. Formation of giant liposomes from lipids in chaotropic ion solutions. Biochim Biophys Acta 1983a; 734:54-61.

Oku N, Macdonald RC. Differential Effects of Alkali Metal Chlorides on Formation of Giant Liposomes by Freezing and Thawing and Dialysis. Biochemistry 1983b; 22:855-863.

Olson F, Hunt, CA, Szoka F, Vail WJ, Papahadjopoulos D. Preparation of liposomes of defined size distribution by extrusion through polycarbonate membranes. Biochim Biophys Acta 1979; 557:9-23.

Olson F, Mayhew E, Maslow D, Rustum Y, Szoka F. Characterisation, toxicity and therapeutic efficacy of adriamycin encapsulated in liposomes. Eur J Cancer Clin Oncol 1982; 18(2):167-176.

Onaga IC, Baillie AJ. The uptake of liposomal and free (^{14}C) chloramphenicol by Tetrahymena pyriformis. Int J Pharm 1982; 12: 323-330.

Osborne MP, Payne JH, Richardson VJ, McCready VR, Ryman BE. The preoperative detection of auxillary lymph node metastases in breast cancer by isotope imaging. Br J Surg 1983; 70:141-144.

Ostro MJ ed. Liposomes, New York: Marcel Dekker, Inc. 1983.

O'Sullivan MJ, Marks V. Methods for the preparation of enzyme-antibody conjugates for use in enzyme immunoassay. In: Methods in Enzymology vol. 73, New York: Academic Press, 1981:147-166.

Ozols RF, Myers CE, Young RC. Intraperitoneal Chemotherapy (Editorial). Ann Int Med 1984; 101(1):118-120.

Pagano RE, Takeichi M. Adhesion of phospholipid vesicles to chinese hamster fibroblasts. Role of cell surface proteins. J Cell Biol 1977; 74:531-546.

Pagano RE, Weinstein JN. Interactions of liposomes with mammalian cells. Ann Rev Biophys Bioeng 1978; 7:435-468.

Page Thomas DP, Phillips NC. Intra-articular liposomal therapy. In: Neuberger A, Tatum EL eds: Front Biol vol 48, Amsterdam: North-Holland Publishing Company, 1979:601-624.

Papahadjopoulos D, Vail WJ, Jacobson K. Poste G. Cochleate lipid cylinders: Formationm by fusion of unilamellar lipid vesicles. Biochim Biophys Acta, 1975; 394:483-491.

Papahadjopoulos D, Poste G, Vail WJ, Biedler JL. Use of lipid vesicles as carriers to introduce Actinomycin D into resistant tumor cells. Cancer Res 1976; 36:2988-2994.

Papahadjopoulos D, ed. Liposomes and their uses in Biology and Medicine. Ann NY Acad Sci vol. 308, New York: The New York Academy of Sciences, 1978.

Papahadjopoulos D, Vail WJ. Incorporation of macromolecules within large unilamellar vesicles (LUV). Ann NY Acad Sci 1978; 308:259-267.

Parker RJ, Hartman KD, Sieber SM. Lymphatic absorption and tissue disposition of liposome entrapped ^{14}C-Adriamycin following intraperitoneal administration to rats. Cancer Res 1981; 41:1311-1317.

Patel HM, Ryman BE. Oral administration of insulin by encapsulation within liposomes. FEBS Lett. 1976; 62:60-63.
Patel KR, Jonah MM, Rahman YE. *In vitro* uptake and therapeutic application of liposome encapsulated methotrexate in mouse hepatoma 129. Eur J Cancer Clin Oncol 1982; 18(9):833-845.

Pick U. Liposomes with a large trapping capacity prepared by freezing and thawing of sonicated phospholipid mixtures. Arch Biochem Biophys 1981; 212(1):186-194.

Pidgeon C, Hunt CA. Calculating number and surface area of liposomes in any suspension. J Pharm Sci 1981; 70(2):173-176.

Pirson P, Steiger RF, Trouet A. Primaquine liposomes in the chemotherapy of experimental murine malaria. Ann Trop Med Parasitol 1980; 74(4):383-391.

Pitt E, Johnson CM, Lewis DA. Encapsulation of drugs in intact erythrocytes: An intravenous delivery system. Biochem Pharmcol 1983; 32(22):3359-3368.

Pool GL, French ME, Edwards RA, Huang L, Lumb RH. Use of radiolabelled hexadecylcholesteryl ether as a liposome marker. Lipids 1982; 17(6):448-452.

Popper TL, Watnick AS. Antiinflammatory steroids. In: Scherrer RA and Whitehouse MW eds. Antiinflammatory Agents Chemistry and Pharmacology Vol. I, New York: Academic Press, 1974:245-290 (de Stevens ed. Medicinal Chemistry Vol. 13-I)

Poste G. Liposome targeting in vivo: Problems and opportunities. Biol Cell 1983; 47(1):19-38.

Poste G, Kirsh R. Site-specific (Targeted) drug delivery in cancer therapy. Biotechnology 1983; 1:869-878.

Proffitt RT, Williams LE, Presant CA et al. Liposomal blockade of the reticuloendothelial system: Improved tumor imaging with small unilamellar vesicles. Science 1983; 220:502-505.

Puisieux F, Benita S. Towards a new galenic dosage form, the liposomes? Biomedicine 1982; 36:4-13.

Puisieux F. Les liposomes. Ann Pharm Fr 1983; 41:3-13.

Rahman YE, Rosenthal MW, Cerny EA, Moretti ES. Preparation and prolonged tissue retention of liposomes encapsulated chelating agents. J Lab Clin Med 1974; 83(4):640-647.

Rahman YE, Hanson WR, Bharucha J, Ainsworth EJ, Jaroslow BN. Mechanisms of reduction of antitumor drug toxicity by liposome encapsulation. Ann NY Acad Sci 1978; 308:325-342.

Rahman YE. Liposomes and chelating agents. In: Gregoriadis G, Allison AC, eds. Liposomes in biological systems. New York: John Wiley and Sons, 1980:265-298.

Rankin EM, McVie JG. Radioimmunodetection of cancer: problems and potential. Br Med J 1983; 287:1402-1404.

Rao LS. Liposome dosage form development - some practical considerations. J Parenteral Sci Tech 1983; 37(3):72-75.

Reynolds JA, Nozaki Y, Tanford C. Gel exclusion chromatography on S-1000 Sephacryl: Application to phospholipid vesicles. Anal Biochem 1983; 130:471-474.

Richardson VJ, Ryman BE. Effect of liposomally trapped antitumor drugs on a drug-resistant mouse lymphoma in vivo. Br J Cancer 1982; 45:552-558.

Richardson VJ. Liposomes in antimicrobial chemotherapy. J Antimicrob Chemother 1983; 12(6):532-534.

Rieger MM. Current aspects of cosmetic science III, Liposomes and their uses. Cosmet Toilet 1981; 96:35-38.

Rogers JA. Recent developments in drug delivery. Can J Hosp Pharm 1982a; 35(6):170-174, 196.

Rogers, MJ. H-2^b bound to egg lecithin liposomes: biochemical and functional properties. Clin Exp Immunol 1982b; 48(3):561-573.

Rowland RN, Woodley JF. The stability of liposomes in vitro to pH, bile salts and pancreatic lipase. Biochim Biophys Acta 1980; 620:400-409.

Rowland RN, Woodley JF. The uptake of distearoyl-phosphatidylcholine/cholesterol liposomes by rat intestinal sacs in vitro. Biochim Biophys Acta 1981; 673:217-223.

Rowland GF. Use of antibodies to target drugs to tumor cells. Clin Immunol Allerg 1983; 3(2):235-257.

Rustum YM, Dave C, Mayhew E, Papahadjopoulos D. Role of liposome type and route of administration in the antitumor activity of liposome-entrapped 1-β-D-arabinofuranosylcytosine against mouse L1210 leukemia. Cancer Res 1979; 39:1390-1395.

Sadee W, Beelan GCM. Methotrexate In: Sadee W, Beelen GCM, eds. Drug level monitoring. Analytical techniques, metabolism and pharmacokinetics. New York; John Wiley and Sons, 1980:327-334.

Schaeffer HE, Krohn D. Liposomes in topical drug delivery. Invest Ophthalmol Vis Sci 1982; 22(2):220-227.

Scherphof G, Roerdink F, Waite M, Parks J. Disintegration of phosphatidylcholine liposomes in plasma as a result of interaction with high-density lipoproteins. Biochim Biophys Acta 1978; 542:296-307.

Schneier H. Liposomen - ein neuartiger Arzneistofftrager. Pharmazie in unserer Zeit 1982; 11(4):97-102.

