Advanced Drug Delivery Reviews, 3 (1989) *267-282*
Elsevier

(14)

ADR 00036

Generating and loading of liposomal systems for drug-delivery applications

P.R. Cullis, L.D. Mayer, M.B. Bally, T.D. Madden and M.J. Hope

The Canadian Liposome Company, North Vancouver, and Biochemistry Department, University of British Columbia, Vancouver, Canada

(Received June 24, 1988)
(Accepted July 5, 1988)

Key words: Liposome; Cancer; Doxorubicin Extrusion; Freeze-thawed liposome ; Passive drug encapsulation; Active drug encapsulation: Remote loading

---

Contents

Summary 268

I. Introductio 268

If. Factors influencing the design of liposomal system s 269
1. Liposome pharmacokinetic ..s 270
2. Mechanism of action of liposomal pharmaceuticals 270
3. Implications for liposomc design 271

III. Methods of generating liposome ..s 271
1. Generation of multilamellar vesicle s 273
2. Generation of large unilametlar vesicle ....s 274
3. Generation of small unilamellar vesicle s 2 7 4

IV. Methods of loading liposomes with drug .s 275
1. Variables in drug loadin g 276
2. Drug loading by passive trapping procedure s 276
3. Drug loading by active trapping procedure ....s 279

Abbreviations: MTP-PE, muramyltripeptid phosphatidylethanolamine; MDP, muramyldipeptide; RES, reticuloendothelial system; MLV. multilamellar vesicle; LUV, large unilamellar vesicle; SUV, small unilametlar vesicle;, FATMLV, frozen and thawed multilamellar vesicle.

Correspondence: P.R. Cullis, The Canadian Liposome Company, Suite 308, 267 West Esplanade, North Vancouver, B.C., Canada V7M 1A5.

0169-409X/89/$03.50 © 1989 Elsevier Science Publishers B.V. (Biomedical Division)

V. Conclusions ... 279
Acknowledgements ... 280
References ... 280

Summary

Techniques involved in generating liposomal-drug systems in a manner compatible with clinical demands are reviewed; Recent advances include extrusion procedures for the rapid and reproducible generation of liposomes and new techniques for the efficient and stable entrapment of drugs at high drug/lipid ratios. Notable examples include the freeze-thaw protocol which can allow drug-trapping efficiencies approaching 90%. Active trapping procedures, utilizing drug uptake in response to ion gradients, can result in extremely high drug/lipid ratios and trapping efficiencies approaching 100%. These and other advances suggest no major difficulties for the manufacture of liposomal drug systems for pharmaceutical applications.

I. Introduction

Since liposomes were initially characterized over 20 years ago, their potential as drug delivery vehicles has been proposed. However, it is only within the last five years that this potential has begun to be realized There are two major reasons for this delay. First, specific therapeutie potentials of liposomal drug delivery are only now becoming apparent. The second reason, which is the subject of this review, is that techniques for generating and loading liposomes in a manner, compatible with pharmaceutical applications have only recently 'been developed.

In order to understand the requirements for liposome generation and loading, it is useful to outline briefly the evolution of liposomes as delivery systems and the likely characteristics of the first wave of liposomal pharmaceuticals. A major thrust of early work was to design liposomes that could be specifically targeted to a particular disease site, employing antibodies or other targeting agents linked to the vesicle exterior. However, this objective has not yet been achieved, mainly due to the rapid uptake of liposomes by the fixed and free macrophages of the reticuloendothehal system, which results in accumulation by organs such as the liver and spleen [1]. The major, serendipitous observation that has led to imminent applications is that significant therapeutic benefits can be achieved by virtue of the different biodistributions achieved by non-targeted liposomal systems. These benefits are primarily reductions in toxicity (while maintaining or increasing efficacy) or reflect benefits arising from. the passive targeting of liposomally encapsulated bioactive molecules.

The three liposomal drug preparations which are currently in clinical trials offer excellent examples of the therapeutic benefits of liposomal drug delivery and the

types of formulations that are required. The first of these is liposomal amphotericin B, developed by Lopez-Bereitein and coworkers [2]. Amphotericin B is, an effective antifungal agent which is also nephrotoxic. Liposomal encapsulation buffers this and other acute toxicities (as indicated by an increase in the LD, from 2 mg per kg to as high as 30 mg per kg [3]) while efficacy is maintained. This leads to a large increase in therapeutic index. With regard to formulation, amphotericin B is a hydrophobic drug which associates with the lipid bilayers of liposomes rather than the interior aqueous space.

The second liposomal formulation in clinical trials is liposomal muramyl tripeptide phosphatidylethanolamine (MTP-FE) [4]. This formulation has evolved from work-on the water soluble compound muramyl dipeptide (MDP) which is a minimal component of bacterial cell membranes which can activate macrophages. Activated macrophages recognize and kill tumour cells in vitro and the liposomal formulations of these. and other macrophage activating factors have demonstrated efficacy in the treatment of metastatic cancer models [S]. The MTP-PE analogue exhibits superior macrophage-activating abilities and can be readily entrapped in liposomes as part of the lipid bilayer:

A third liposomal drug formulation in clinical trials employs the anticancer drug doxorubicin. Doxorubicin is the most widely employed drug in cancer chemotherapy, activeagainst a wide range of solid and ascitic tumours. However, administration of this drug causes serious acute toxicities, including myelosuppression and gastrointestinal toxicity as well as a cumulative, dose limiting cardiotoxicity [6]. The acute toxicity of doxorubicin (as reflected by LD_{50} values),and the cardiotoxicity can be ameliorated by delivery in liposomes while anticancer efficacy is maintained or even increased [7]. This drug, which is soluble to some extent in -both aqueous and membrane environments, represents a third kind of encapsulation problem.

In summary, the first generation of liposomal formulations of drugs which result in reduced toxicity, enhanced efficacy or which act to stimulate immune defenses are in advanced stages of development. As indicated for the liposomal preparations summarized above, these drugs exhibit quite different physical properties. These properties markedly influence the design and method of loading of the liposomal carriers, a theme that is further developed in the following sections.

