

COMMENTARY: LIPOSOMES BY ACCIDENT¹

Pieter R. Cullis*

Department of Biochemistry & Molecular Biology, University of
British Columbia, Vancouver, BC, Canada V6T 1Z3; Inex
Pharmaceuticals Corporation, 100 – 8900 Glenlyon Parkway,
Burnaby, BC, Canada V5J 5J8

INTRODUCTION

It was a great honor to receive the Bangham award and a particular pleasure that Alec Bangham himself was there to present the award. Subsequent to receiving the award and giving the associated lecture, Andy Janoff convinced me that I should put together a summary of my remarks for publication in the Journal of Liposome Research. As readers who were at the conference may remember, I discussed advances that I have been associated with from the perspective that most of them were accidental, as the research initiatives that led to these advances usually had quite different objectives. This gave rise to the title and dictated the organization of the talk.

Briefly, much of our work has turned out to be useful to developing liposomes as drug delivery systems for in vivo applications. As noted in Figure 1, an ideal drug delivery system contains encapsulated drug at a high drug-to-lipid ratio, has a small, homogeneous size so that it is able to extravasate from the circulation on reaching a disease site such as a tumour site and, if intracellular delivery is required, can associate with a target cell and deliver its contents to the cell interior. Some of our work that has been helpful to achieve this ideal delivery system includes the extrusion method for producing small homogenous liposomes (1,2), the pH method for efficiently loading liposomes with drugs (3,4), detergent

*Address correspondence to: P. Cullis at the University of BC address, Phone: (604) 822-4144, Fax: (604) 822-4843, Email: pieterc@unixg.ubc.ca

¹**Editor's Note:** This contribution represents excerpts from the Alec Bangham Award Lecture given by the recipient at the 7th International Liposome Research Days Conference, Napa, CA.

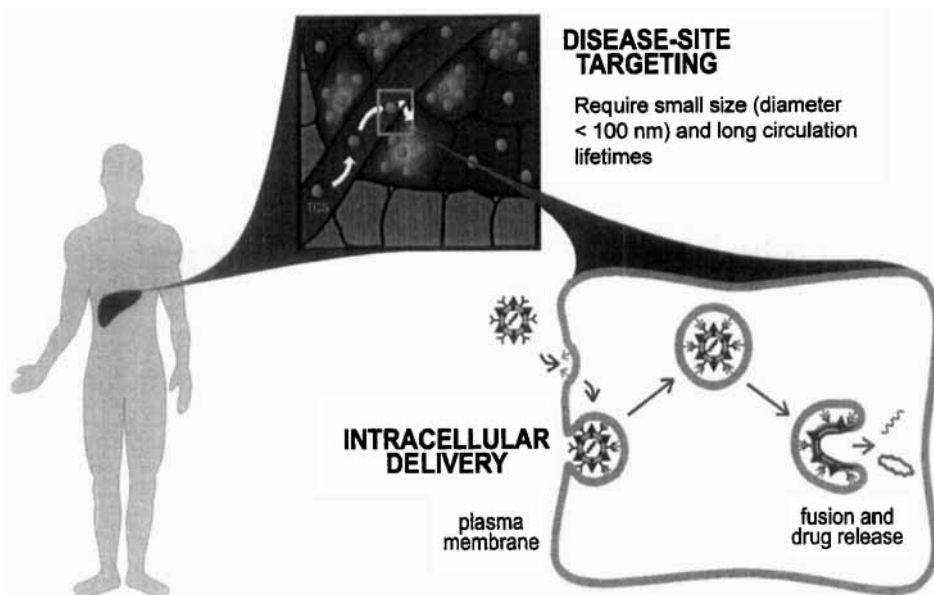


Figure 1. Characteristics of an ideal liposomal drug delivery system. Following systemic (i.v.) administration, small, long circulating liposomes exhibit “passive” or “disease-site” targeting resulting in preferential accumulation at disease sites such as tumour sites. The liposomes must then be able to deliver their contents to the interior of target cells. This may require the presence of ligands that interact with cell surface receptors and facilitate uptake as well as liposome components that promote fusion with the endosomal membrane following uptake.

dialysis methods for encapsulating macromolecules such as plasmids (5) and methods to promote intracellular delivery of liposome contents (6,7). Below I summarize this work, indicating the original initiative that led to the “accidental” discovery.

Lipid Asymmetry and Extrusion Procedures for Making Liposomes

Much of our work in the 80’s and early 90’s was focussed on understanding factors giving rise to the lipid asymmetry observed in biological membranes and the functional consequences of this asymmetry. Our original, completely erroneous, hypothesis was that lipid asymmetry in membranes may depend, in part, on membrane potentials. The origin of this idea was simple. Biological membranes exhibit substantial membrane potentials, which correspond to very large electrical fields across the lipid bilayer. For example, a membrane potential of 100 mV correspond to an electrical field of $\sim 10^5$ volts per cm, higher than the breakdown potential of air. It therefore seemed possible that the transbilayer distribution of lipids, particularly charged lipids, could be influenced by the membrane potential. However, in order to pursue this question, it became clear that a well-defined unilamellar vesicle system would be required. In particular, if we could generate a large

unilamellar vesicle (LUV) that contained charged and uncharged lipids and that also possessed a membrane potential, it would be possible to look for induced lipid asymmetry. Thus we required a method to generate LUVs as well as methods to generate and measure membrane potentials.

In the early 80's the methods available for the generation of large unilamellar vesicles included dispersion of lipids from organic solvents (8), sonication (9), detergent dialysis (10), reversed phase evaporation (11) among others, and all have serious drawbacks. For example, injection of lipid-ethanol solutions into aqueous buffer gives rise to vesicles with a large size distribution. The production of vesicles by sonication leads to small unilamellar vesicles (SUV) that are often unstable and have a very small aqueous trapped volume. Detergent dialysis methods are time consuming and always leave residual detergent that can perturb membrane properties. What we required was a method for forming LUV that was rapid, simple, and gave rise to a liposomes with a well-defined size distribution. The solution came from an extension of the work of Papahadjopoulos, Szoka and co-workers (12) that showed that the sequential extrusion of MLV through Nuclepore filters with pore sizes of 1.0 μ , 0.8 μ , 0.6 μ , 0.4 μ , and 0.2 μ using a maximum pressure of 50 psi could give rise to LUV systems. We reasoned that extrusion under higher pressures should eliminate the need for the sequential extrusions through filters with decreasing pore size and possibly allow the direct extrusion of MLV through 100 nm filters to produce 100 nm diameter LUV. This turned out to be the case (1,2). A purpose-built extrusion system was constructed in which MLV could be extruded through polycarbonate filters under high pressure (up to 500 psi). As shown in Figure 2, by using this method, LUV with diameters ranging from 50 nm to 200 nm can be generated by extrusion through filters with appropriate pore sizes. The procedure is rapid, taking on the order of 15 min, and gives rise to LUV with relatively narrow size distributions. It is now the preferred laboratory method of making LUV.