Schroit AJ, Madsen JW. Synthesis and properties of radioiodinated phospholipid analogues that spontaneously undergo vesicle-vesicle and vesicle-cell transfer. Biochemistry 1983; 22:3617-3623.

Schroit AJ, Galligioni E, Fidler IJ. Factors influencing the in situ activation of macrophages by liposomes containing muramyl dipeptide. Biol Cell 1983; 47:87-94.

Schwinke DL, Ganesan MG, Weiner ND. In situ jejunal uptake from liposomal systems. Int J Pharm 1984; 20:119-127.

Segal AW, Gregoriadis G, Black CDV. Liposomes as vehicles for the local release of drugs. Clin Sci Molec Med 1975; 49:99-106.

Senior J, Gregoriadis G. Stability of small unilamellar liposomes in serum and clearance from the circulation: the effect of the phospholipid and cholesterol components. Life Sci 1982; 30(24):2123-2136.

Shaw IH, Knight CG, Dingle JT. Liposomal retention of modified anti-inflammatory steroid. Biochem J 1976; 156:473-476.

Shaw IH, Dingle JT, Phillips NC, Page Thomas PD, Knight CG. Liposomes in the treatment of experimental arthritis. Ann. NY Acad Sci 1978; 308:435-436.

Shaw IH, Dingle JT. Liposomes as steroid carriers in the intra-articular therapy of rheumatoid arthritis. In: Gregoriadis G, Allison AC eds. Liposomes in Biological Systems. New York: John Wiley, 1980:299-324.

Shen D, Huang A, Huang L. An improved method for covalent attachment of antibody to liposomes. Biochim Biophys Acta 1982; 689:31-37.

Singh K. Ocular distribution of liposome encapsulated drugs. [Master's thesis] Halifax, Nova Scotia: Dalhousie Unviersity, 1982.

Singh K, Mezei M. Liposomal ophthalmic drug delivery system I. Triamcinolone acetonide. Int J Pharm 1983; 16:339-344.

Singh K, Mezei M. Liposomal ophthalmic drug delivery system II. Dihydrostreptomycin sulfate. Int J Pharm 1984; 19(3):263-269.

Sinha D, Karush F. Attachment to membranes of exogenous immunoglobulin conjugated to a hydrophobic anchor. Biochem Biophys Res Commun 1979; 90(2):554-560.

Sinha D, Karush F. Specific reactivity of lipid vesicles conjugated with oriented anti-lactose antibody fragments. Biochim Biophys Acta 1982; 684:187-194.

Smith JE, Pirson P, Sinden RE. Studies on the kinetics of uptake and distribution of free and liposome-entrapped primaquine and of sporozites by isolated perfused rat liver. Ann Trop Med Parasitol 1983; 77(4):379-386.

Smolin G, Okumoto M, Feiler S, Condon D. Idoxuridine-liposome therapy for herpes simplex keratitis. Am J Ophthalmol 1981; 91:220-225.

Souhami RL, Patel HM, Ryman BE. The effect of reticuloendothelial blockade on the blood clearance and tissue distribution of liposomes. Biochim Biophys Acta 1981; 674:354-371.

Spjer HH, Scherphof GL. Targeting of lactosylceramide-containing liposomes to hepatocytes in vivo. Biochim Biophys Acta 1983; 734:40-47.

Stamp D, Juliano RL. Factors affecting the encapsulation of drugs within liposomes. Can J Physiol Pharmacol 1979; 57:535-539.

Stevenson RW, Patel HM, Parsons JA, Ryman BE. Prolonged hypoglycemic effect in diabetic dogs due to subcutaneous administration of insulin in liposomes. Diabetes 1982; 31:506-511.

Stratford RE Jr, Kang DC, Redell MA, Lee VHL. Effects of topically applied liposomes on disposition of epinephrine and inulin in the albino rabbit eye. Int J Pharm 1983; 13:263-272.

Strauss G, Ingenito EP. Stabilization of liposome bilayers to freezing and thawing: Effects of cryoprotective agents and membrane proteins. Cryobiology 1980; 17:508-515.

Sur B, Ray RR, Sur P, Roy DK. Effect of liposomal encapsulation of cis-platinum diamminodichloride in the treatment of Ehrlich ascites carcinoma. Oncology 1983; 40:372-376.

Szoka F Jr., Papahadjopoulos D. Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. Proc Natl Acad Sci USA 1978; 75(9):4194-4198.

Szoka F Jr., Papahadjopoulos D. Comparative properties and methods of preparation of lipid vesicles (liposomes). Ann Rev Biophys Bioeng 1980; 9:467-508.

Szoka F, Olson F, Heath T, Vail W, Mayhew E, Papahadjopoulos D. Preparation of unilamellar liposomes of intermediate size (0.1-0.2 μm) by a combination of reverse phase evaporation and extrusion through polycarbonate membranes. Biochim Biophys Acta 1980; 601:559-571.

Szoka F, Papahadjopoulos D. Liposomes: Preparation and characterization. In: Knight CG, ed. Liposomes: From physical structure to therapeutic applications. New York: Elsevier Biomedical Press, 1981.

Szoka F, Magnusson K-E, Wojcieszyn J, Hou Y, Derzko Z, Jacobson K. Use of lectins and polyethylene glycol for fusion of glycolipid-containing liposomes with eukaryotic cells. Proc Natl Acad Sci USA 1981; 78(3):1685-1689.

Szoka FC Jr., Mayhew E. Alteration of liposome disposition in vivo by bilayer situated carbohydrates. Biochem Biophys Res Commun 1983; 110(1):140-146.

Todd JA, Levine AM, Tokes ZA. Liposome encapsulated methotrexate interactions with human chronic lymphocytic leukemia cells. JNCI 1980; 64(4):715-719.

Todd JA, Modest EJ, Rossow PW, Tokes ZA. Liposome encapsulation enhancement of methotrexate sensitivity in a transport resistant human leukemic cell line. Biochem Pharmacol 1982; 31(4):541-546.

Todorov DK, Deliconstantinos G. Incorporation of the antitumor alkaloid thaliblastine in liposomes enhances its cytotoxic activity in vitro. Experienta 1982; 38(7):857-859.

Tomasi M, Gitman AG, Friedlander B, Loyter A. Conjugation of specific antibodies to Sendai virus particles. FEBS Lett 1982; 143(2):252-256.

Toonen PAHM, Crommelin DJA. Immunoglobulins as targeting agents for liposome encapsualted drugs. Pharm Weekbl Sci Ed 1983; 5:269-280.

Torchilin VP, Khaw BA, Smirnov VH, Haber E. Preservation of antimyosin antibody activity after covalent coupling to liposomes. Biochem Biophys Res Commun 1979; 89(4):1114-1119.

Trouet A, Baurain R, Campeneere DD, Layton D, Masquelier M. DNA, liposomes and proteins as carriers for antitumoral drugs. Recent Results Cancer Res 1981; 75(2):229-235.

Tsujii K. Sunamoto J, Fendler JH. Improved entrapment of drugs in modified liposomes. Life Sci 1976; 19:1743-1750.

Tsukada K. Okada R, Hisaoka M, Morioka T. Liposome-encapsulated carboquone. I. Method of preparation and carboquone release. Chem Pharm Bull 1982; 30(10):3679-3684.

Tyrrell DA, Heath TD, Colley CM, Ryman BE. New aspects of liposomes. Biochim Biophys Acta 1976; 475:259-302.

Ueno M, Nakasaki T, Horikoshi I, Sakuragawa N. Oral administration of liposomally-entrapped heparin to beagle dogs. Chem Pharm Bull 1982; 30(6):2245-2247.

van Bommel I, Crommelin DJA. Integrity of liposomes on storage in aqueous solution or after freezing or freeze-drying. In: FIP 83 Abstracts, 43rd International Congress of Pharmaceutical Sciences, Montreux, Switzerland, September 1983:325.

Van Renswoude AJBM, Blumenthal R, Weinstein JN. Thin-layer chromatography with agarose gels. A quick, simple method for evaluating liposome size. Biochim Biophys Acta 1980; 595:151-156.

Van Rooijen N, van Nieuwmegen R. Immunoadjuant properties of liposomes. In: Gregoriadis G, Senior J, Trouet A, eds. Targeting of drugs. NATO ASI, series A, New York: Plenum press, 1982:301-326.

Weingarten C, Moufti A, Desjeux JR, Luong TT, Durand G, Devissaguet JP, Puisieux F. Oral ingestion of insulin liposomes: Effects of the administration route. Life Sci 1981; 28:2747-2752.

Weinstein JN, Yoshikami S, Henkart P, Blumenthal R, Hagins WA. Liposome-cell Interaction: Transfer and intracellular release of a trapped fluorescent marker. Science 1977; 195:489-491

Weinstein JN, Blumenthal R, Sharrow SO, Henkart PA. Antibody-mediated targeting of liposomes. Binding to lymphocytes does not ensure incorporation of vesicle contents into the cells. Biochim Biophys Acta 1978; 509:272-278.