II. Factors influencing the design of liposomal systems

Within the context of drug delivery systems, liposomes possess two general characteristics which make them particularly useful. First, these carrier systems are biocompatible and nontoxic. Second, they are remarkably flexible. Liposomes can be large or small (0.025-10 μm diameter) and can have ‘a large variety of lipid compositions which markedly affect drug retention and stability properties.

Variations in liposome size, lipid composition and drug-to-lipid ratio can markedly affect the therapeutic benefits. arising from liposomal drug delivery (Refs.. 3 and 9; see also Mayer, L,D., ‘Tai, L.C., Ko, D.S.C., Masin, D., Ginsberg, R.S., Cullis, P.R. and Bally, M.B., unpublished results).This obviousIy places demands

on the type of liposomal-drug complex that one may wish to generate. Here we briefly review the pharmacokinetics and mechanism of action of liposomal drug formulations and the resulting implications for liposome design.

II.1. *Liposome pharmacokinetics*

As indicated above, intravenously injected liposomes are eventually sequestered into the organs and fixed and free phagocytic cells (macrophages) of the reticuloendothelial system (RES). Thus liposomes accumulate primarily in the liver and spleen and, to a lesser extent, in the lung, lymph nodes and bone marrow. Within this overall picture, however, different liposomes can exhibit quite different behaviour. First, in the absence of cholesterol, liposomes leak substantially when introduced intravenously. This can be largely inhibited by the presence of cholesterol [1] and long-chain saturated lipids;' **Second,** for the same lipid dose, smaller vesicles are cleared more slowly than their large counterparts. Blood residence times can be increased from minutes to hours for large (greater than 0.4 μm) liposomes as compared to small (smaller than 0.025 μm) systems [10]. Third, circulation times are sensitive to the lipid dose, higher doses lead to longer circulation times. For 0.1 μm systems, for example,, the 'circulation half-time increases from 20 min to 3 h on increasing the lipid dose from 0.4 to 40 mg per kg [10,11]. Finally, charged liposomal systems are cleared more rapidly 'than uncharged systems, which may be related to their greater affinity for serum proteins [12].

The implications of these observations are clear in that different types of liposomes are required for different applications. Large charged systems may provide optimum delivery to organs such as the liver and spleen, for example, whereas small, stable, neutral liposomes may be more useful in applications requiring slow extended leakage in the circulation. It would appear. however, that the presence of cholesterol is invariably required to prevent leakage of water-soluble compounds.

11.2. *Mechanism of action of liposomal pharmaceuticals*

This is an area of considerable interest and controversy. The central problem is inherent in the increasingly general observation that liposomal encapsulation of drugs can reduce the acute toxicity of the drug while maintaining or even increasing efficacy. The reduction in toxicity is straightforward to rationalize in that encapsulation of a drug in a carrier would be expected to reduce availability to susceptible tissues, reducing toxic effects. The reasons why efficacy is maintained are more difficult to understand, and have been proposed to involve benefits involved in extended drug payout, uptake by macrophages either associated with a disease site or subsequently mobilized to it or the presence of a leaky vasculature at the disease site which results in preferential sequestration of liposomes at the desired location. Whatever the mechanism, there is increasingly compelling evidence supporting preferential accumulation of liposomally encapsulated molecules at sites of inflammation [13], infection [14] and certain solid tumours

11.3. Implications for liposome design

There are three major variables in the design of liposomal drug carriers. These include liposome size, lipid composition and drug-to-lipid ratio. In the absence of a general understanding of the mechanisms whereby liposome delivery is of therapeutic benefit, it is presently difficult to make general statements regarding the properties of a therapeutically optimized liposomal drug delivery system. Optimum methods of liposome generation and loading will therefore exhibit considerable flexibility with regard to size, lipid composition and drug content to allow optimization for a particular application.

III. **Methods of generating liposomes**

The three major categories of liposomes are illustrated in Fig. 1. These include multilamellar vesicles (MLVs) which contain two or more concentric lamellae arranged in an onion skin configuration and which can range in size from 0.2 to 10 μm. Large unilamellar vesicles (LUVs) on the other hand have a single bilayer and a size distribution which usually falls in the range 0.05-0.2 μm. Small unilamellar vesicles (SUVs), which are often referred to as 'limit size' vesicles can range in size from 0.02 to 0.05 μm. The common procedures for generating these lipo-