Membrane Potentials and Drug Loading

The second stage of our program to determine whether membrane potentials could influence lipid asymmetry concerned developing vesicles that possessed a sizeable membrane potential. Our original work in this area utilized a membrane potential generated by a K^+ -gradient (achieved by trapping K^+ ions inside and exchanging the outer medium for a Na^+ buffer) in the presence of the K^+ ionophore valinomycin (13). However we soon found (14) that a convenient way of generating a membrane potential was in LUV that exhibited a transmembrane pH gradient (ΔpH), as shown in Figure 3. The membrane potential ($\Delta \Psi$) originates from the relatively high permeability of H^+ ion through lipid bilayers. The higher concentration of H^+ ions on the inside of an LUV with an acidic interior, for example, leads to an efflux of protons until the electric field generated by the transbilayer movement of charge is large enough that further efflux is inhibited. The magnitude of the membrane potential generated is then given by the relation-

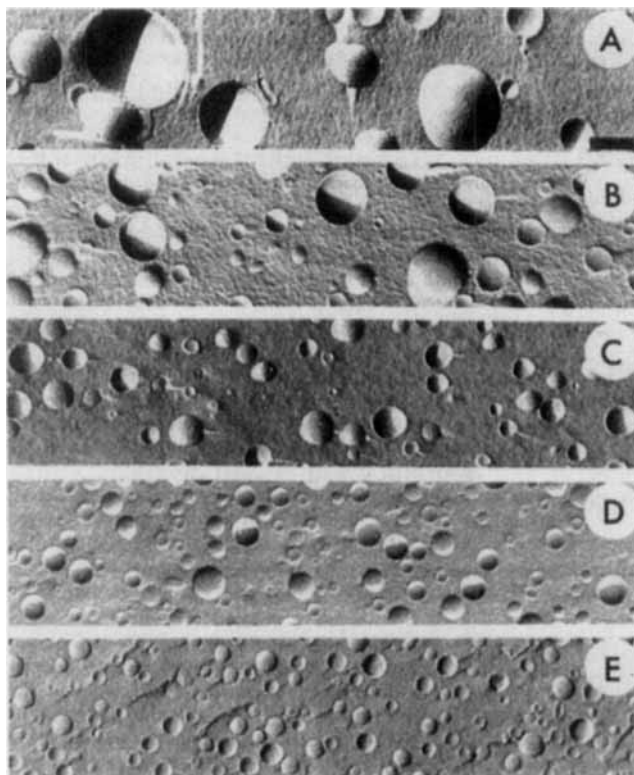


Figure 2. Freeze-fracture electron micrographs of egg phosphatidylcholine LUV following extrusion of MLV through filters with various pore sizes. The pore sizes of the filters were (A) 400 nm; (B) 200 nm; (C) 100 nm; (D) 50 nm; (E) 30 nm. The bar in panel A represents 150 nm.

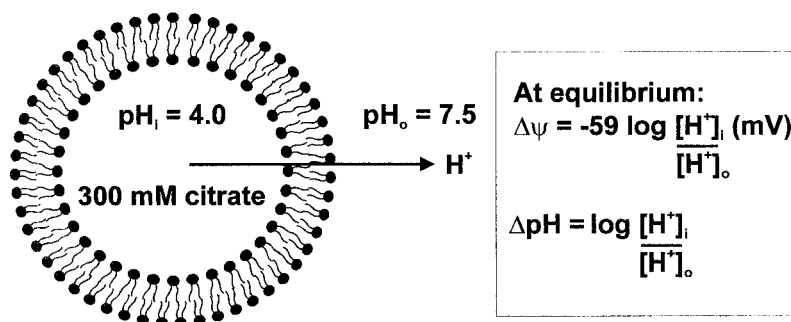


Figure 3. LUV exhibiting a transmembrane pH gradient (ΔpH) and an induced membrane potential ($\Delta\psi$). The ΔpH can be generated by producing LUV in a low pH buffer (e.g. a citrate buffer at pH 4) and then adjusting the external pH to higher values or exchanging the external medium for a higher pH buffer. Due to the relatively high permeability of H^+ ions through lipid bilayers, protons will leak out giving rise to a membrane potential ($\Delta\psi$).

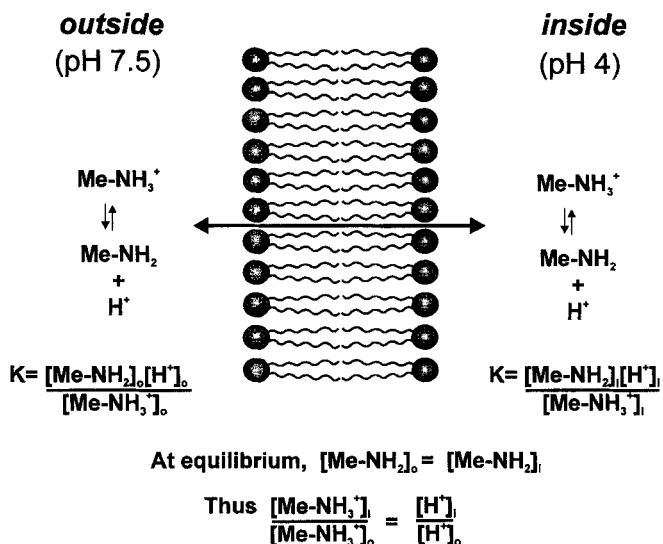


Figure 4. Measurement of ΔpH using methylamine. Methylamine can exist in a charged, protonated, membrane impermeable form and a neutral, deprotonated, membrane permeable form. On addition to LUV with acidic interiors the deprotonated form will diffuse across the membrane and become protonated in the acidic interior of the LUV. This process continues until, at equilibrium, the interior and exterior concentrations of the deprotonated form are equal. Determination of the internal and external methylamine concentrations at equilibrium yields a quantitative measure of ΔpH .