Weinstein JN, Magin RL, Yatvin MB, Zaharko DS. Liposomes and local hyperthermia: Selective delivery of methotrexate to heated tumors. Science 1979; 204:188-191.

Weinstein JN, Magin RL, Cysyk RL, Zaharko DS. Treatment of L1210 murine tumors with local hyperthermia and temperature sensitive liposomes containing methotrexate. Cancer Res 1980; 40:1388-1395.

Weinstein JN, Leserman LD. Liposomes as drug carriers in cancer chemotherapy. Pharmac Ther 1984; 24:207-233.

Weissmann G, Finkelstein M. Uptake of enzyme bearing liposomes by cells in vivo and in vitro. In: Gregoriadis G, Allison AC, eds. Liposomes in Biological Systems, New York: John Wiley, 1980; 153-178.

Weldon JS, Munnell JF, Hanson WL, Alving CR. Liposomal chemotherapy in visceral leishmaniasis: An ultrastructural study of an intracellular pathway. Z. Parasitenkd 1983; 69:415-424.

Whelan WJ. Use of liposomes in medicine. Biochem Soc Trans 1984; 12(2):333-335.

Widder KJ, Senyei AE, Sears B. Experimental methods in cancer therapeutics. J Pharm Sci 1982; 71(4):379-387.

Wilschut J, Duzgunes N, Hong K, Hoekstra D, Papahadjopoulos D. Retention of aqueous contents during divalent cation-induced fusion of phospholipid vesicles. Biochim Biophys Acta 1983; 734:309-318.

Wold F. Bifunctional reagents. In: Colowick SP, Kaplan NO eds. Methods in Enzymology vol. 25, New York: Academic Press, 1982:623-651.

Wu P-S, Tin GW, Baldeschwieler JD. Phagocytosis of carbohydrate-modified phospholipid vesicles by macrophage. Proc Natl Acad Sci USA 1981; 78(4):2033-2037.

Yatvin MB, Kreutz W, Horwitz BA, Shinitzky M. pH sensitive liposomes: Possible clinical implications. Science 1980; 210:1253-1255.

Yatvin MB, Lelkes PI. Clinical prospects for liposomes. Med Phys 1982; 9(2):149-175.

Yatsuyanagi T, Nonomura M, Ikeda K. Ultrasonic size reduction of liposomes and volume estimation of internal aqueous phase. J Parenteral Sci Tech 1981; 35(6):271-275.

Zachman RD, Tsao FHC. Pulmonary uptake of liposomal phosphatidylcholine. Pediat Res 1980; 14:24-27.

Zaharko DS, Przybylski M, Oliverio VT. Binding of anticancer drugs to carrier molecules. In: DeVita VT Jr, Busch H eds. Methods in cancer research vol. 16. New York: Academic Press, 1979:347-380.

Zumbuehl O, Weder HG. Liposomes of controllable size in the range of 40 to 180 nm by defined dialysis of lipid/detergent mixed micelles. Biochim Biophys Acta 1981; 640:252-262.

APPENDICES

Appendix 1

Formulae for different solutions

1. Bray's solutions (Bray GA, Anal Biochem 1960; 1: 279-285):

Omnifluor	4 g
Naphthalene	30 g
Ethylene glycol	10 ml
Methanol	50 ml
1,4-dioxane q.s.	500 ml

2. 0.01 M Phosphate buffered saline (0.01 M PBS):

$NaH_2PO_4.H_2O$	18 g
Na_2HPO_4	38.1 g
NaCl	340 g
Distilled water	4 liter
pH	7

(Dilute the solution 10 times with distilled water to obtain 0.01M PBS)

3. Acetate buffer pH 5:

1 M acetic acid	84 ml
Anhydrous sodium acetate	17.2 g
or	
Sodium acetate $3H_2O$	27.2 g
Distilled water q.s.	4 liter
pH	5

1M acetic acid:

Glacial acetic acid	57.7 ml
Distilled water q.s.	1000 ml

4. 0.1 M Sodium phosphate buffer, 0.15 M NaCl, pH 7.5:

0.2 M Na_2HPO_4	420 ml
0.2 M NaH_2PO_4	80 ml
NaCl	8.5 g
Distilled water q.s.	1000 ml
pH	7.5

0.2 M Na_2HPO_4: Na_2HPO_4 28.4 g/liter

0.2 M NaH_2PO_4: $NaH_2PO_4.H_2O$ 27.6 g/liter

5. 0.1 M Acetate buffer, 0.15 M NaCl, pH 4.5:

Glacial acetic acid	5.72 ml
1N NaOH q.s.	pH 4.5
NaCl	8.5 g
Distilled water q.s.	1000 ml

6. 0.5% Sodium dodecyl sulfate buffered solution:
(Johnson LF, Fuhrman CL, Abelson HT. Cancer Res 1978; 38:2408-2412)

Tris HCl	1.58 g (0.01M)
NaCl	5.845 g (0.1M)
Sodium dodecyl sulfate	5 g (0.5%)
EDTA	0.292 g (0.001M)
Distilled water	1000 ml
pH	7.4

7. EDTA solution for tissue culture:

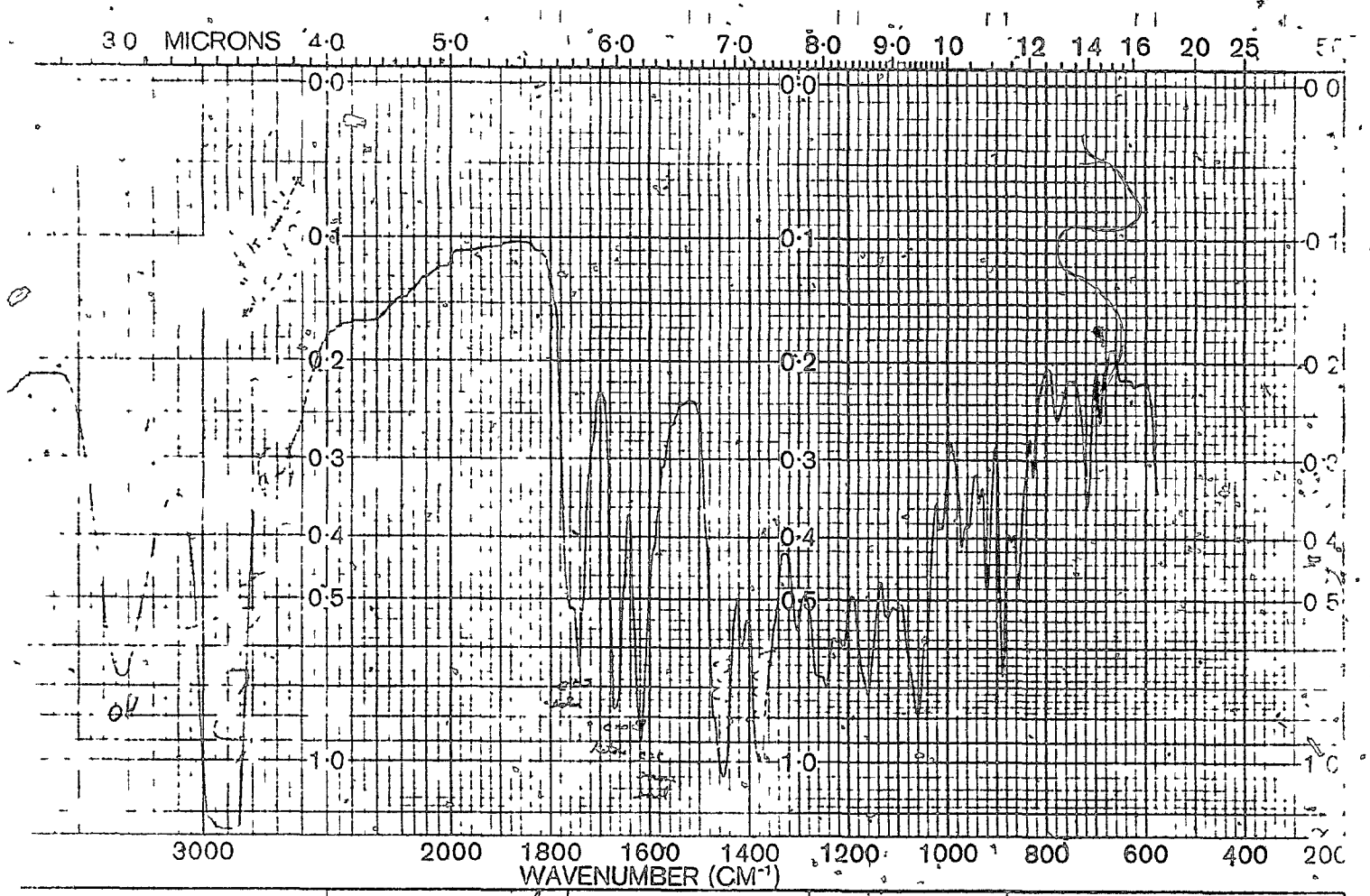
EDTA (sodium salt)	0.2 g
KCl	0.4 g
NaCl	8.0 g
$NaHCO_3$	0.35 g
Glucose	1.0 g
Phenol red	0.005 g
Distilled water	1000 ml

8. Alkaline water:

Sodium citrate	2 g
Sodium bicarbonate	2 g
Tap water q.s.	1000 ml

Appendix 2

The IR spectrum of triamcinolone acetonide-21-palmitate.



