LIPOSOMES: MODELS OF BIOLOGICAL MEMBRANES AND DRUG DELIVERY SYSTEMS

by David B. Fenske and Pieter R. Cullis

Liposomes were originally constructed and characterized in order to gain insight into the physical properties and functional roles of lipids in biological membranes [1]. The diversity of lipids and proteins in biological membranes usually precludes the direct observation of the properties of individual lipid species and thus liposomes, which can be constructed from single or mixed lipid systems as desired, provide a convenient model system. A remarkable feature of this model building approach to understand biological membranes is that it has directly led to the construction of liposomal drug delivery systems with considerable therapeutic potential [2]. These systems contain drugs, such as anticancer drugs, and are designed to exhibit extended circulation lifetimes following intravenous injection, which leads to preferential accumulation at disease sites such as tumour sites. More sophisticated versions of these nanoparticles are under development that can associate with a specific target cell and deliver the liposome contents to the cell interior (Figure 1). In this review, we focus on the applications of liposomes as drug delivery agents and the contributions of our group to this field, which originated from our interest in developing more accurate models of biological membranes rather than developing drug delivery systems per se. These contributions include the extrusion method for producing small homogenous liposomes [3,4], the pH-gradient method for efficiently loading liposomes with drugs [5,6], detergent dialysis methods for encapsulating macromolecules such as plasmids [7], and methods to promote intracellular delivery of liposome contents [8,9].

We focus on the applications of liposomes as drug delivery agents and the contributions of our group to this field, which originated from our interest in developing more accurate models of biological membranes rather than developing drug delivery systems.

Liposomes were first described by Bangham in 1965 [10]. The first liposomes to be characterized, termed multilamellar vesicles (MLV), are large (1-10 μ diameter) onion-skinned structures consisting of concentric rings of lipid bilayers and form spontaneously upon mechanical agitation of lipids with water. The physical properties of lipids in MLVs have been characterized in some detail by a wide variety of physical techniques, including ^{2}H-NMR [11,12], ^{31}P-NMR [12,13], ESR [14], fluorescence [15], and DSC [16], detailing features of lipid order and motion in bilayer structures, the polymorphic phase preferences of lipids, and gel-liquid crystalline lipid phase transitions. MLV are not suitable for most drug delivery applications, as their large size results in rapid clearance from the circulation. Early techniques for producing smaller (unilamellar) liposomal systems included dispersion of lipids from organic solvents [17], sonication [18], detergent dialysis [19], and reversed phase evaporation [20]. These techniques all have their drawbacks. Injection of lipid-ethanol solutions into aqueous buffer gives rise to vesicles with large size distributions, whereas 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 was needed was a method to prepare vesicles that would be large enough to carry a significant payload, and yet be small enough to extravasate from the circulation through tumour vasculature so that tumour accumulation could occur. As it turned out, this demands liposomes of approximately 100 nm diameter. Liposomes in this size range are usually termed large unilamellar vesicles, or LUV.

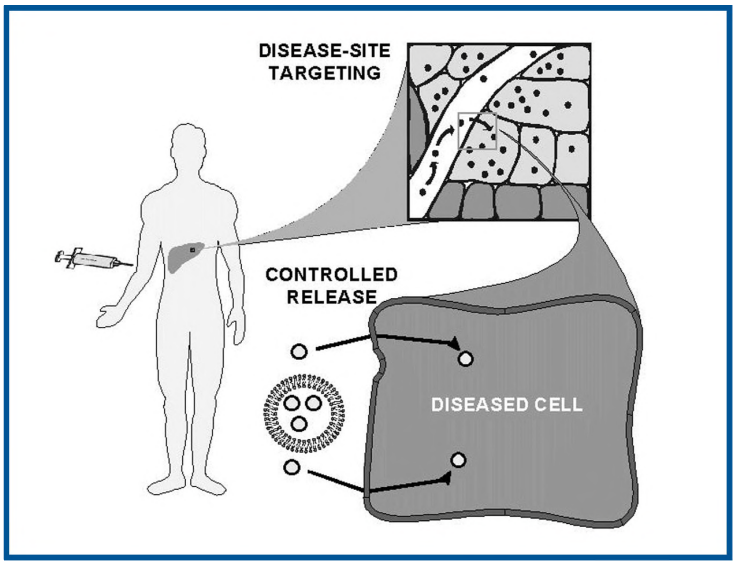

Fig. 1 Characteristics of an ideal liposomal drug delivery system. Following systemic (intravenous) administration, small, long-circulating liposomes exhibit "passive" or "disease-site" targeting, resulting in preferential accumulation at tumours and other disease sites. The liposomes must then be able to deliver their contents to the interior of target cells. There are several ways this can be achieved, including (1) designing liposomes that release their contents at an optimized rate, (2) including ligands that interact with cell surface receptors and facilitate uptake, or (3) including liposome components that promote fusion with the endosomal membrane following uptake.