ship $\Delta\Psi = -59\log\{[\text{H}^+]_i/[\text{H}^+]_o\}$. These gradients can be quantified by measuring the transmembrane distribution of certain lipophilic cations. For example, addition of the permanently charged lipophilic cation methytriphenylphosphonium (MTPP) to the exterior of LUV with a $\Delta\Psi$ (inside negative) results in accumulation of MTPP in the interior of the LUV until the electrical potential is balanced by the chemical potential of MTPP according to the relationship $\Delta\Psi = -59\log\{[\text{MTPP}]_i/[\text{MTPP}]_o\}$. Measurement of the equilibrium transbilayer concentrations of MTPP thus provides an accurate measure of $\Delta\Psi$.

Weak bases, such as methylamine (MeAm), can be used to measure the ΔpH . Analysis of the response of MeAm to ΔpH gives important insight into how pH gradients can be used to load liposomes with drugs. We consider the LUV shown in Figure 3 possessing a ΔpH of 3.5 units ($\text{pH}_o = 7.5$; $\text{pH}_i = 4$), to which is added an external aliquot of methylamine. A (small) fraction of the methylamine will be in the neutral (deprotonated) form, which is highly membrane permeable. The neutral methylamine can then diffuse across the membrane, however once inside the liposome, it will be protonated and unable to escape, as lipid bilayers are highly impermeable to charged molecules. As indicated in Figure 4, at equilibrium, when the concentration of the neutral form of MeAm is equal both inside and outside the LUV, the ratio of the internal and external MeAm concentrations equals the proton gradient. For our ΔpH of 3.5 units this corresponds to a concentration of

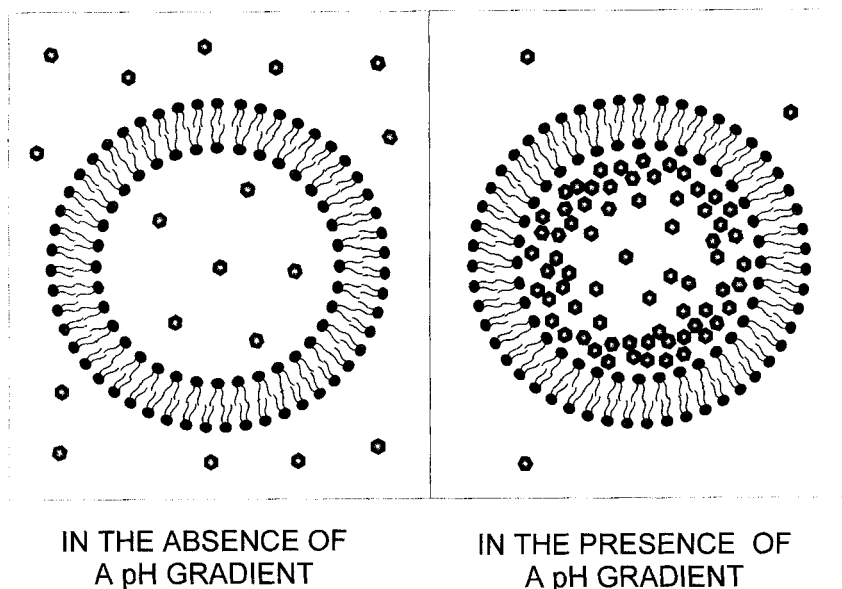


Figure 5. Cartoon describing the uptake of drugs that are lipophilic weak bases into LUV exhibiting a transmembrane pH gradient (inside acidic). At equilibrium the ratio of the internal concentration of the drug to the external concentration of the drug will equal the proton concentration gradient. For $\Delta\text{pH} > 3$ units, this leads to interior drug concentrations that are greater than 10^3 -fold higher than the exterior drug concentrations. Further, by using entrapped media with high buffering capacities, conditions can be achieved that lead to essentially 100% uptake of drug to achieve drug-to-lipid ratios > 0.2 (wt/wt).

methylamine on the inside of the LUV that is 3.2×10^3 -fold higher than the concentration outside, which is rather an impressive level of accumulation.

This line of investigation did lead to generation of lipid asymmetry in LUV. For example, it was found that asymmetric transbilayer distributions of acidic lipids such as fatty acids (15), phosphatidic acid (16) and phosphatidylglycerol (17,18) could be generated in response to transmembrane pH gradients, although these induced asymmetries were due to ΔpH rather than $\Delta\Psi$. However, the most exciting results came from wondering whether drugs that were weak bases would respond to ΔpH in the same way as methylamine, as Deamer and colleagues had shown for catecholamines (19). An obvious candidate was the anticancer drug doxorubicin, a lipophilic amine with a single amino group and a $\text{pK}_a = 8.6$. It was found (20) that under appropriate conditions the addition of doxorubicin to LUV exhibiting a transmembrane pH gradient could result in essentially complete uptake of doxorubicin to achieve doxorubicin loaded liposomes with very high drug-to-lipid ratios. Further, it was found that a wide variety of commonly used pharmaceuticals could be loaded into liposomes in response to ΔpH (4) as illustrated in cartoon form in Figure 5. Subsequent work showed that pH gradients giving rise to drug uptake could be generated in a variety of ways, including utilizing NH_4SO_4

gradients (21) or MgSO_4 gradients (22) (in combination with ionophores). Drug loading in response to ΔpH is now the preferred method of loading small molecule drugs into LUV for drug delivery applications.