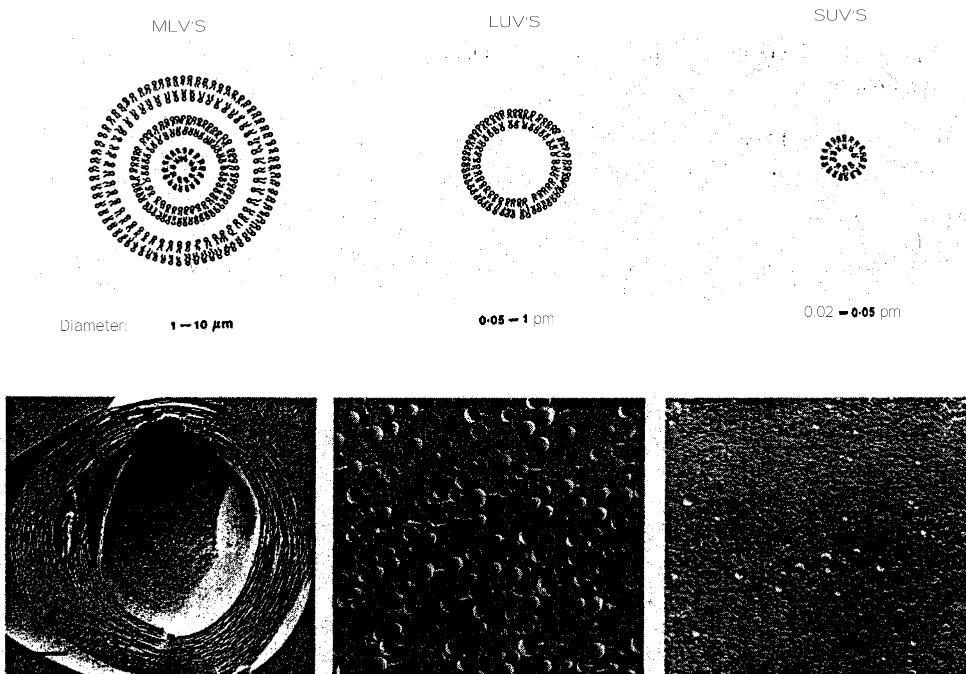

Fig. 1. Egg phosphatidyicholine multilamellar vesicles (MLV), large unilamellar vesicles (LUV) and small unilamellar vesicles (SUV) visualized by freeze-fracture electron microscopy. The bar represents 200 nm.

TABLE I

COMMON PROCEDURES FOR THE GENERATION OF MULTILAMELLAR AND UNILAMELLAR VESICLES

Category and technique	Type	Trapped volume (l/mol lipid)	Advantages	Disadvantages	Refs.
Direct hydration					
aqueous medium added to dry lipid	MLV	0.5	fast procedure	low trapped volume: low trapping efficiency; unequal distribution of solute	17,18
plus freeze-thaw	FATMLV	5-10	fast procedure; high trapped volume; high trapping efficiency	solute-dependent	17,21
MLV extruded through 0.1 m polycarbonate filter	LUV	1-2	high trapping efficiency for extrusion techniques; no detergents or solvents used; fast procedures	trapped volumes relatively low unless freeze-thaw protocol is employed	20
plus freeze-thaw	LUV	1-10	idem	idem	23,24,34,40,41
	suv	0.2-0.5	idem	idem	
press plus freeze-thaw		1-10	idem	idem	
Hydration from organic					
SPLV technique	MLV	1-10	high trapping efticiency	limited by lipid solubility in organic phase; residual organic solvent	17
reverse-phase evaporation	LUV	10	idem	idem	22
ether evaporation	LUV	lo-20	idem	idem	
Detergent removal					
cholate (deoxycholate) dialysis/gel filtration	LUV	0.5-5	reconstitution of proteins possible; high trapped volumes	detergents difficult to remove completely; procedures lengthy; generally low trapping efficiency limited to certain lipid mixtures	34,35
octylglucoside	LUV	10	idem	idem	

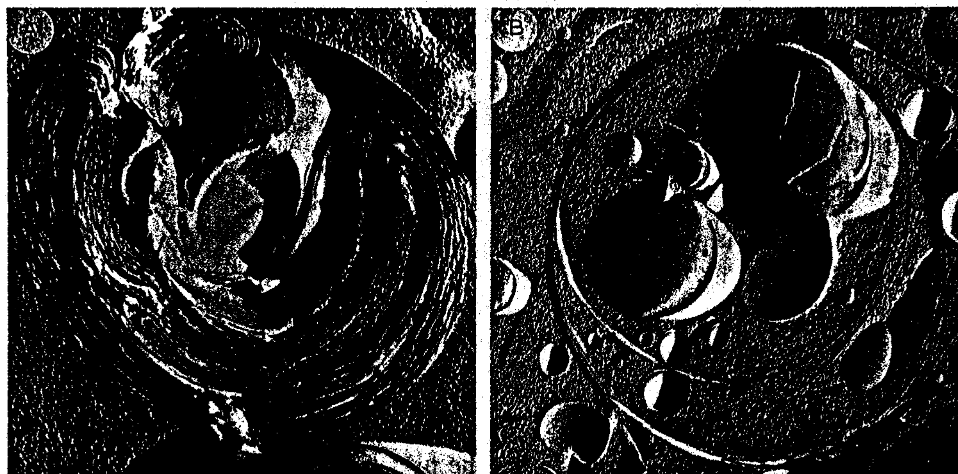

Fig. 2. Freeze-fracture -electron micrograph of egg phosphatidylcholine MLVs (A) before and (B) after five freeze-thaw cycles. The bar represents 140 nm. For further details, see Ref. 18.

somes are summarized in Table I and include three general techniques. Two of these involve the initial solubilization of lipids in organic solvents or detergents which must be removed subsequently, whereas the third includes those procedures which do not require solubilizing agents.

III.1 *Generation of multilamellar vesicles*

The classical procedure for generating MLVs involves the simple addition of water to a lipid film followed by dispersal by mechanical agitation [16]. Recently, it has been shown that this technique gives rise to low trapping efficiencies for water-soluble agents [17,18]. Specifically, the concentrations of solute in the aqueous interbilayer spaces inside the liposomes can be substantially lower than in the external bulk aqueous phase, referred to as non-equilibrium solute distributions.

TABLE II

TRAPPED VOLUMES AND TRAPPING EFFICIENCIES OF FROZEN AND THAWED MLV (FATMLV) AT VARIOUS LIPID CONCENTRATIONS (EGG PC)

Egg PC MLV were prepared by mechanical dispersion of dry lipid in the presence of an aqueous buffer. The FATMLVs were prepared employing five freeze-thaw cycles. For details, see Ref. 18

Sample	Lipid concn. (mg/ml)	Trapped volume (μl/μmol lipid)	Trapping efficiency
MLV	100	0.47 ± 0.03	5.8
FATMLV	50	5.02 ± 0.04	31.3
FATMLV	100	5.27 ± 0.17	65.9
FATMLV	200	3.07 ± 0.05	76.7
FATMLV	400	1.77 ± 0.09	88.6

Dramatic increases in the trapping efficiencies (defined as the percentage of aqueous solute that is entrapped) and trapped volumes can be achieved by simple procedures such as freeze-thawing [18] of the preformed liposomes which produces FATMLVs as illustrated in Fig. 2. As shown in Table II, the use of high lipid concentrations in conjunction with freeze-thaw techniques can result in trapping efficiencies as high as 88%.