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EXTRUSION PROCEDURES FOR MAKING LUV

Our interest in developing new procedures to make LUV developed from a desire to determine whether membrane potentials and pH gradients commonly observed to exist across biological membranes could play a role in driving the asymmetric transbilayer distribution of lipids observed in most biological membranes. This required well-defined unilamellar vesicle systems exhibiting transbilayer ion gradients leading to membrane potentials and pH gradients similar to those observed across cell and organelle membranes. However, as discussed above, the methods then available for the rapid generation of large unilamellar vesicles (LUV) with a well-defined size distribution were all unsuitable. The solution came from an extension of the work of Papahad-jopoulos, Szoka and coworkers [21] who 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 with a diameter of approximately 200 nm. We showed that extrusion under higher pressures (up to 500 psi) eliminated the need for sequential extrusions through filters with decreasing pore size, and allowed the direct generation of 100 nm diameter LUV by extrusion of MLV through 0.1 μ filters [3,4]. As shown in Figure 2, LUV with diameters ranging from 50 nm to 200 nm can be generated by extrusion through filters with appropriate pore sizes. The procedure can be performed in less than 20 min, and gives rise to LUV with relatively narrow size distributions. Larger vesicles (400 nm - 1000 nm) can also be formed by this method, but they contain bilamellar and multilamellar vesicles [4].

Subsequent work demonstrated that these LUV could maintain stable membrane potentials and pH gradients, although this ability is dependent on the lipid composition. LUV containing saturated phospholipids such as distearoylphosphatiylcholine (DSPC) will exhibit reduced membrane permeabilities (and thus more stable transbilayer ion gradients) compared to unsaturated lipids such as dioleoylphosphatidylcholine. The addition of cholesterol to approximately equimolar levels also reduces the membrane permeability for unsaturated lipid systems and has the additional benefit of removing gel-liquid crystalline phase transitions that can cause leakage of liposomal contents. Improved retention can also be achieved by employing sphingomyelin in place of phosphatidy-cholines. This has been attributed to the greater hydrogen bonding effects in the headgroup region for sphingomyelin as compared to phosphatidylcholines.

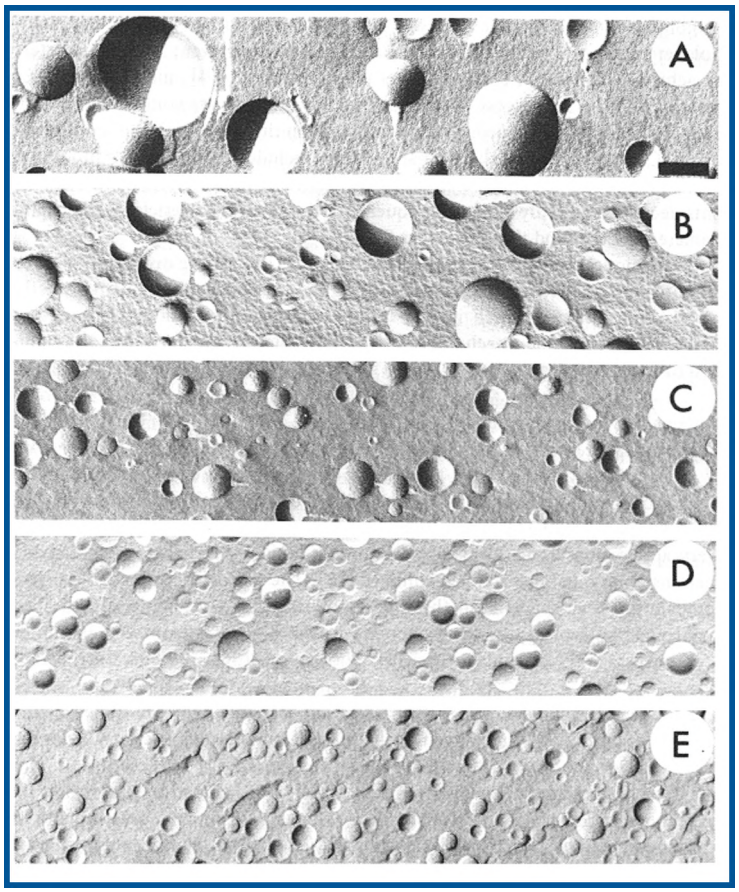

Fig. 2 Freeze-fracture electron micrographs of egg phosphati-dylcholine vesicles following extrusion of MLV through filters with pore sizes of (A) 400 nm; (B) 200 nm; (C) 100 nm; (D) 50 nm; (E) 30 nm. The bar in panel A represents 150 nm. Reproduced from M.J. Hope, M.B. Bally, L.D. Mayer, A.S. Janoff and P.R. Cullis, "Generation of multilamellar and unilamellar phospholipid Vesicles", Chem. Phys. Lipids 40: 89-108 (1986), with permission.