Applications of Extrusion and ΔpH Loading: Liposomal Vincristine

The extrusion technique and pH-gradient loading allowed the development of liposomal drugs with clinical utility. Homogeneous, small liposomes could be rapidly generated using the extrusion procedure. By employing the ΔpH loading process, these liposomes could then be readily loaded with drugs such as doxorubicin to achieve high drug-to-lipid ratios. For example, drug-to-lipid ratios for doxorubicin (0.2 wt:wt), corresponding to $\sim 40,000$ drug molecules per 100 nm diameter liposome can be readily achieved. Finally, by varying the lipid composition, excellent drug retention and regulated release could be achieved *in vivo*. This has led to liposomal formulations of doxorubicin (23,24) that exhibit properties of reduced toxicity that are now approved for clinical use. However, our subsequent work showed that not all drugs benefit to the same extent from liposomal encapsulation. Liposomal formulations of drugs such as doxorubicin and daunorubicin result in reduced toxicities relative to the free drug whereas others, such as vincristine, also display enhanced efficacy (25). I believe that it is for drugs such as vincristine, where administration of liposomal drug can give rise to increased efficacy as compared to the same dose of free drug, that liposomal encapsulation is likely to show the greatest clinical benefit.

Vincristine is the most widely used anticancer agent with indications for lymphomas, lung cancer, leukemia, and breast cancer (26). Due to its lack of myelotoxicity it is widely used in combination chemotherapy, as most other anticancer drugs are highly bone marrow suppressive. In common with liposomal formulations of other drugs, liposomal encapsulation of vincristine can lead to dramatically different pharmacokinetics following *i.v.* injection, and a circulation half-life of 12 h or longer can readily be achieved for the liposomal systems. This leads to a "passive targeting" or "disease-site targeting" phenomenon, which is perhaps the most important discovery made in the liposome field in the last 20 years. Briefly, liposomes with small size and long circulation lifetimes tend to accumulate at sites of disease such as tumours, infection or inflammation due to the increased permeability of the vasculature in these regions (27,28,29). This can lead to remarkable enhancements of 50-fold or more in the amounts of drug that are delivered to tumour sites as compared to administration of the same dose of free drug. In turn, this can lead to dramatic improvements in efficacy.

For drugs such as vincristine, optimizing the payout rate is crucial to achieve maximum efficacy. The basic reasons for this are straightforward. If the drug leaks out of the vesicle at a rapid rate, it will all leak out before getting to the tumour and no therapeutic benefit over free drug will be seen. On the other hand, if the drug leaks out of the liposome very slowly, the drug will get to the tumour but will leak out so slowly that the levels of free drug never reach therapeutic concen-

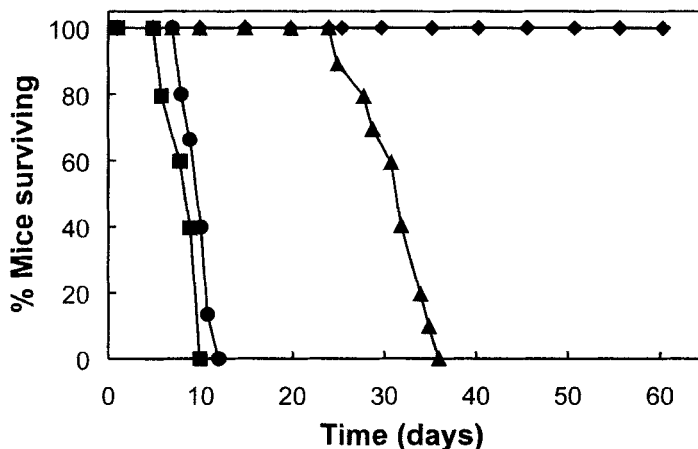


Figure 6. Influence of intravesicular pH on the efficacy of vincristine encapsulated in DSPC/Chol LUV against P388 tumors. BDF1 mice bearing peritoneal ascites P388 tumors were untreated (■) or were treated with free vincristine (●) or LUVs of DSPC/Chol with encapsulated vincristine and intravesicular pH of either 4.0 (▲) or 2.0 (◆). For further information see ref. (25).

trations. The sensitivity of the antitumour efficacy to vincristine payout rates can be illustrated for two distearoylphosphatidylcholine (DSPC)/cholesterol preparations of liposomal vincristine that differ only in the internal pH of the liposomes (30). Lower internal pH values lead to reduced efflux rates when the pH loading technique is employed (31). As shown in Figure 6, the formulation with the lowest internal pH (pH = 2) gives rise to dramatically improved efficacy over the formulation with an interior pH of 4. Subsequent work (32) has identified a sphingomyelin/cholesterol formulation that is more chemically stable and has similar payout rates as the DSPC/cholesterol pH 2 formulation, and this is currently in pivotal clinical trials against relapsed lymphoma. It is interesting to note that the increased efficacy observed in the preclinical studies appears to also be observed in the clinical setting (33).

Encapsulation of Plasmid by Detergent Dialysis

As indicated above encapsulation of small molecules in liposomes with small size and extended circulation lifetimes leads to preferential delivery of drug to sites of disease such as tumour sites. This suggests that encapsulation of macromolecules such as plasmid DNA in small, long circulating liposomes should also lead to disease site targeting of plasmid following systemic (i.v.) administration, possibly resulting in preferential gene expression at disease sites such as tumor sites. The resulting effort to develop a systemic gene delivery system has occupied most of my time for the last five years. The need for such a systemic gene therapy vector is clear- both viral vectors and non-viral vectors such as plasmid DNA-cationic lipid "lipoplexes" or plasmid DNA-cationic polymer "polyplexes" exhibit very short circulation lifetimes following i.v. injection. As a result, they only reach "first-pass" organs such as the lung, liver and spleen. This has restricted

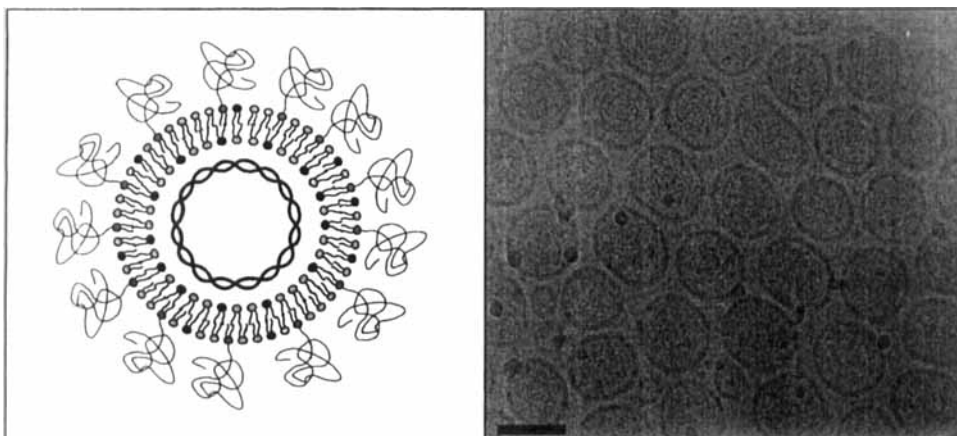


Figure 7. Cartoon and cryo-electron micrograph of stabilized plasmid-lipid particle (SPLP) produced by detergent dialysis. As depicted, the SPLP is composed of the “fusogenic” lipid dioleoylphosphatidylethanolamine (DOPE; white headgroups), the cationic lipid DODAC (black headgroups), and the stabilizing PEG lipid (PET-CerC₂₀; grey headgroups). The SPLP shown in the cryo-electron micrograph have been purified by removal of untrapped plasmid and empty vesicles formed during detergent dialysis as described in reference (5).