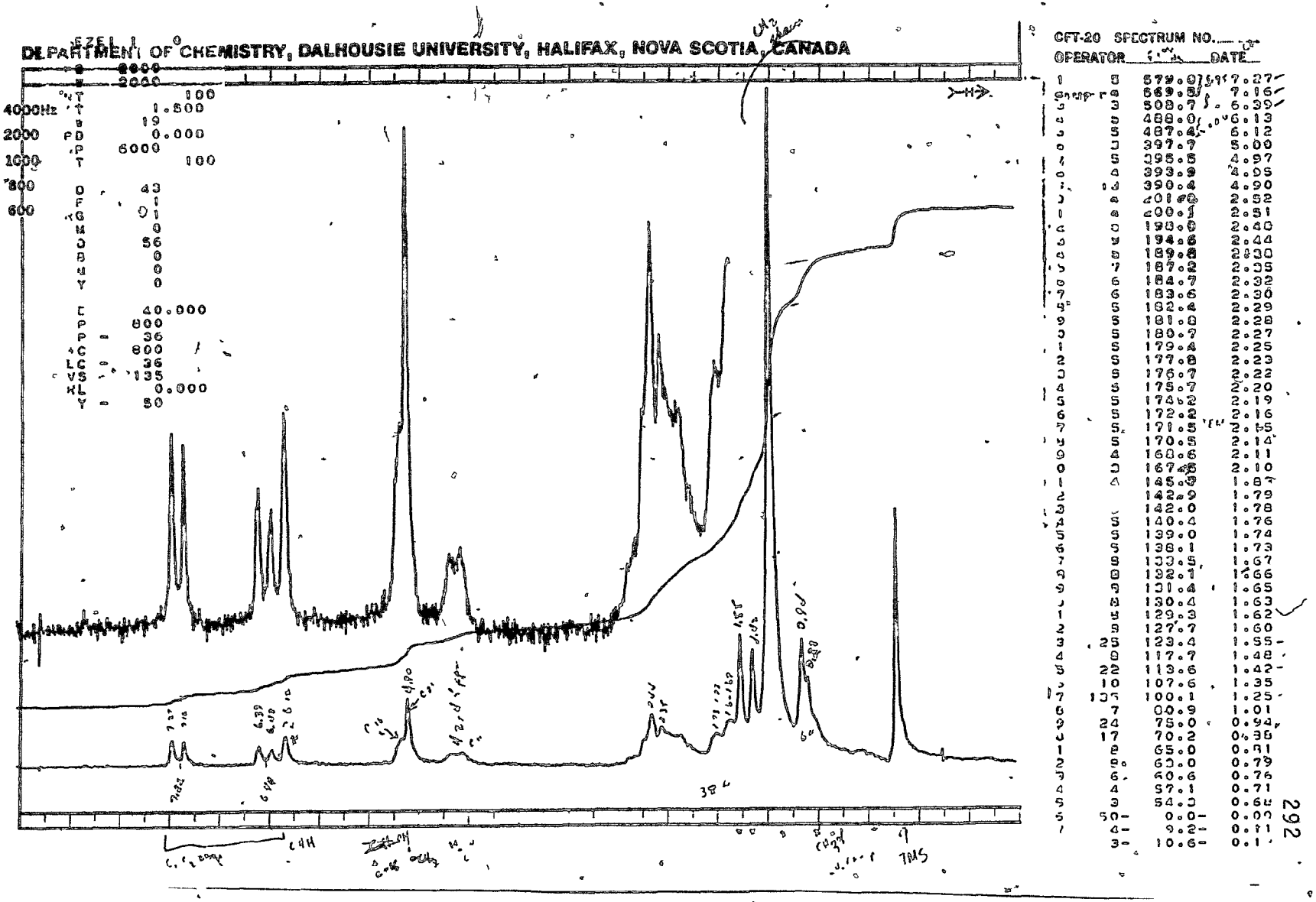
TRMA-21-palmitate

SOLVENT Nujol	SCAN 3 mins	SINGLE B
CONC	SLIT	T.D. SPEED
CELL PATH Film	OPERATOR	ORD. EXP.
REFERENCE	DATE July 21, 81	T CONST
PERKIN ELMER	PART No 5102 1001	REF No

REMARKS

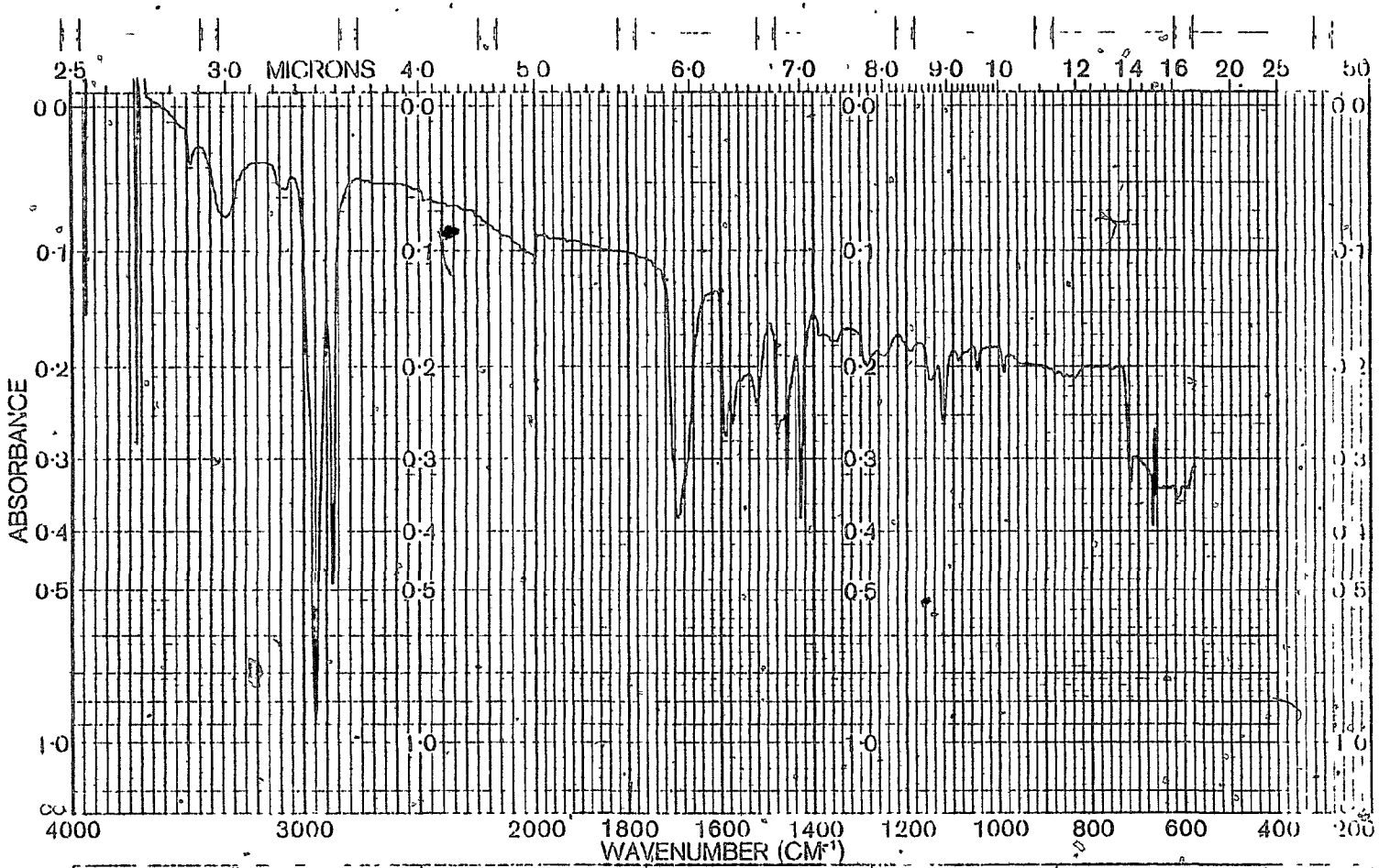
Appendix 3

The ^{1}H NMR spectrum of triamcinolone acetonide-21-palmitate.