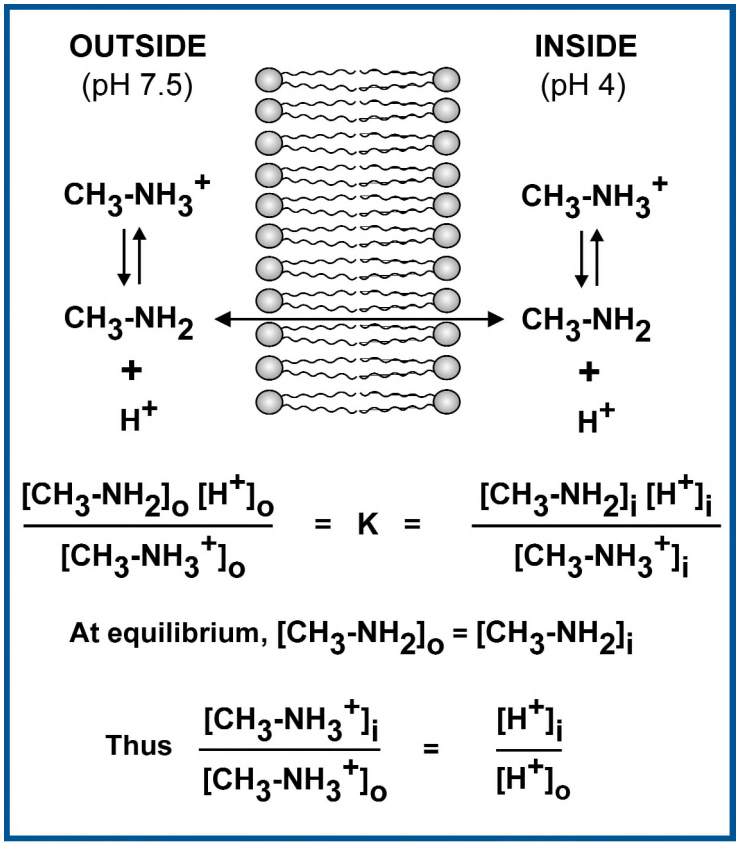

Fig. 3 Measurement of DpH using [14C]methylamine. Methylamine can exist in a charged, protonated, membrane impermeable form and in a neutral, deprotonated, membrane permeable form. When added to LUV possessing a DpH (interior acidic), the neutral form will diffuse across the membrane and become protonated in the acidic interior of the vesicle. This process continues until (at equilibrium) the concentrations of the neutral form on either side of the membrane are equal. Determination of the interior and exterior methylamine concentrations at equilibrium provides a quantitative measure of DpH. Reproduced from reference 39, with permission.

PH GRADIENTS AND DRUG LOADING

Transmembrane pH (DpH) gradients are straightforward to generate in LUV systems. For example, the LUV can be prepared in a buffer at a low pH and then the exterior pH raised by addition of a base. In addition to a pH gradient, such systems also give rise to a membrane potential due to the ability of the H+ ions to permeate out [22,23]. It was found that asymmetric transbilayer distributions of lipids such as fatty acids [24], phosphatidic acid [25] and phosphatidylglycerol [26] could be generated in response to pH gradients, however the more interesting observations concerned the techniques used to measure the ΔpH [27]. Briefly, the magnitude of the ΔpH can be determined using weak bases, such as methylamine (MeAm), as illustrated in Figure 3. An analysis of the response of MeAm to ΔpH gave important insight into how pH gradients could be used to load liposomes with drugs. Consider an LUV possessing a ΔpH of three units (pH_o = 7; pH_i = 4), to which is added an external aliquot of MeAm. 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 3, assuming the dissociation constants for MeAm are the same both inside and outside the LUV, this process will continue until the ratio of the internal to external methylamine concentration mirrors the proton gradient. For our ΔpH of 3 units this corresponds to a concentration of methylamine on the inside of the LUV that is 10^3-fold higher than the concentration outside.