use of these vectors to direct (e.g. intratumoral) injection or regional (e.g. intraperitoneal) injection. However treatment of systemic diseases such as cancer or inflammatory diseases clearly requires systemic therapy.

The first step in this process was to encapsulate plasmid DNA in small liposomal systems. We were influenced by an observation of Bally and co-workers (34), who showed that plasmid could be taken up into organic solvents when cationic lipid was present, presumably forming inverted micelles in the organic solvent with the plasmid DNA inside the inverted micelle and the acyl chains of the cationic lipid oriented towards the organic phase. We reasoned that we might be able to solubilize such inverted micelles in aqueous media containing detergent and then substitute phospholipid for detergent by adding phospholipid and dialyzing away the detergent. Such detergent dialysis techniques are commonly employed to reconstitute integral membrane proteins such as cytochrome oxidase into LUV systems (35). Although our original reasoning turned out to be wrong, it did lead to the discovery that plasmid could be encapsulated in LUV with a diameter of 70 nm employing a detergent dialysis process (5).

The process involves the incubation of plasmid DNA, cationic lipid, the “fusogenic” lipid dioleoylphosphatidylethanolamine (DOPE) and a polyethyleneglycol (PEG) lipid (PEG-Ceramide) and the non-ionic detergent octylglucopyranoside. Dialysis then results in the formation of “stabilized plasmid-lipid particles” (SPLP), empty vesicles and untrapped plasmid. The untrapped plasmid and empty vesicles can be removed, leaving purified SPLP that are shown in Figure 7. The SPLP are small (diameter = 70 nm), homogeneous, stable, well-defined systems containing one plasmid per particle (5). Further, SPLP exhibit

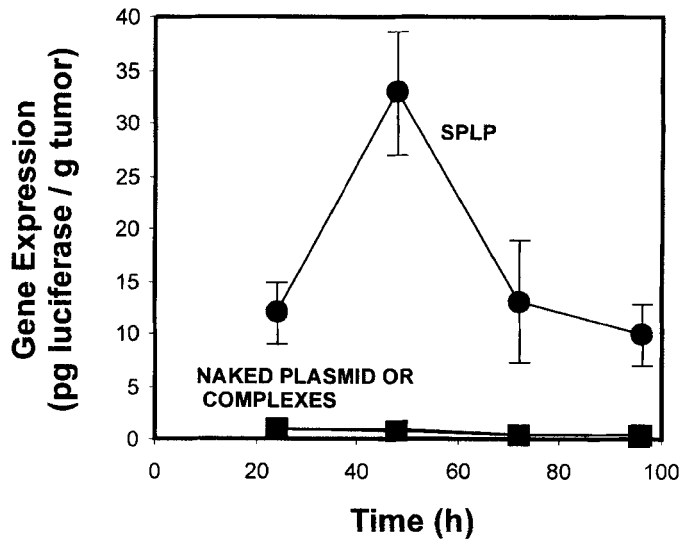


Figure 8. Transgene expression at a distal tumor site following intravenous injection of naked plasmid DNA (■) or plasmid DNA-cationic lipid complexes and SPLP (●). Mice bearing subcutaneous Lewis lung tumors on the hind flank were injected i.v. with doses containing 100 μ g of cCMVluc. Tumors were harvested at the indicated times and assayed for luciferase activity. The level of transgene expression reported is normalized for the weight of the tumor tissue. For further details see ref. (36).

excellent pharmacokinetic properties (36) following i.v. injection, with long circulation lifetimes similar to those achieved for liposomal formulations of conventional drugs (36). In contrast, when free plasmid DNA, or plasmid DNA in lipoplexes is injected into mice, the plasmid is cleared from the circulation almost immediately (36).

The small size and long circulation lifetimes exhibited by SPLP would be expected to lead to preferential accumulation at tumour sites following i.v. administration, as observed for liposomal vincristine. This has proven to be the case. Tumour levels corresponding to 6% of the total injected dose of plasmid per gram of tumour tissue are observed 10 h following injection, which corresponds to more than 1000 intact plasmid molecules per tumour cell. Administration of the same dose of plasmid DNA in the free form or in lipoplexes resulted in essentially no plasmid delivery to the tumour. As shown in Figure 8, the preferential delivery of plasmid to the tumour also results in reporter gene expression (36), whereas naked plasmid DNA and plasmid in lipoplex form were completely ineffective.

The levels of reporter gene expression at the tumour site induced by administration of SPLP are significant and superior to that achievable with other vectors, however such levels may not be sufficient for therapeutic effects employing certain genes. Efforts in my laboratory are now focusing on how to increase gene expression by increasing plasmid escape from the endosome following uptake of

SPLP. Again, these efforts have benefited substantially from accidental observations. In particular, Ismail Hafez, a graduate student in my laboratory was investigating the pH sensitive fusion properties of LUV composed of mixtures of cationic lipid and anionic lipid and found that equimolar mixtures would not form LUV at all, resulting only in massive precipitates on hydration. Curious as to what the structure of these precipitates may be we conducted freeze-fracture electron microscopy and ^{31}P NMR studies and found that these mixtures were largely in the non-bilayer hexagonal H_{II} phase (37). As detailed below, this provided a major clue as to the mechanism of action of cationic lipids, which are relatively effective intracellular delivery agents for macromolecules such as plasmid DNA. This observation also fitted well with a research program on lipid polymorphism that Ben de Kruijff and I initiated 25 years ago while we were both postdocs at the University of Oxford.