DEPARTMENT OF CHEMISTRY, DALHOUSIE UNIVERSITY, HALIFAX, NOVA SCOTIA, CANADA
CFT-20 SPECTRUM NO.
OPERATOR
DATE
4000Hz
2000
1000
800
600
TMS

Appendix 4

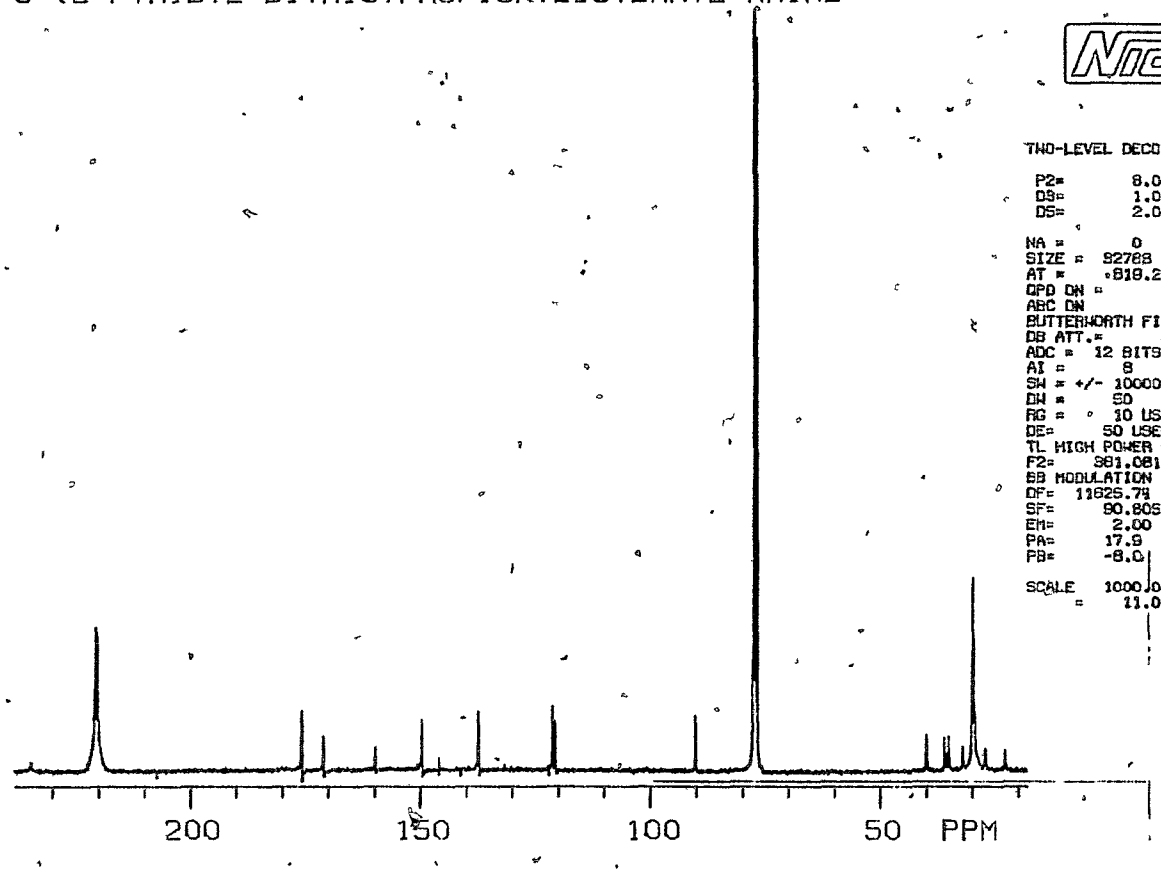
The IR spectrum of N-[3-(2-pyridyldithio)-propionyl]stearylamine (PDP-SA).


2·5
3·0
MICRONS
4·0
5·0
6·0
7·0
8·0
9·0
10
12
14
16
20
25
50
ABSORBANCE
0·0
0·1
0·2
0·3
0·4
0·5
1·0
4000
3000
2000
1800
1600
1400
1200
1000
800
600
400
200
WAVENUMBER (CM⁻¹)
SAMPLE
SOLVENT
CONC
CELL PATH
REFERENCE
ORIGIN
PERKIN ELMER
SCAN
SLIT
OPERATOR
DATE
PART No 6102 1001
SINGLE B.
TD SPEED
ORD EXP.
T CONST
REF No
REMARKS

Appendix 5

The ^{13}C NMR spectrum of N-[3-(2-pyridyldithio) propionyl]stearylamine (PDP-SA).

MEZEI . 001 DLH 03DEC82
3
N-
3-(2-PYRIDYL DITHIO)PROPIONYL-STEARYL AMINE



TWO-LEVEL DECOUPLING

P2= 8.00 USEC
DS= 1.00 MSEC
DS= 2.00 SEC

NA = 0
SIZE = 32768
AT = 819.20 MSEC
QPD ON = 1
ABC ON
BUTTERWORTH FILTER ON
DB ATT.= 8
ADC = 12 BITS
AI = 8
SW = +/- 10000.0
DW = 50
RG = 10 USEC
DE= 50 USEC
TL HIGH POWER ON
F2= 381.081802
SB MODULATION ON
OF= 11825.7
SF= 90.805071
EM= 2.00
PA= 17.9
PB= -8.0

SCALE 1000.00 HZ/CM
= 11.0128 PPM/CM

Appendix 6

Publications

Reprinted from the Journal of Pharmaceutical Sciences.
Vol. 73, No. 6, June 1984, © 1984 by the American Pharmaceutical Association.

Chemical Modification of Triamcinolone Acetonide to Improve Liposomal Encapsulation

ASHOK GOUNDALKAR and MICHAEL MEZEI ×

Received February 7, 1983, from the *College of Pharmacy, Dalhousie University, Halifax, N.S. B3H 3J5, Canada* Accepted for publication April 18, 1983

Abstract □ The 21 palmitate of triamcinolone acetonide was synthesized to aid in the liposomal encapsulation of the drug. Encapsulation efficiency of triamcinolone acetonide 21-palmitate was 85%, compared with 5% for the parent drug.

Keyphrases □ Triamcinolone acetonide—synthesis of the 21 palmitate ester, liposomal encapsulation □ Liposomes—encapsulation of triamcinolone acetonide-21-palmitate, synthesis

Liposomes are microscopic vesicles composed of phospholipid bilayers separated by aqueous compartments. Their potential as temporal and spatiotemporal drug delivery systems is being widely investigated (1–3). The applicability of liposomes as selective drug delivery systems for cutaneous administration of drugs has been studied (4, 5) in this laboratory using triamcinolone acetonide as the model drug. Attempts to test various types of liposomes with the same drug revealed a low level of encapsulation of the drug in the multilamellar liposomes.

A relationship between liposomal encapsulation and the structure of the derivatives of another corticosteroid, hydrocortisone, has been reported by Shaw *et al.* (6). The observation that among a variety of derivatives, hydrocortisone-21-palmitate had the maximum encapsulation in the liposomes led us to study a similar approach for enhancing encapsulation of triamcinolone acetonide. The synthesis and purification of triamcinolone acetonide-21-palmitate, based on the principles of the methods reported by Shaw *et al.* (6) and Diamanti and Bianchi (7), is described in this report.

EXPERIMENTAL

9-Fluoro-21-(1-oxohexadecoxy)-11β-hydroxy-16,17-[(1-methylethylidene)-bis(oxy)]-pregna-1,4-diene-3,20-dione—Triamcinolone acetonide (435 mg, 1 mmol) was dissolved in 4 mL of *N,N*-dimethylformamide. Pyridine (160 mg, 2 mmol) and palmitoyl chloride (550 mg, 2 mmol) were added, the mixture was stirred magnetically at room temperature for 22 h, and then was poured into 0.5 M sulfuric acid (1600 mL) and stirred vigorously. The product was removed by filtration and was purified by column chromatography on silica gel (toluene–ethyl acetate–acetic acid, 90:10:1). Recrystallization from methanol gave 471 mg (70% yield) of triamcinolone acetonide-21-palmitate, mp 143–147°C. IR (Nujol): 1760 (ester C=O), 1740 (ketone C=O), 1670 (α,β, α',β'-unsaturated ketone C=O), 1620 (C=C conjugated with ketone), and 890 cm^{-1} (*cis*-CH of Δ-1,4 system). ^{1}H-NMR ($CDCl_3$): δ 0.88 (t, 3, terminal CH_3), 0.94 (s, 3, 18 CH_3), 1.25 (narrow m, 27, acetonide β CH_3 and CH_2 chain), 1.42 (s, 3, acetonide α-CH_3), 1.55 (s, 3, 19 CH_3), 1.6–2.5 (m, 4, $CH_2CH_2CO_2$), 4.90 (s, 2, 21 CH_2O), 4.97 (br, 1, 16-CHO), 6.12 (d, 1, $J_{2,4}$ = 0.6 Hz, 4-CH), 6.44 (dd, 1, $J_{2,4}$ = 0.6 Hz, $J_{1,2}$ = 9.5 Hz, 2-CH), and 7.22 ppm (d, 1, $J_{1,2}$ = 9.5 Hz, 1-CH).