MLVs with equilibrium solute distributions and high trapping efficiencies can also be obtained by the use of 'reverse phase' procedures; which involve the initial solubilization of the lipid in organic solvent, which is subsequently diluted or evaporated in the presence of aqueous buffer [17].

III.2. *Generation of large. unilamellar vesicles*

Generation, of LUVs can now be conveniently accomplished without the use of organic solvents or solubilizing agents. The primary technique involves extrusion of preformed MLVs through polycarbonate filters with defined pore size. As originally practised [19] this involved the sequential extrusion of vesicles through gradually decreasing pore size under low pressure (less than 550 kPa). More recently, a rapid extrusion procedure employing a purpose-built high-pressure extrusion device has been developed [20,21]. This procedure allows the direct extrusion of preformed MLVs at pressures up to 5.5 MPa through filters with pore size of 0.03 pm or larger, giving rise to the rapid production of reproducible LUV preparations in the size range of 50-200 nm (see Fig. 3). Further, high lipid concentrations (up to 400 mg/ml) can be employed, enabling trapping efficiencies of 60% to be achieved for 100 nm systems [21]. LUVs can also be produced by the injection of lipids solubilized in ethanol or ether into an aqueous buffer (for a review, see Ref. 22). These latter procedures suffer certain drawbacks, including a lack of reproducibility and the often limited solubility of certain lipids in the organic solvent. Diethyl ether, petroleum ether, pentane or methanol can be used to overcome such problems [22]. However, given the general applicability of the extrusion process and the difficulties involved in subsequently removing solvent, these procedures are increasingly less attractive.

III.3. *Generation of small unilamellar vesicles*

Small unilamellar vesicles (SUVs) can be produced by sonication [23], French press [24] and homogenization [25] procedures. The small size of these systems (e.g., 0.025 μm for egg phosphatidylcholine) results in very small trapped volumes and trapping efficiencies (see Table I) and can exhibit instability, fusing to form larger structures. For drug delivery or model membrane studies it is therefore generally preferable to employ LUV systems.

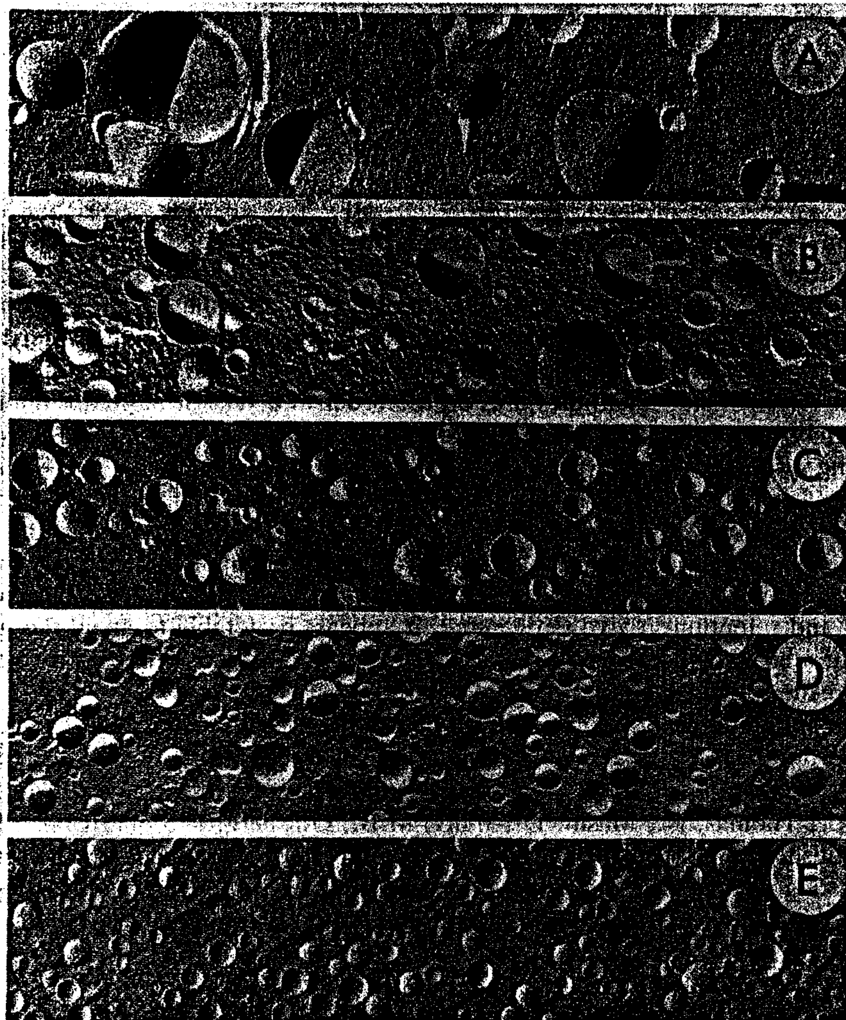

Fig. 3. Freeze-fracture electron micrographs of frozen and thawed egg phosphatidylcholine MLVs passed 20 times through filters of various pore sizes. The pore sizes of the filters were (A) 400 nm, (B) 200 nm, (C) 100 nm, (D) 50 nm and (E) 30 nm. The bar in panel A represents 150 nm. For further details, see Ref. 21.

IV. Methods of loading liposomes with drugs

The physical and chemical properties of drugs vary considerably. Here we consider drugs which are relatively soluble in an aqueous environment. Techniques for trapping insoluble hydrophobic drugs are not considered in detail. In general it should be noted that such hydrophobic drugs (e.g., amphotericin B, steroids and some alkylating agents) can usually be co-solubilized in an organic solvent with the

lipid, and subsequently dispersed in aqueous buffer either after removing the solvent or by a reverse-phase procedure. Trapping efficiencies of 100% are usually achievable; however, the drug/lipid ratio and lipid composition required for a stable preparation can vary significantly.