Our early studies on lipid polymorphism identified ^{31}P NMR as a useful technique to monitor the different structural preferences of aqueous dispersions of lipids and showed that a large proportion of lipids in biological membranes preferred the non-bilayer hexagonal H_{II} phase in isolation (38). This applied particularly to unsaturated species of PE, to lipids such as cholesterol that induce H_{II} organization in mixed lipid systems as well as charged lipids such as cardiolipin, which adopt the H_{II} phase in the presence of Ca^{2+} . The ability of ^{31}P NMR and freeze-fracture to identify lipid polymorphism is illustrated in Figure 9.

The discovery that many membrane lipids had a preference for non-bilayer structures such as the H_{II} phase led naturally to questions concerning the functional roles of non-bilayer lipid structures in membrane mediated phenomena. Membrane fusion was a logical candidate as it is a topological impossibility for two membrane bound bodies to fuse without a local departure from bilayer structure. Further, we showed that factors that induced fusion in membranes also tended to induce non-bilayer lipid structures. We proposed (39,40) that membrane fusion required a non-bilayer intermediate structure as shown in Figure 10. While the actual type of intermediate may differ from the inverted micellar intermediate pictured (current models favour the 'stalk' intermediate (41)), it is now generally accepted that the ability of lipids to adopt non-bilayer 'inverted' structures plays a basic role in membrane fusion phenomena. Most notably, factors that promote hexagonal phase structure also promote membrane fusion, and vice versa. H_{II} phase promoters include divalent cations such as Ca^{2+} , increased acyl chain unsaturation, dehydration of the membrane, and increased temperatures.

The observation that cationic lipids induce H_{II} phase in combination with anionic lipids such as phosphatidylserine has obvious implications for the mechanism whereby cationic lipids promote the intracellular delivery of plasmid in plasmid DNA-cationic lipid complexes. Following uptake into the cell Szoka and colleagues (42) suggest that the cationic lipid is displaced from the nucleic acid by negatively charged lipids from the endosomal membrane. The fact that the cationic lipid-anionic lipid pairs thus formed promote H_{II} phase organization then suggests that the displaced cationic lipid can then play a direct role in encouraging

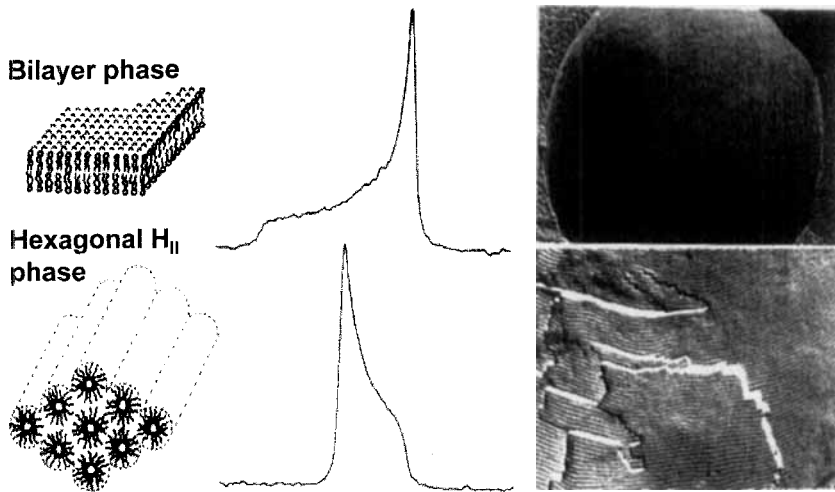


Figure 9. ^{31}P NMR and freeze-fracture characteristics of aqueous dispersions of phospholipids in the bilayer and hexagonal H_{II} phases. ^{31}P NMR spectra from lipids in the bilayer phase (egg yolk phosphatidylcholine) exhibit a low field shoulder and high field peak, whereas lipids in the hexagonal H_{II} phase (soy bean phosphatidylethanolamine) exhibit spectra that have reversed asymmetry and are a factor of two narrower. Freeze-fracture micrographs obtained from lipids in the bilayer organization display smooth fracture faces, whereas lipids in the H_{II} phase exhibit a corrugated pattern as the fracture face cleaves between the tubes of hexagonal phase lipid.

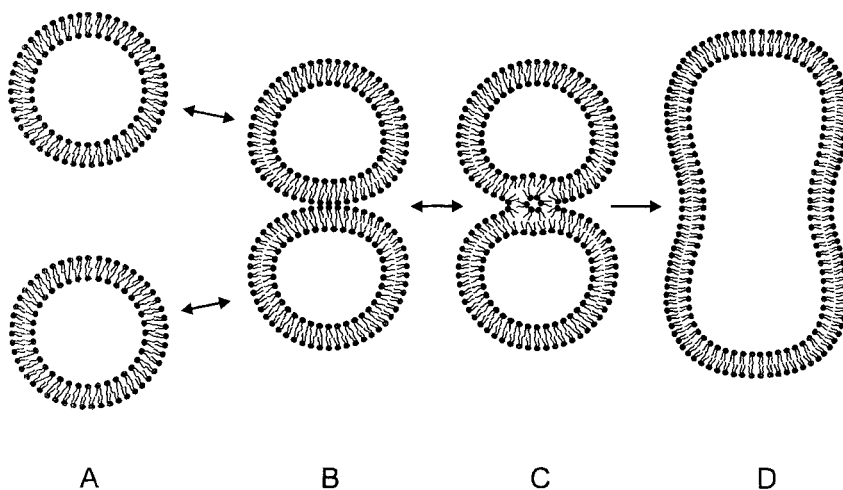


Figure 10. Model of membrane fusion relying on the formation of non-bilayer intermediates. The inverted micellar intermediate (IMI) pictured here was the initial intermediate suggested (39,40). Current models indicate that the most likely intermediate is a stalk structure (41).

the membrane fusion or disruption event that is crucial to the plasmid escaping from the endosome into the cell interior.