Anal.—Calc. for $C_{24}H_{61}FO_7$: C, 71.39; H, 9.44; F, 2.82. Found: C, 71.26; H, 9.25; F, 2.80.

Table I—Comparative Data for Liposomal Encapsulation of Triamcinolone Acetonide and Triamcinolone Acetonide-21-Palmitate

Procedure	Fraction	Concentration, mg/22 mL: Triamcinolone Acetonide	Concentration, mg/22 mL: Triamcinolone Acetonide 21 Palmitate
Preparation	Crude product	20	20
Filtration	Filtrate	7	19
Centrifugation	Supernatant	6	2
	Pellet (liposomes)	1	17

Preparation of Liposomes—To reliably determine encapsulation efficiency, radioactive triamcinolone acetonide 21 palmitate was prepared as above from [6,7-^{3}H]triamcinolone acetonide. Multilamellar liposomes were prepared as described by Mezei and Gulasekharam (4). Dipalmitoyl phosphatidyl choline (160 mg) and triamcinolone acetonide-21 palmitate (22 mg, 350 μCi) in chloroform–methanol (2:1) were evaporated using a rotary evaporator. The film was then dispersed with 22 mL of aqueous 8 mM $CaCl_2$ solution at 60°C. The preparation was evaluated microscopically and then filtered through a 12 μm polycarbonate filter. The filtrate was then centrifuged at 22,000×*g* for 25 min. Radioactivity in each fraction was measured after each treatment. Triamcinolone acetonide liposomes were prepared in a similar manner.

RESULTS AND DISCUSSION

Results presented in Table I indicate considerable improvement in liposomal encapsulation of triamcinolone acetonide as a result of palmitoylation. In the case of the triamcinolone acetonide liposomal preparation the first major loss occurred after filtration through a 12-μm polycarbonate filter. This filtration step is necessary with the liposomal encapsulation of a lipophilic drug. Since the drug is insoluble or very slightly soluble in the aqueous medium, only that portion is encapsulated that is in solution and intimately associated with the lipid bilayers, the remaining portion is in solid form which, although it is unencapsulated, would be present in the liposomal fraction after centrifugation. The loss of 13 mg (*i.e.*, 65%) of triamcinolone acetonide by the filtration process was due mainly to unencapsulated crystals observed in the crude preparation. Such crystals were absent in the filtrate. No crystals were seen even in the unfiltered triamcinolone acetonide-21-palmitate liposomal fraction, consequently only 1 mg (*i.e.*, 5%) was lost by the filtration. This suggests that the palmitate form has a stronger association with the lipid layers and that almost complete encapsulation is achieved.

The second major loss in the case of triamcinolone acetonide liposomes was in the supernatant after centrifugation of the filtrate. This is due mainly to the drug in solution but not associated with liposomes. Evidence for this was demonstrated by gel filtration chromatography. Thus, the overall encapsulation of triamcinolone acetonide, determined in the final purified liposomal fraction, was only 5% while that of its palmitate derivative was found to be 85%. The encapsulation of the palmitate derivative could be close to 100%, if one considers the unavoidable loss due to the filtration and centrifugation procedures, *i.e.*, by adsorption to the filter and glassware. Another minor but inherent loss of liposomes could be due to the presence of small (<0.5 μm) liposomes, which are not completely sedimented by centrifugation. However, for comparison purposes, both preparations were analyzed by the same procedures, and consequently, the same considerations should be applied.

A possible reason for increased encapsulation of the palmitate derivative could be the change in the partition coefficient. For lipid-soluble compounds a logarithmic partition coefficient (log P) between 1.7 and 4 is unfavorable for liposomal encapsulation (8). Triamcinolone acetonide has a log P of 2.53 (9). Palmitoylation would increase this to ~11 as calculated using substituent constants (9).

The mechanism for increased encapsulation could be predicted to be analogous to that of hydrocortisone-21-palmitate (10). The palmitoyl chain may act as "hydrophobic anchor" holding the steroid head group on the surface of the lipid bilayer.

One of the drawbacks in liposomal drug delivery systems is the poor encapsulation of the drug in the liposomes. Chemical modification is likely to be a powerful approach to overcome such an obstacle, as evidenced here and elsewhere (6). Although only a small amount of the drug was associated with the liposomes, the drug disposition was altered favorably on dermal application of a triamcinolone acetonide liposomal preparation (4, 5). In light of this, the potential usefulness of this highly concentrated liposomal triamcinolone

0022-3549/84/0600-0034$01.00/0

acetonide-21-palmitate preparation as a selective drug delivery system for cutaneous administration may be optimal

REFERENCES

(1) G Gregoriadis, in "Liposomes in Biological Systems," G Gregoriadis and A C Allison, Eds , Wiley, New York, N Y , 1980 p 377
(2) R L Juliano and D Layton, in "Drug Delivery Systems," R L Juliano, Ed , Oxford University Press, New York, N Y , 1980, p 189
(3) M D Yatvin and P I Lelkes, *Life Sci* , 9, 149 (1982)
(4) M Mezei and V Gulasekharam, *Life Sci* , 26, 1473 (1980)
(5) M Mezei and V Gulasekharam, *J Pharm Pharmacol* 34, 473 (1982)
(6) J H Shaw, C G Knight, and J T Dingle, *Biochem J* , 153, 473 (1976)
(7) E Diamanti and G E Bianchi *Arzneim -Forsch* , 21, 251 (1971)
(8) F Defrise Quertain P Chatelain, J M Ruysschaert, and M Delmelle, *Biochim Biophys Acta* 628, 57 (1980)
(9) C Hansch and A Leo, "Substituent Constants For Correlation Analysis in Chemistry and Biology," Wiley, New York, N Y , 1979 pp 19 315
(10) F J T Fildes and J E Oliver, *J Pharm Pharmacol* ; 30, 337 (1978)

ACKNOWLEDGMENTS

This study was supported by the Medical Research Council of Canada (MA 6664) The authors thank Dr P S Farmer, College of Pharmacy, Dalhousie University, Halifax, Nova Scotia, for his advice and assistance during the course of this project

J Pharm. Pharmacol. 1984, 36. 465–466
Communicated November 10, 1983

Covalent binding of antibodies to liposomes using a novel lipid derivative

ASHOK GOUNDALKAR, TARUN GHOSE†, MICHAEL MEZEI*, *College of Pharmacy, Faculty of Health Professions and †Department of Pathology, Faculty of Medicine, Dalhousie University, Halifax, N S Canada B3H 3J5*

N-[3-(2-Pyridyldithio) propionyl] stearylamine (PDP-SA) was synthesized from a reaction between stearylamine and the heterobifunctional reagent *N*-succinimidyl-3-(2-pyridyldithio) propionate. Use of this PDP-SA to covalently couple antibodies to liposomes was investigated The binding efficiency was found to be 24–32%. The antibodies bound to liposomes were shown to retain the specific antibody activity. This new procedure of coupling antibodies to lipsosomes could be an efficient means to deliver drugs to selected target organs, especially in cancer chemotherapy.

Several methods to couple antibodies to liposomes via a stable covalent linkage have been reported (Barbet et al 1981; Endoh et al 1981; Jansons & Mallett 1981; Martin et al 1981; Shen et al 1982; Goldmacher 1983). Martin et al (1981) have described a method which has a higher efficiency of binding than achieved by the other methods and which avoids dialysis in the presence of a detergent. However, because of the use of an unsaturated phosphatidylethanolamine derivative, the long-term stability of the product might be questionable due to the risk of oxidation. We report here the successful linkage of a rabbit anti-BSA IgG to liposomes using a stearylamine derivative of *N*-succinimidyl-3-(2-pyridyldithio) propionate (SPDP). This method has a high efficiency of binding without substantially interfering with the antibody activity of the bound IgG and the product does not have the risk of undergoing oxidation.