IV.1. Variables in drug loading

Primary variables to consider.are trapping efficiency, drug retention and drug-to-lipid ratio. With regard to trapping efficiencies,. an optimum loading procedure would trap 90% or more of available drug, thus avoiding the need to remove untrapped material subsequently. The need for drug retention, on the other hand, may vary according to the application and other considerations. For storage purposes, for example, retention times on the order of a year or more are required.' This may be achieved by employing dehydrated preparations (26,271 or by employing systems with high (more than 90%) passive trapping efficiencies as may be achieved for high lipid concentrations. On the other hand, in vivo characteristics may require release half-times ranging from hours to days. As previously indi cated, this can be approached by employing different lipid compositions, where the inclusion of cholesterol or long-chain saturated lipids will lead to enhanced stability and retention in vivo. In general, it appears preferable to avoid the use of unsaturated, negatively charged lipids such as phosphatidylserine and cardiolipin due to their high cost and often labile nature.

The optimum drug-to-lipid ratio will be dictated by therapeutic efficacy and toxicity properties. There are indications that high drug lipid ratios lead to reduced toxicity for amphotericin B [3] and doxorubicin preparations (Mayer, L.D., Ko, D.S.C., Thomas, N., Masin, D., Bally, M.B., Ginsberg, R.S. and Cullis, P.R., unpublished results); however, there is currently insufficient evidence to establish this as a general trend. From the practical point of view, the highest drug-to-lipid ratios are clearly preferable due to the reduced lipid requirements.

IV.2. *Drug loading by passive trapping procedures*

Passive trapping procedures include all techniques where the lipid and drug are codispersed in an aqueous buffer, thus achieving entrapment while the liposomes are being formed. A synopsis of the trapping efficiencies available for a variety of water-soluble drugs is presented in Table III. In general, for procedures which do not involve organic solvents or detergents, it may be noted that trapping efficiencies are usually less than 50% and often less than 10%. Only the freeze-thaw [18] and dehydration-rehydration procedures [44] can provide trapping efficiencies approaching 90%, and that only where high lipid concentrations are used. Thus unless the agent to be entrapped is extremely water soluble (e.g., 100 mg/ml) high drug-to-lipid ratios are difficult to achieve in conjunction with reasonable trapping efficiencies for passive trapping procedures. Maximum trapping efficiencies for procedures requiring the use of organic solvents such as the stable pleurilamellar vesicle [17] MLV preparations can exceed 50% as can reverse-phase evaporation procedures

TABLE III

TRAPPING PROPERTIES OF LIPOSOME FORMULATIONS

Vesicle type	Preparation procedure	Vesicle diameter (pm)	Entrapped agent	Trapping efficiency (%)	Refs.
SUV	sonication	0.025-0.040	cytosine arabinoside methotrexate, carboxyfluorescein	1-5	38,39
SUV	French press	0.020-0.050	carboxyfluorescein, inulin, trypsin, bovine serum albumin	5-25	40
SUV	detergent removal	0.036-0.050	carboxyfluorescein, inulin	12	41
LUV	detergent removal	0.1-10.0	inulin, cytochrome c, carboxyfluorescein	12-42	36,41,42
LUV	reverse-phase	0.2-1 .0	carboxyfluorescein, cytosine, arabinoside, 25 sRNA, DNA, insulin, albumin	28-45	38
LUV	solvent evaporation	0.1-0.5	inorganic phosphate, chromate, glucose, soy bean trypsin inhibitor, DNA	2-45	22,43
LUV	extrusion	0.056-0.2	^{22}Na, inulin, methotrexate, cytosine arabinoside, DNA	15-60	20,21
MLV	mechanical mixing	0.4-3.5	^{22}Na, carboxyfluorescein, glucose, albumin, DNA	1-8.5	44
MLV	sonicate-freeze-thaw	0.17-0.26	asparaginase	50-56	44
MLV	freeze-thaw	0.5-5.0	^{22}Na, inulin	35-88	18
MLV	sonicate-dehydrate-rehydrate	0.3-2.0	carboxyfluorescein, sucrose albumin, factor III, ATP, vincristine, melphalan	27-54	45
MLV	solvent-evaporation-sonication	0.3-2.0	inulin, streptomycin sulfate, chloramphenicol, oxytetracycline, sulfamerazine	6.3-38	17

TABLE IV

CHARACTERISTICS OF LIPOSOME ENCAPSULATED DOXORUBICIN PREPARATIONS
PC, phosphatidylcholine; PS, phosphatidylserine; C, cholesterol; CL, cardiolipin; DCP, dicetylphosphate; SA, stearylamine; PG, phosphatidylglycerol; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearylphosphatidylcholine; n.d., not determined