These observations led to the conjecture that if cationic lipids promote intracellular delivery by promoting non-bilayer organization following uptake into cells, then other factors that promote H_{II} organization should also enhance the intracellular delivery of plasmid and associated gene transfer properties. For example, Ca^{2+} can trigger bilayer-to-hexagonal phase transitions in lipid mixtures containing negatively charged lipids (38). In agreement with the hypothesis, it was found that Ca^{2+} increases the transfection potency of SPLP in BHK cells by some 600-fold (43). The effect of Ca^{++} on transfection was also observed for SPLP that contain a cationic PEG-lipid (CPL) to enhance cell uptake (7). In the absence of Ca^{2+} , very little gene expression was observed, whereas in the presence of Ca^{2+} , increases up to 10^5 -fold were observed. These and other data suggest that rational design of systems giving rise to optimized intracellular delivery can now be achieved by maximizing the ability of these systems to promote H_{II} phase structure following uptake into cells.

In summary, it is clear that the most useful observations that we have made concerning the development of liposomes as drug delivery agents has come from research initiatives aimed at achieving a basic understanding of the physical properties and functional roles of lipids in membranes. This applies to development of the extrusion technique, pH loading procedures, encapsulation of macromolecules and increasingly, optimization of the intracellular delivery capabilities of liposomes. I expect that this will continue and that increased understanding of the biochemistry and biophysics of membrane lipids will also lead to the next major breakthroughs in liposome drug delivery.

ACKNOWLEDGEMENTS

I have many people to thank, beginning with Myer Bloom, who first introduced me to membrane biophysics, Ben de Kruijff who really taught me what lipids and membranes were all about and to members of the Royal Lipex Ballet, who helped me form a team in Vancouver in the early 80's that, to my great satisfaction, is still here 20 years later. This team includes Mick Hope, Marcel Bally, Tom Madden and Lawrence Mayer and it has been, and continues to be, a terrific pleasure to work with them. I also have to thank over 20 graduate students and over 30 postdoctoral fellows who have contributed so much over the last 20 years. Finally I have to thank Andy Janoff for many things including introducing me to the torments of corporate life and Dave Fenske for his crucial assistance in putting this manuscript together.

REFERENCES

1. M.J. Hope, M.B. Bally, G. Webb and P.R. Cullis, 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 (1985)
2. L.D. Mayer, M.J. Hope and P.R. Cullis, Vesicles of Variable Sizes Produced by a Rapid Extrusion Procedure, *Biochim. Biophys. Acta* 858, 161-168 (1986)
 3. L.D. Mayer, M.B. Bally and P.R. Cullis, Uptake of Adriamycin into Large Unilamellar Vesicles in Response to a pH Gradient, *Biochim. Biophys. Acta* 857, 123-126 (1986)
 4. T.D. Madden, P.R. Harrigan, L.C. Tai, M.B. Bally, L.D. Mayer, T.E. Redelmeier, H.C. Loughrey, C.P.S. Tilcock, L.W. Reinish and P.R. Cullis, The Accumulation of Drugs within Large Unilamellar Vesicles Exhibiting a Proton Gradient: A Survey, *Chem. Phys. Lipids* 53, 37-46 (1990)
 5. J.J. Wheeler, L. Palmer, M. Ossanlou, I. MacLachlan, R.W. Graham, M.J. Hope, P. Scherrer and P.R. Cullis, Stabilized Plasmid-Lipid Particles: Construction and Characterization, *Gene Therapy* 6, 271-281 (1999)
 6. I.M. Hafez, S. Ansell and P.R. Cullis, Tunable pH Sensitive Liposomes Composed of Mixtures of Cationic and Anionic Lipids, *Biophysical J.*, in press (2000)
 7. L.R. Palmer, T. Chen, A.M.I. Lam, D.B. Fenske, K.F. Wong and P.R. Cullis, Stabilized plasmid-lipid particles containing cationic PEG lipids exhibit enhanced transfection potencies, *Gene Therapy*, submitted (2000)
 8. S. Batzri and E.D. Korn, Single bilayer liposomes prepared without sonication, *Biochim. Biophys. Acta* 298, 1015-1020 (1973)
 9. C.H. Huang, Studies on phosphatidylcholine vesicles. Formation and physical characteristics, *Biochemistry* 8, 344-351 (1969)
 10. L.T. Mimms, G. Zampighi, G. Nozaki, C. Tanford and J.A. Reynolds Phospholipid vesicle formation and transmembrane protein incorporation using octylglucoside *Biochemistry* 20, 833-840 (1981)
 11. F. Szoka and D. Papahadjopoulos, Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation, *Proc. Nat. Acad. Sci. USA* 75, 4194-4198 (1978)
 12. F. Olson, C.A. Hunt, F. Szoka, W.J. Vail and D. Papahadjopoulos, Preparation of liposomes of defined size distribution by extrusion through polycarbonate membranes, *Biochim. Biophys. Acta* 557, 9-20 (1979)
 13. M.B. Bally, M.J. Hope, C.J.A. van Echteld and P.R. Cullis, Uptake of Safranin and Other Lipophilic Cations into Vesicles in Response to a Membrane Potential, *Biochim. Biophys. Acta* 812, 66-76 (1985)
 14. T.E. Redelmeier, L.D. Mayer, K.F. Wong, M.B. Bally and P.R. Cullis, Proton Flux in Large Unilamellar Vesicles in Response to Electrical Potentials and pH Gradients, *Biophys. J.*, 56, 385-393 (1989)
 15. M.J. Hope, P.R. Cullis, Lipid Asymmetry Induced by Transmembrane pH Gradients in Large Unilamellar Vesicles, *J. Biol. Chem.* 262, 4360-4366 (1987)
 16. S.J. Eastman, M.J. Hope and P.R. Cullis, Transbilayer transport of phosphatidic acid in response to transmembrane pH gradients, *Biochemistry* 30, 1740-1745 (1991)
 17. M.J. Hope, T.E. Redelmeier, K.F. Wong, W. Rodriguez and P.R. Cullis, Phospholipid Asymmetry in Large Unilamellar Vesicles Induced by Transmembrane pH Gradients, *Biochemistry* 28, 4181-4187 (1989)
 18. T.E. Redelmeier, M.J. Hope and P.R. Cullis, On the Mechanism of Transbilayer Transport of Phosphatidylglycerol in Response to Transmembrane pH Gradients, *Biochemistry* 29, 3046-3053 (1990)