Methods

Synthesis of N-[3-(2-pyridyldithio) propionyl] stearylamine (PDP-SA). Stearylamine[a] (SA) (260 mg, 965 μmol) and *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) (200 mg, 640 μmol) were dissolved separately in 12 ml portions of absolute methanol. The solution of SPDP was added dropwise to the stearylamine solution and the mixture was stirred at room temperature (20 °C) for 30 min. Methanol was then removed under reduced pressure. The crude product was purified by column chromatography on silica gel; eluting with ethyl acetate–light petroleum (40:60) (282 mg, 94%): mp 61 °C; ir (CH_2Cl_2): 3450 (–NH–), 2950 [$(CH_2)_{10}$], 1690 (Amide I) and 1525 cm^{-1} (Amide II); [^{13}C]-nmr ($CDCl_3$): 175, 170 (*cis* and *trans* CO), 160–120 (5, pyridyl), 40–27 (several, CH_2), 23 ppm (CH_3). Anal.-Calc. for $C_{26}H_{46}N_2S_2O$: C, 66·90; H, 9·93; N, 6·00; S, 13·74. Found C, 67·28. H, 9·89; N, 5·81; S, 13·75.

Preparation of liposomes. Preparations containing large unilamellar and oligolamellar liposomes were obtained by the reverse-phase evaporation method described by Szoka & Papahadjopoulos (1978) using DL-α-dipalmitoyl phosphatidyl choline (DPPC), cholesterol, SA, and PDP-SA in a molar ratio of 4:4:1:1. Specifically, DPPC (80 mg, 109 μmol), cholesterol (42 mg, 109 μmol), SA (7·5 mg, 27·8 μmol) and PDP-SA (12·7 mg, 27·3 μmol) were dissolved in a mixture of chloroform (4·5 ml) and diethyl ether (8 ml). Next, 4·2 ml of 0·5% $NaHCO_3$ (pH 8) was added, after which, the lipid solution was sonicated at 0–5 °C for 5 min in a bath-type sonicator. Organic solvents were removed from the resulting emulsion-like dispersion by a rotary vacuum evaporator. The aqueous dispersion was then shaken at 55 °C for half an hour to obtain liposomes.

To incorporate methotrexate (MTX) into the liposomes a 20 mM solution of MTX in 0·5% $NaHCO_3$ was used for entrapment. Crude liposomes thus obtained were washed twice with 0·5% $NaHCO_3$ by centrifugation at 22 000*g* for 25 min. The washed pellet, which consisted of MTX liposomes free from unentrapped MTX, was finally dispersed in a volume of 0·5% $NaHCO_3$. The leakage of entrapped MTX during antibody-liposome coupling was calculated as the difference between liposomal MTX content (determined spectrophotometrically at 303 nm in methanol) before and after attachment of thiolated normal rabbit IgG to the liposomes.

Preparation of PDP-IgG. Rabbit anti-BSA IgG was fractionated with 33% saturated ammonium sulphate from immune sera and its antibody activity was assayed by radial immunodiffusion as described by Kulkarni et al (1981). Pyridyl disulphide moieties were introduced into IgG, using the heterobifunctional reagent SPDP by the method of Carlsson et al (1978). Ten moles of SPDP was reacted with 1 mol of IgG in 0·1 m sodium phosphate buffer containing 0·1 M NaCl, pH 7·5, for 30 min. The reaction mixture was dialysed extensively against the above buffer at 4 °C in order to remove low-molecular-weight substances. The content of 2-pyridyl disulphide groups, as determined by the method of Carlsson et al (1978), was 6–8·2 per mole of

* Correspondence.

IgG. The modified IgG was stored at −20 °C until needed

Covalent coupling of antibodies to liposomes. PDP-IgG was bound to liposomes by the method of Barbet et al (1981). PDP-IgG was reduced with dithiothreitol (DTT) in 0·1 M sodium acetate buffer containing 0·1 M NaCl, pH 4·5 for 40 min. Excess DTT and pyridine-2-thione were removed by gel filtration on a Sephadex G-25 M column equilibrated with 0·5% $NaHCO_3$. Thiolated IgG (8–12·6 mg) and liposomes (26 μmol of phospholipid) were stirred together overnight at room temperature and pH 8. Liposomes were separated from unbound antibody twice by centrifugation at 22 000*g* for 25 min followed by washing of the pellet with 0·5% $NaHCO_3$. In order to determine non-specific adherence of antibodies to liposomes, 9 mg of PDP-IgG that had not been reduced by DTT was mixed with liposomes as above. The protein content of the liposome fraction was analysed by the method of Lowry et al (1951).

Determination of antibody activity. The antigen-binding capacity of the anti-BSA IgG was determined using [^{125}I]BSA as antigen by a method based on Farr assay (Hudson & Hay 1980). For determining the antigen-binding capacity of the IgG linked to liposomes, an aliquot of liposome-anti-BSA IgG containing 314 μg of IgG was incubated with an excess of [^{125}I]BSA (400 μg, 55 000 counts min^{-1}) for 2 h at 37 °C with continuous shaking. This was followed by incubation at 4 °C for an additional 2 h. Liposome-antibody-antigen complex was then sedimented by centrifugation at 22 000*g* for 30 min. The pellet was washed twice with 0·1 M PBS. The amount of BSA bound to liposomes was calculated by determining the ^{125}I associated with the pellet. In control experiments the non-specific binding of [^{125}I]BSA to normal rabbit IgG or normal rabbit IgG-liposomes was determined. Values for specific binding to anti-BSA IgG were corrected by subtracting the amounts of radioactivity that bound non-specifically.

Results and discussion

About 24–32% of the thiolated IgG became bound to liposomes (96–123 μg IgG μmol^{-1} phospholipid), a binding efficiency comparable to that reported by other investigators (Barbet et al 1981; Martin et al 1981). The non-specific adherence of antibodies to liposomes was only 0·9% (3·1 μg IgG μmol^{-1} phospholipid). This confirms that the chemical reaction between thiolated IgG and liposomes (i.e. the thiol-disulfide exchange reaction) was responsible for increased binding of IgG to liposomes.

For immunospecific liposomal targeting it is essential that the liposome-bound antibody retain its immunological reactivity. The antigen-binding capacity of anti-BSA IgG before incorporation in liposomes was found to be 0·1641 μg BSA/μg anti-BSA IgG. The antigen-binding capacity of the rabbit anti-BSA IgG was reduced by only about 20% after its linkage to liposomes.

By virtue of the primary amino group, stearylamine can react readily with the heterobifunctional reagent SPDP to give rise to another reactive compound: PDP-SA.

Stearylamine, in contrast to phosphatidylethanolamine, has a well-defined and simple molecular structure, i.e. it has a single hydrocarbon chain whereas phosphatidylethanolamine (PE) has two fatty acyl chains of varying length and saturation. Because of its single chain, PDP-SA may have a better fit into the lipid bilayers of the liposomes than the two-'tailed' PDP-PE in which the length of the two chains are often unequal. Further, because its chain is saturated, PDP-SA is likely to be more stable than PDP-PE with its unsaturated fatty acyl chains. An oxidative reaction involving the PDP-PE could affect the integrity of liposomes and the stability of their linkage to antibodies. Other advantages of using PDP-SA for coupling antibodies to liposomes are the low cost of SA, the simple synthesis procedure and the almost quantitative conversion of SA to PDP-SA. Only about 20% of entrapped MTX leaked during the overnight binding reaction. Thus, by the linkage of appropriate antitumour antibodies to MTX-containing liposomes, it may be possible to add to the tumour-inhibitory effect of MTX linked directly to anti-tumour IgG (Kulkarni et al 1981).

We thank Dr P. S. Farmer, College of Pharmacy, Dalhousie University, for his advice in the chemical synthesis aspect of this project and Mr Peter King, Editorial Services, Kellogg Library for his assistance with the preparation of this manuscript. Supported by the Medical Research Council of Canada (MA 6664 and MT 6922).

REFERENCES

Barbet, J., Machy, P., Leserman, L. D. (1981) J. Supramolec. Struct. Cell. Biochem 16: 243–258

Carlsson, J., Drevin, H., Axen, R. (1978) Biochem. J. 173: 723–737

Endoh, H., Suzuki, Y., Hashimoto, Y. (1981) J. Immunol. Methods 44: 79–85

Goldmacher, V. S. (1983) Biochem. Pharmacol 32: 1207–1210

Hudson, L., Hay, F. C. (1980) Practical Immunology. Blackwell Scientific Publications, Oxford, 2nd ed., pp 94–98

Jansons, V. K., Mallett, P. L. (1981) Anal. Biochem. 111: 54–59

Kulkarni, P. N., Blair, A. H., Ghose, T. I. (1981) Cancer Res. 41: 2700–2706

Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. (1951) J. Biol. Chem 193: 265–275

Martin, F. J., Hubbell, W. L., Papahadjopoulos, D (1981) Biochemistry 20: 4229–4238

Shen, D., Huang, A., Huang, L. (1982) Biochim. Biophys. Acta 689: 31–37

Szoka, F. Jr., Papahadjopoulos, D. (1978) Proc. Natl. Acad. Sci. USA 75: 4194–4198

spleen of a BBUF rat by stimulating splenic T cells (obtained from a diabetic rat) in vitro with islet cell antigens for three days, recovering T cell blasts on a Percoll density gradient, and growing such blasts with interleukin 2 (IL-2). A similar autoreactive T cell line was derived directly from the pancreas of a diabetic BB rat. Islets of Langerhans were recovered from the pancreas by collagenase digestion, then a cell monolayer was prepared by further digestion with trypsin. T cells could be cultured from this monolayer by adding IL-2. These T cells rapidly overgrew all other cell types, and showed specificity for islet cell antigens. The isolation and long term growth of autoreactive T cell clones will allow detailed studies of the role that T cells play in the pathogenesis of IDDM.