Liposome type	Size (nm)	Composition	Ratio (mol/mol)	Drug/Lipid (wt/wt)	Trapping efficiency (%)	Refs.
Preparations employing passive trapping procedures						
SUV	135 ± 70	PS/PC/C	3:7:10	0.05:1	25	46
MLV	n.d.	PC/C	1:1	0.028:1	14	53
MLV	n.d.	PC		0.022: 1	10	
MLV	n.d.	CL/PC/C	1:4:5	0.039:1	62	
MLV	n.d.	CL/PC	1:4	0.040:1	58	
MLV	n.d.	PS/PC/C	3:7: 10	0.040: 1	42	
SUV	n.d.	PC/C	1:1	0.066:1	15	
SUV	n.d.	CL/C	5:2:5	0.027:1	90	
SUV	n.d.	CL/PC/C	1:4:2	0.033: 1	47	
SUV	n.d.	CL/PC/C	1:4:5	0.031:1	45	
SUV	n.d.	PS/PC/C	3:7:10	0.021:1	22	
SUV	n.d.	PC/C	7:2	0.006:1	6.6	48
SUV	n.d.	PC/C/DCP	7:2:1	0.021:1	25.7	
SUV	n.d.	PC/C/SA	7:2:1	0.004:1	4.0	
SUV	n.d.	PC/C/PS	0:4:1	0.069:1	55:	49
SUV	n.d.	PC/C/SA	10:4:3	0.049:1	35	
SUV	90 ± 20	CL/PC/C/SA	1:5:3.5:2	0.068:1	55	50
LUV	150	PG/DCP/C	1:4:5	0.031:1	50	51
LUV	300	PC/PS/C	10:1:4	0.039: 1	57	52
LUV	730	DPPC/DPPG/C	10:1:10	0.022: 1	27	
Preparation employing active trapping procedures						
LUV	158 ± 37	PC		0. 29:1	>99.0	32
LUV	10 ± 28	PC/C	55:45	0.251	98.8	
MLV	1440 ± 400	PC/C	55:45	0.28:1	>99.0	
LUV	773 ± 140	DSPUC	55:45	0.281	98.7	
LUV	192 ± 67	PC/PG/C	0.95:0.05:1	0.30:1	>99.0	

In the case of the large category of drugs which are lipophilic and positively charged (lipophilic cations), higher trapping efficiencies, drug-to-lipid ratios and enhanced retention can be achieved by incorporating acidic (negatively charged) lipids into the liposome carrier. This procedure has been explored most extensively for the antineoplastic agent doxorubicin (adriamycin). As summarized in Table IV, trapping efficiencies can be enhanced from 10% or less for neutral phosphatidylcholine systems to greater than SO%, particularly when cardiolipin is employed. This is accompanied by an enhancement in the drug-to-lipid ratios, to values as high as 0.07:1 (wt/wt).

IV.3. Drug loading by active trapping procedures

Active trapping procedures refer to those techniques where the drug is loaded after the liposomes have been formed. In such procedures liposomes exhibiting a membrane potential ($\Delta\psi$) or transmembrane pH gradients are generated, which can subsequently accumulate many drugs which are lipophilic amines. For drug-delivery applications, loading in response to pH gradients (inside acidic) is the preferable procedure as ionophores, such as the K+ ionophore valinomycin (a potentially toxic compound), are required to establish $\Delta\psi$ [29]:Uptake in response to pH gradients is relatively straightforward to perform [30], involving manufacturing the liposomes in a low pH buffer (e.g., sodium citrate, pH=4.0) and subsequently adjusting the external pH to 7 or higher. This can be accomplished directly by the addition of base to the liposome solution or by exchanging the external medium for a high pH buffer. Uptake of lipophilic cations is then simply achieved by addition of drug and a short incubation, and has been most thoroughly explored for doxorubicin where drug uptake levels as high as 0.29 to 1 (drug to lipid, wt/wt) can be achieved in combination with trapping efficiencies of 98% or higher (see Table IV). Although the mechanism involved in the case of uptake in response to $\Delta\psi$ is not yet clear 131], uptake in response to ΔpH is consistent with uptake of the neutral form of the drug. Equilibrium levels corresponding to $[drug]_{in}/[drug]_{out} = [H^+]_{in}/[H^+]_{out}$ are then predicted. Thus for a pH gradient of three units, interior concentrations 1000-fold higher than exterior concentrations are achievable, with excellent drug retention properties (Ref. 30; see also Mayer, L.D., Tai, C.L.C., Bally, M.B., Mitilenes,, G.N., Ginsberg, R.S. and Cullis, P.R., unpublished results).

Uptake in response to $\Delta\psi$ or ΔpH has been demonstrated for a range of lipophilic amino-containing drugs in addition to doxorubicin, including vinblastine [29], dibucaine [31], dopamine [33], serotonin [33], epinephrine [33] among others. Such uptake is independent of lipid composition, and the advantages of high drug/lipid ratios, excellent trapping efficiencies and enhanced retention properties suggest that this is the technique of choice **for** appropriate drugs. Further, the fact that the-drugs can be entrapped subsequent to formation of the liposome allows encapsulation to be performed immediately prior to use, avoiding the requirement for retention during extended storage periods.

V. Conclusions

This review presents a brief summary of recent advances in the generation and loading of liposomes for drug delivery applications. As indicated in Section III, the properties of an optimum delivery system in terms of size, lipid composition and drug-to-lipid ratio are not yet known. It is likely that different formulations will be required for different applications. However, it should be clear that means now exist to generate and load liposomal systems with considerable flexibility. In the opinion of the authors, there are no major conceptual difficulties involved in adapting the liposome delivery vehicle to exhibit characteristics appropriate to a

given application. The next challenge is to determine the characteristics of those delivery systems which provide optimized therapeutic benefits.

Acknowledgements

The research of the authors is supported by the Medical Research Council of Canada, The National Cancer Institute of Canada and The Liposome Company, Inc.