19. P.R. Harrigan, M.J. Hope, T.E. Redelmeier and P.R. Cullis, Determination of transmembrane pH gradients and membrane potentials in liposomes, *Biophys. J.* 63, 1336-1345 (1992)
20. P.R. Cullis, L.D. Mayer, M.B. Bally, T.D. Madden and M.J. Hope Generation and Loading of Liposomal Systems for Drug Delivery Applications, *Advanced Drug Delivery Reviews*, 3, 267-282 (1989)
21. G. Haran, R. Cohen, L.K. Bar and Y. Barenholz, Transmembrane ammonium sulphate gradients in liposomes produce efficient and stable entrapment of amphipathic weak bases, *Biochim. Biophys. Acta* 1151, 201-215 (1993)
22. D.B. Fenske, K.F. Wong, E. Maurer, N. Maurer, J.M. Leenhouts, N. Boman, L. Amankwa and P.R. Cullis Ionophore-Mediated Uptake of Ciprofloxacin and Vincristine into Large Unilamellar Vesicles Exhibiting Transmembrane Ion Gradients, *Biochim. Biophys. Acta* 1414, 188-204 (1998)
23. A. Chonn and P.R. Cullis, Recent advances in liposomal drug delivery systems, *Curr. Op. Biotechnol.* 6, 698-708 (1995)
24. M. Harrison, D. Tomlinson, S. Stewart, Liposomal-entrapped doxorubicin: an active agent in AIDS-related Kaposi's sarcoma, *J. Clin. Oncol.* 13, 914-920 (1995)
25. N.L. Boman, D. Masin, L.D. Mayer, P.R. Cullis and M.B. Bally Liposomal Vincristine Which Exhibits Increased Drug Retention and Increased Circulation Longevity Cures Mice Bearing P388 Tumors, *Cancer Res.* 54, 2830-2833 (1994)
26. S.M. Sieber, J.A.R. Mead and R.H. Adamson Pharmacology of antitumour agents from higher plants *Cancer Treat. Rep.* 60, 1127-1139 (1976)
27. J.R. Morgan, L.A. Williams and C.B. Howard, Technetium-labelled liposome imaging for deep-seated infection, *B. J. Radiol.* 58, 35-39 (1985)
28. C.A. Pressant, R.T. Profitt and J.D. Smith, Evidence for solid tumour accumulation of intravenously injected lipid vesicles in patients, *Proc. Am. Acad. Cancer Res.* 27, 158 (1986)
29. S. Kohn, J.A. Nagy, H.F. Dvorak and A.M. Dvorak, Pathways of macromolecular tracer transport across venules and small veins. Structural basis for the hyperpermeability of tumour blood vessels, *Lab. Invest.* 67, 596-607 (1992)
30. N.L. Boman, M.B. Bally, P.R. Cullis, L.D. Mayer, M.S. Webb, Encapsulation of vincristine in liposomes reduces its toxicity and improves its antitumor efficacy, *J. Liposome Research* 5, 523-541(1995)
31. P.R. Cullis, M.J. Hope, M.B. Bally, T.D. Madden, L.D. Mayer and D.B. Fenske, Influence of pH Gradients on the Transbilayer Transport of Drugs, Lipids, Peptides and Metal Ions into Large Unilamellar Vesicles, *Biochim Biophys. Acta*, 1331, 187-211 (1997)
32. M.S. Webb, T.O. Harasym, D. Masin, M.B. Bally and L.D. Mayer, Sphingomylin-cholesterol liposomes significantly enhance the pharmacokinetic and therapeutic properties of vincristine in murine and human tumour models, *Br. J. Cancer* 72, 896-904 (1995)
33. A.H. Sarris, F. Hagemester, J. Romaguera, M.A. Rodrigues, P. McLaughlin, A.M. Tsimberidou, L.J. Medeiros, B. Samuels, O. Pate, M. Oholendt, H. Kantarjian, C. Burge and F. Cabanillas, Liposomal vincristine in relapsed non-Hogkin's lymphomas: early results of an ongoing clinical trial, *Annals of Oncology* 11, 69-72 (2000)
34. D.L. Reimer, Y.P. Zhang, S. Kong, J.J. Wheeler, R.G. Graham and M.B. Bally, Formation of novel hydrophobic complexes between cationic lipids and plasmid DNA, *Biochemistry* 34, 12877012883 (1995)

35. T.D. Madden, M.J. Hope and P.R. Cullis, Influence of Vesicle Size and Oxidase Content on Respiratory Control in Reconstituted Cytochrome Oxidase Vesicles, *Biochemistry* 23, 1413-1418 (1984)
36. P. Tam, M. Monck, D. Lee, O. Ludkovski, E.C. Leng, K. Clow, H. Stark, P. Scherrer, R.W. Graham and P.R. Cullis, Stabilized Plasmid-Lipid Particles for Systemic Gene Therapy, *Gene Therapy*, submitted (2000)
37. I.M. Hafez, S. Ansell and P.R. Cullis, Tunable pH Sensitive Liposomes Composed of Mixtures of Cationic and Anionic Lipids, *Biophysical J.*, in press (2000)
38. P.R. Cullis and B. de Kruijff, Lipid Polymorphism and the Functional Roles of Lipids in Biological Membranes, *Biochim. Biophys. Acta* 559, 399-420 (1979)
39. P.R. Cullis and M.J. Hope, Effects of Fusogenic Agents on the Membrane Structure of Erythrocyte Ghosts and the Mechanism of Membrane Fusion, *Nature* 271, 672-675 (1978)
40. A.J. Verkleij, C. Mombers, W.J. Gerritsen, J. Leunissen-Bijvelt and P.R. Cullis, Fusion of phospholipid vesicles in association with the appearance of lipidic particles as visualized by freeze-fracturing, *Biochim. Biophys. Acta* 555, 358-362 (1979)
41. D.P. Siegel, The modified stalk mechanism of lamellar/inverted phase transitions and its implications for membrane fusion. *Biophys. J.* 76, 291-313 (1999)
42. Y. Xu and F.C. Szoka, Mechanism of DNA release from cationic liposome/DNA complexes used in cell transfection *Biochemistry* 35, 5616-5623 (1996)
43. A.M.I. Lam, L.R. Palmer and P.R. Cullis, Calcium dramatically enhances the transfection potency of stabilized plasmid-lipid particles, *Molec. Therapeutics*, submitted 2000