59 RELEASE OF ACTIVE METHOTREXATE CONTAINING FRAGMENTS FROM CARRIER ANTITUMOR IMMUNOGLOBULINS. P. Uadia[1], A.H. Blair[1], T. Ghose[2], Departments of Biochemistry[1] and Pathology[2], Faculty of Medicine, Dalhousie University, Halifax, Nova Scotia, B3H 4H7.

Methotrexate (MTX) coupled to IgG antibodies against tumor-associated antigens on mouse EL4 lymphoma or human H21 melanoma cells inhibits tumor growth in vivo better than free MTX, free antibody or MTX linked to normal rabbit IgG (NRG). Either in vitro or in vivo, tumor cells accumulated more MTX when it is conjugated to antitumor antibodies than the free drug. However, free MTX is more effective in inhibiting dihydrofolate reductase (DHFR) in vitro than MTX-IgG conjugates.

To determine if a conjugate is hydrolysed releasing free MTX or fully active MTX-containing fragments, we have incubated MTX-NRG with liver homogenates and subjected the MTX-antiH21 IgG conjugate to intracellular catabolism by H21 cells in vitro. Liver homogenates produced a low molecular weight product isolated by gel and DEAE-cellulose chromatography. Catabolism was optimal at pH 4.6, activated by dithiothreitol and inhibited by antipain and tosyllysine chloromethyl ketone, implicating lysosomal enzymes. When H21 cells preloaded with MTX-antiH21 IgG were incubated in conjugate-free medium, a low molecular weight fraction containing MTX was obtained from both medium and cell homogenate. Production was inhibited by chloroquine, again implicating lysosomal enzymes. This fraction was resolved into two peaks by DEAE-cellulose chromatography. All three catabolites were as effective in inhibiting DHFR as the original conjugate.

60 PREPARATION OF TARGETED LIPOSOMES AND THEIR INTERACTION WITH HUMAN MELANOMA CELLS. A. Goundalkar, M. Nezei,[1] T. Ghose,[2] [1]College of Pharmacy and [2]Department of Pathology, Faculty of Medicine, Dalhousie University, Halifax, N.S. B3H 4H7

Liposomes, i.e. artificial phospholipid vesicles, are being widely investigated as carriers of anticancer drugs. Antitumor antibodies may improve the homing capability of drug carrying liposomes to tumor cells. In order to covalently bind IgG to liposomes, a novel lipid derivative N-[3-(2-pyridyldithio)propionyl] stearylamine (PDP-SA) was synthesized. Thiol disulfide exchange reaction was used for the linkage of IgG to liposomes. Such chemical linkage did not significantly alter the antibody activity as shown by radioimmunoassay.

The interaction between methotrexate-carrying liposomes linked to anticolorectum IgG and target melanoma cells was studied using membrane immunofluorescence. Microscopic examination revealed that larger numbers of liposomes bound to H21 cells when the liposomes were linked to anticolorectum IgG than when they were linked to nonspecific IgG. However, whether liposome-encapsulated drugs are taken up by target cells remains to be investigated.

61 LYMPH NODE METASTASIS: ULTRASTRUCTURAL STUDIES ON THE RAT 13762 MAMMARY CARCINOMA AND WALKER CARCINOMA. I. Carr and M. Levy, Dept. of Pathology, University of Manitoba and St. Boniface General Hospital, Winnipeg, Manitoba R2H 2A6.

Two types of rat tumor cells have been studied while spreading on a plastic surface in vitro; their behavior as reflected principally in their ultrastructure has been compared with the behavior of the same tumors metastasizing from the footpad to the popliteal lymph node as seen by transmission electron microscopy. It has been found that 13762 rat mammary tumor cells do not spread readily on a plastic surface and do not migrate actively through the sinusoidal wall. On the other hand Walker rat carcinoma cells protrude filopodia and spread readily on a plastic surface and also protrude filopodia and migrate readily through the sinusoidal wall, both into the pulp of the lymph node and into its capsule. These findings suggest that cell movement is important in lymph node metastasis, to a varying degree in different tumors.
Supported by National Cancer Institute of Canada and St. Boniface General Hospital Research Foundation.

62 GENERATION OF MONOCLONAL ANTIBODIES TO HUMAN RENAL CELL CARCINOMA P Dioun, S Jothy. Department of Pathology, McGill University, Montreal, P.Q. H3A 2B4.

The purpose of this study was to develop investigative and diagnostic probes to study human renal cell carcinoma (RCC). Monoclonal antibodies (MAb) were generated after immunization of mice with microsomal and lipidic fractions of fresh RCC tissue. Screening was optimized to identify cells secreting IgG antibodies based upon the difference in their reactivity with RCC and normal kidney (NK). Culture supernatants were tested by ELISA for binding of IgG to adsorbed RCC and NK tissue extracts; secreting wells were then systematically tested by indirect immunofluorescence (I.IF) on paired frozen sections of RCC and NK. Selected hybridomas, exhibiting differential staining, were then cloned and expanded by induction of ascites.

From three immunized mice the hybridoma growth rate ranged from 60 to 73%. Out of 320 microculture supernatants tested by ELISA 48 gave a high differential ratio of RCC/NK binding activity. I IF screening showed that 10 of these supernatants contained IgG antibodies with differential staining of RCC vs NK, no detectable staining of NK was found with 2 MAb. Formalin fixed paraffin embedded tissue studied by immunoperoxidase was shown to be usable with 2 MAb.

In conclusion a systematic comparison between normal and tumor tissues by immunohistochemistry on frozen sections yields sensitive and specific information which allows a highly selective screening of MAb for their potential use in investigative and diagnostic tests of RCC.

Supported in part by the Ministère de la Science et de la Technologie, Québec, the Medical Research Council of Canada and the Cedars Cancer Fund.

63 Production of a Progressive Autoimmune Nephritis in the Rat by Multiple Intravenous Injections of Heterologous Anti-rat Kidney F3 Antiserum. R. LAMARCAN, J. CORNISH and A.I. BARABAS. University of Calgary, Calgary, Alberta.

Ten rats received 5 weekly IV injections of 1 ml of a rabbit anti-rat kidney fraction 3 antibody. One control group received a single IV injection of 1 ml of the antiserum followed by four weekly injections of 1 ml normal rabbit serum. A second control group received 5 weekly injections of normal rabbit serum. The experiment was terminated at 12 weeks. The test group of animals were severely proteinuric (+ 100 mg/dl). Animals in the control groups had no proteinuria. By immunofluorescence the test groups showed intense beaded fluorescence (++++). In the control group receiving 1 ml of the antiserum there was less beaded fluorescence (++) and the second control group was negative. Gammaglobulin eluted from the glomeruli of the test animals reacted with the brush border zone of the proximal convoluted tubules of normal rat kidneys when stained for rat gammaglobulin. The two control groups were negative.

We conclude that multiple injections of the antiserum produced a progressive immune complex disease as the result of an autoimmune process.

64 ACCUMULATION OF FAT IN LIVER CELLS OF RATS GIVEN CHOLINE-METHIONINE DEFICIENT DIET. R. Padmore, A. Chouhal, T. Rushmore and E. Farber. Dept. of Pathology, University of Toronto, Toronto, Ontario, M5S 1A8.

Experimental diets deficient in choline have been reported to initially cause severe steatosis about the central vein followed eventually by fibrosis originating around the central vein. This study by Hartroft (Hartroft W.S. (1950) Anat. Rec. 106,61), was done at a time when the diet preparation used may well have contained significant amounts of aflatoxin and/or may have been deficient in folic acid and Vitamin B_{12}.

In contrast, we report the accumulation of fat in a periportal distribution, with relative sparing of Zone III hepatocytes, in Fischer 344 rats fed a diet deficient in choline and low in methionine. This preferential Zone I fat accumulation was most marked in the first week of feeding the diet. Long term follow up of animals on this diet for 12-29