The use of weak bases to measure the magnitude of ΔpH in LUV systems clearly leads to high concentrations of weak base inside the LUV when the interior is acidic. The relevance of this observation to the use of liposomes as drug delivery vehicles stemmed from the observation that a large proportion of commonly employed drugs are themselves weak bases. For example, the anti-cancer drug doxorubicin is a lipophilic amine with a single amino group and a pK_a = 8.6. It was found [28] 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 a similar way. 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 [29] or $MgSO_4$ gradients in combination with ionophores [30]. 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 PH 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 DpH loading process, these liposomes could then be readily loaded with drugs such as doxorubicin to achieve high drug-to-lipid ratios (0.2 mol:mol), corresponding to ~40,000 drug molecules per 100 nm diameter liposome. 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 [31,32] 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 [33]. Liposomal encapsulation is likely to show the greatest clinical benefit 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.

Vincristine is the most widely used anticancer agent with indications for lymphomas, lung cancer, leukemia, and breast cancer [34]. 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, as the amount in the circulation is increased by ~10^5-fold compared to injection of the same amount of the free drug. 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 [35,36,37]. 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.

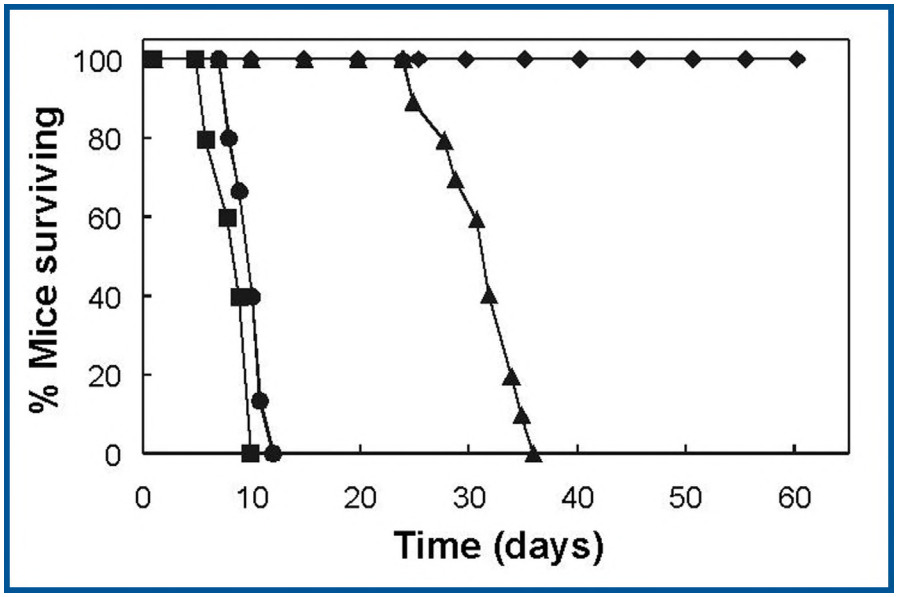

Fig. 4 **Influence of intravesicular pH on the efficacy of vincristine encapsulated in DSPC/Chol LUV against P388 tumors. BDF1 mice bearing peritoneal ascitis P388 tumors were untreated (n), treated with free vincristine (l), or were treated with LUVs containing vincristine and an intravesicular pH of either 4.0 (s) or 2.0 (u). Vesicles with lower internal pH values exhibit much improved drug retention properties. Reproduced from reference 38, with permission.**

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 concentrations. 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 [38]. Lower internal pH values lead to reduced efflux rates when the pH loading technique is employed [39]. As shown in Figure 4, 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 [40] has identified a sphingomyelin/cholesterol formulation that is more chemically stable and has similar payout rates as the DSPC/cholesterol pH 2 formulation. This formulation of liposomal vincristine has recently completed human clinical trials for treatment of relapsed lymphoma [41] and a New Drug Application to the US FDA has been submitted for clinical approval of this drug .

ENCAPSULATION OF PLASMID BY DETERGENT DIALYSIS FOR GENE THERAPY APPLICATIONS

The fact that 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 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. If the plasmid contains a therapeutic gene the possibility then exists for preferential expression of the therapeutic gene at the disease site. The need for such a systemic gene therapy vector is clear- both viral gene therapy vectors and non-viral vectors such as plasmid DNA-cationic lipid "lipoplexes" or plasmid DNA-cationic polymer "polyplexes" exhibit very short circulation life-times following i.v. injection, only reach "first-pass" organs such as the lung, liver and spleen and are highly toxic. This has restricted 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.