References

1 Gregoriadis, G. (1976) The carrier potential of liposomes in biology and medicine, N. Engl. J. Med. 295, 705-707.
2 Lopez-Berestein, G. and Juliano, R.L. (1987) Application of liposomes to the delivery of antifungal agents. In: M.J. Ostro (Ed.), Liposomes: From Biophysics to Therapeutics, Marcel Dekker, New York, pp. 253-276.
3 Janoff, A.S., Boni, L.T., Popescu, M.C., Minchey, S.R., Cullis, P.R., Madden, T.D., Taraschi, T., Gruner, S.M., Shyamsunder, E., Tate, M.W., Mendelsohn, R. and Bonner, D. (1988) Lipid stabilized amphotericin B. Aggregates: a molecular rationale for toxicity attenuation, Proc. Natl. Acad. Sci. USA, in press.
4 Fidler, I.J., Jessup, J.M., Fogler, W.E., Staerkel, R. and Mazumder, A. (1986) Activation of tumoricidal properties in peripheral blood monocytes of patients with colorectal carcinoma, Cancer Res. 46, 994-998.
5 Fidler, I.J., Raz, A., Fogler, W.E., Bugelshi, P. and Poste, G. (1980) Design of liposomes to improve delivery of macrophage augmenting agents to alveolar macrophage, Cancer Res. 40,4460-70.
6 Lum, B.L., Svec, J.M. and Forti, F.M. (1985) Doxorubicin: alteration of dose scheduling as a means of reducing cardiotoxicity, Drug Intelligence Clin. Pharm. 19,259-272.
7 Rahman, A., White, G., More, N. and Schein, P.S. (1985) Pharmacological toxicological and therapeutic evaluation in mice of doxorubicin entrapped in cardiolipin liposomes, Cancer Res. 45, 796-804.
8 Reference deleted.
9 Balazsovits, J., Mayer, L.D., Bally, M.B.,Cullis, P.R., Ginsberg, R.S.'and Falk, R. (1989) Analysis of the effect of liposome encapsulation on the vesicant properties, acute and cardiac toxicities and antitumour efficacy of doxorubicin, Cancer Chemother. Pharmacol. 23, 81-86.
10 Senior, J., Crawley, J.C.W. and Gregoriadis, G. (1985) Tissue distribution of liposomes exhibiting long half-lives in the circulation after intravenous injection, Biochim. Biophys. Acta 839, l-8.
11 Sommerman, E.F. (1986) Factors influencing the biodistribution of liposomal systems, Ph.D. Thesis, University of British Columbia, Vancouver.
12 Juliano, R.L. and Lin, G. (1980) The interaction of plasma proteins with liposomes: Protein binding and affects on the clotting and complement systems. In: H.J. Baldwin and H.R. Six (Eds.), Liposomes and Immunology, Elsevier/North-Holland, Amsterdam, pp. 49-84.
13 Williams, B.D., O'Sullivan, .M., Saggu, G.S., Williams, K.E., Williams, L.A. and Morgan, J.R. (1987) Synovial accumulation of technetium-labelled liposomes in rheumatoid arthritis,Ann. Rheum. Dis. 46, 314-318.
14 Morgan,J.R., Williams, L.A. and Howard, C.B. (1985) Technetium-labelled penetration of ceftriaxone into synovial fluid of the inflamed joint, Br. J. Radiol. 58, 35-41.
15 Profitt, R.T., Williams, L.E., Presant, C.A., Tin, G.W., Uliana, J.A., Gamble, R.C. and Baldeschwieler, J.D. (1983) Liposomal blockade of the reticuloendothelial system: improved tumor imaging with small unilamellar vesicles, Science 220, 502-505.
16 Bangham, A.D., Standish, M.M. and Watkins, J.C. (1965) Diffusion of univalent ions across the lamellae of swollen phospholipids, J. Mol. Biol. 13, 238-252.
17 Gruner, S.M., Lenk, R.P., A.S. and Ostro, M.J. (1985) Novel multilayered lipid vesicles:

Comparison of physical characteristics of multilamellar liposomes and stable pleurilamellar vesicles, Biochemistry 24, 2833-2842.
18 Mayer, L.D., Hope, M.J., Cullis, P.R. and Janoff, A.S. (1985) Solute distributions and trapping efficiencies observed in freeze-thawed multilamellar vesicles, Biochim. Biophys. Acta 817,193-196.
19 Olson, F., Hunt, C.A., Szoka, F.C., Vail, W.J. and Papahadjopoulos. D. (1979) Preparation of liposomes of defined size distribution by extrusion through polycarbonate membranes, Biochim. Biophys. Acta 557, 9-23.
20 Hope, M.J., Bally, M.B., Webb, G. and Cullis, P.R. (1985) Production of large unilamellar vesicles by a rapid extrusion procedure: characterization of size, trapped volume and ability to maintain a membrane potential, Biochim. Biophys. Acta 812, 55-65.
21 Mayer, L.D., Hope, M.J. and Cullis, P.R. (1986) Vesicles of variable sizes produced by a rapid extrusion procedure, Biochim. Biophys. Acta 858, 161-168.
22 Deamer, D.W. (1984) Preparation of solvent vaporization liposomes. In: G.,Gregoriadis (Ed.); Liposome technology, Vol. 1, CRC Press, Boca Raton, FL, pp. 29-35.
23 Huang, C.H. (1969) Studies on phosphatidylcholine vesicles.. Formation and physical characteristics, Biochemistry 8, 344-351.
24 Barenholz, Y., Amselem, S. and Lichtenberg, D. (1979) A new method for preparation of phospholipid vesicles (liposomes), FEBS Lett. 99,210-215.
25 Hirota, S. and Kikuchi, M. (1985) Lipid membrane structures incorporating a lactose mono fatty acid ester or amide for use as drug carrier, Japanese Patent No. 7934-1985.
26 Crowe, L.M., Crowe, J.H., Rudolph, A., Womersley, C. and Appel, L. (1985) Preservation of freeze-dried Iiposomes by trehalose, Arch. Biochem. Biophys. 242, 240-250.
27 Madden, T.D., Bally, M.B., Hope, M.J., Cullis, P.R., Schieren, H.P. and Janoff, A.S. (1985) Protection of large unilamellar vesicles by trehalose during lyophilization: retention of vesicle contents, Biochim. Biophys. Acta 817, 67-75.
28 Szoka, F.C. and Papahadjopoulos, D. (1978) Procedure for preparation of Iiposomes with large internal aqueous space and high capture by reverse-phase evaporation, Proc. Natl. Acad. Sci. USA 75, 4194-4198.
29 Bally, M.B., Hope, M.J,, van Echteld, C.J.A. and Cullis, P.R. (1985) Uptake of Safranine and other lipophilic cations into vesicles in response to a membrane potential, Biochim. Biophys. Acta 812,66-76.
30 Mayer, L.D., Bally, M.B. and Cullis, P.R. (1986) Uptake of adriamycin into large unilamellar vesicles in response to a pH gradient, Biochim. Biophys. Acta 857, 123-126.
31 Mayer, L.D., Wong, K.F., Menon, K., Chong, C., Harrigan, P.R. and Cullis, P.R. (1988) Influence of ion gradients on the transbilayer distribution of dibucaine in large unilamellar vesicles, Biochemistry 27, 2053-2060.
32 Reference deleted.
33 Bally, M.B., Mayer, L.D., Loughrey, H., Redelmeier, T., Madden, T.D., Wong, K., Hope, M.J. and Cullis, P.R. (1988) Dopamine accumulation in large unilamellar vesicle systems by transmembrane ion gradients, Chem. Phys. Lipids 47, 97-107.
34 Enoch, H.G. and Strittmatter (1979) Formation and properties of **1000-Å-diameter,** single-bilayer phospholipid vesicles, Proc. Natl. Acad. Sci. USA 76, 145-149.
35 Kagawa, Y. and Racker, E. (1971) Partial resolution of the enzymes catalyzing oxidative phosphorylation part 15 reconstitution of vesicles catalyzing phosphorus-32 ortho phosphate ATP exchange, J. Biol. Chem. 246, 5477-5487.
36 Mimms, L.T., Zampighi, G., Nozaki, Y., Tanford, C. and Reynolds, J.A. (1981) Phospholipid vesicle formation and transmembrane protein incorporation using octyl glucoside, Biochemistry 20, 833-840.
37 Helenius, A., Fries, E. and Kartenbeck, J. (1977) Reconstitution of Semliki forest virus membrane, Cell Biol. 75, 866880.
38 Szoka, F. and Papahadjopoulos, D. (1980) Comparative properties and methods of preparation of lipid vesicles (liposomes), Annu. Rev. Biophys. Bioenerg. 9, 467-508.
39 Mayhew, E., Lazo, R. and Vail, W.J. (1984) Preparation of liposomes entrapping cancer chemotherapeutic agents for experimental in vivo and in vitro studies. In: G. Gregoriadis (Ed.), Liposome Technology, Vol. 2, CRC Press, Boca Raton, FL, pp. 19-31.

40 Lelkes, P.I. (1984) The use of french pressed vesicles for efficient incorporation of bioactive macromolecules and as drug carriers in vitro and in vivo. In: G. Gregoriadii (Ed.), Liposome Technology, Vol. 1, CRC Press, Boca Raton, FL, pp. 51-65.
41 Weder, H.G. and Zumbuehl, 0. (1984) The preparation of variably sized homogeneous liposomes for laboratory, clinical, and industrial use by controlled detergent dialysis. In: G. Gregoriadis (Ed.), Liposome Technology, Vol. 1, CRC Press, Boca Raton, FL, pp. 79-107.
42 Cudd, A. and Nicolau, C. (1984) Entrapment of recombinant DNA in liposomes and its transfer and expression in eukaryotic cells. In: G. Gregoriadis (Ed.), Liposome Technology, Vol. 2. CRC Press, Boca Raton, FL, pp. 207-221.
43 Ostro, M.J., Giacomoni, D., Lavelle, D., Paxton, W. and Draz, S. (1978) Evidence for translation of rabbit globin mRNA after liposome mediated insertion into a human cell line. Nature 274,912-923.'
44 Kirby, C.J. and Gregoriadis, G. (1984) A simple procedure for preparing liposomes capable of high encapsulation efficiency under mild conditions. In: G. Gregoriadis (Ed.), Liposome Technology, Vol. 1. CRC Press, Boca Raton, FL, pp. 19-27.
45 Ohsawa, T., Miura, H. and Harada, K. (1985) Evaluation of a new liposome preparation technique, the freeze-thawing method, using L-asparaginase as a model drug, Chem.. Pharm. Bull. 33, 2916-2923.
46 Gabizon, A., Goren, D., Fuks, Z., Burenholz, Y., Dagan, A. and Meshoren, A. (1983) Enhancement of adriamycin delivery to liver metastatic cells with increased tumoricidal effect using liposomes as drug carriers, Cancer Res. 43, 4730-4735.
47 Forssen, E.A. and Tokes, Z.A. (1981) Use of anionic liposomes for the reduction of chronic doxotubicin-induced cardiotoxicity, Proc. **Natl.. Acad.** Sci. USA 78, 1873-1877.
48 Shinozawa, S., Araki, Y. and Oda, T. (1981) Tissue distribution and antitumour effect of liposome-entrapped doxorubicin (adriamycin) in Ehrlich solid tumour-bearing mouse, Acta Med. Okayama 35,395-405.
49 Rahman, A., Kessler, A., More, N., Sikic, B., Rowden, G., Woolley, P. and Schein, P.S. (1980) Liposomal protection of adriamycin-induced cardiotoxicity in mice, Cancer Res. 40, 1532-1537.
50 Herman, E.H., Rahman, A., Ferrans, V.J., Vick, J.A. and Schein, P.S. (1983) Prevention of chronic doxorubicin cardiotoxicity in beagles by liposomal encapsulation, Cancer Res. 43, 5427-5432.
51 Olson, F., Mayhew, E., Maslow, D., Rustum, Y. and Szoka, F. (1982) Characterization, toxicity and therapeutic efficacy of adriamycin encapsulated in liposomes, Eur. J. Cancer Clin. Oncol. 18, 167-176.
52 Storm, G., Roerdink, F.H., Steerenberg, P.A., De Jong, W.H. and Crommelin, D.J.A. (1987) Influence of lipid composition on the antitumouractivity exerted by doxorubicin-containing liposomes in a rat solid tumour model, Cancer Res. 47.3366-3372.
53 Gabizon, A., Dagan, A., Goren, D., Barenholz, Y. and Fuks, Z. (1982) Liposomes as in vivo carriers of adriamycin: reduced cardiac uptake and preserved antitumour activity in mice, Cancer Res. 42,47364744.