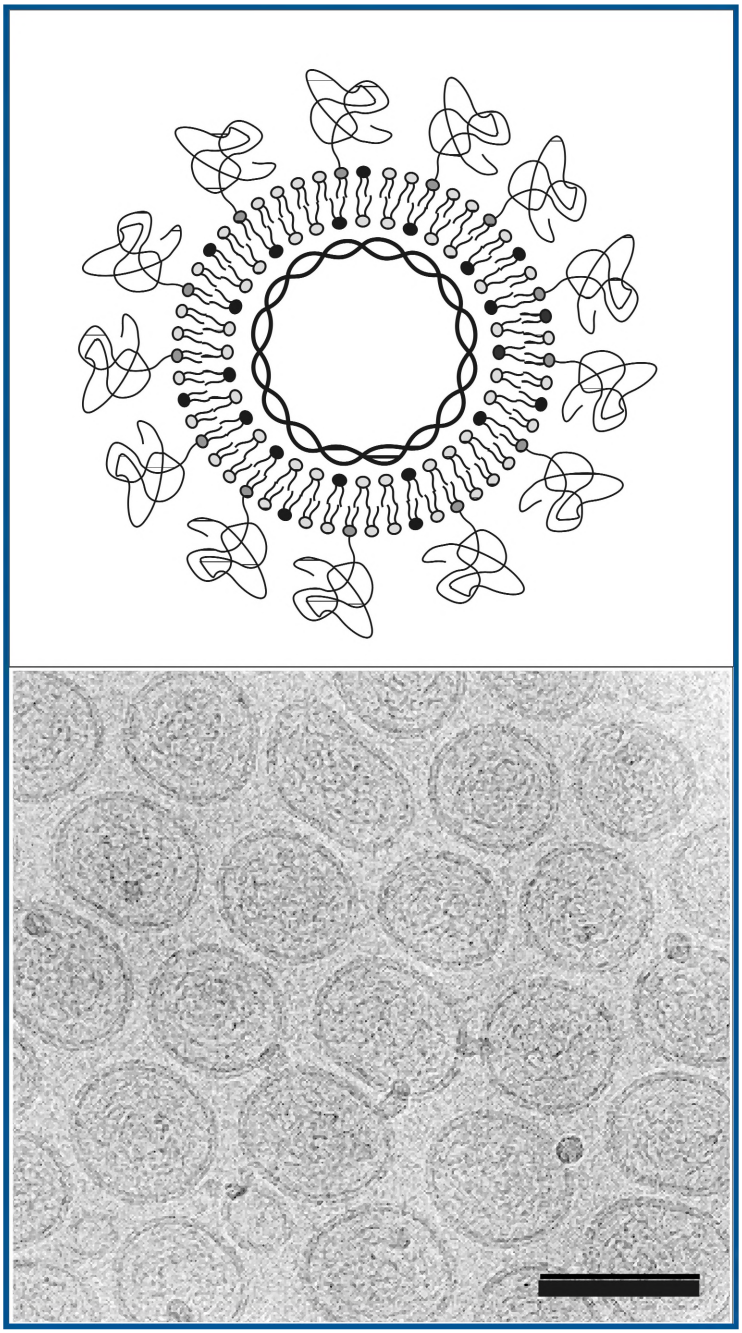

Fig. 5 Cartoon (top) and cryo-electron micrograph (bottom) of stabilized plasmid-lipid particle (SPLP) produced by detergent dialysis. The SPLP is composed of the fusogenic lipid dioleoyl-phosphatidylethanolamine (DOPE; light headgroups), the cationic lipid DODAC (black headgroups), and a stabilizing PEG-lipid (such as PEG-ceramide; dark grey headgroups).

The first step in this process was to encapsulate plasmid DNA in small liposomal systems. This was a challenging problem, as standard encapsulation techniques (pH gradients or passive entrapment) would not work on large, highly charged, impermeable polymers such as plasmid DNA. Still, there were suggestions that alternate approaches may prove fruitful. Bally and co-workers [42] had observed that cationic lipid could facilitate the uptake of plasmid into organic solvents, presumably by the formation of 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. This led to attempts to encapsulate DNA by forming plasmid DNA/cationic lipid inverted micelles in aqueous media using detergent, and then adding phospholipid to replace the detergent as it was removed by dialysis. Such detergent dialysis techniques are commonly employed to reconstitute integral membrane proteins such as cytochrome oxidase into LUV systems [43]. Although our ideas regarding the putative structure of these particles and how to form them 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 [7].

The process involves the incubation of plasmid DNA, the cationic lipid *N*,*N*-dioleyl-*N*,*N*-dimethyl-ammonium chloride (DODAC), the "fusogenic" lipid dioleoylphosphatidylethanolamine (DOPE), a polyethyleneglycol (PEG) lipid (PEG-ceramide) and the non-ionic detergent octylglu-

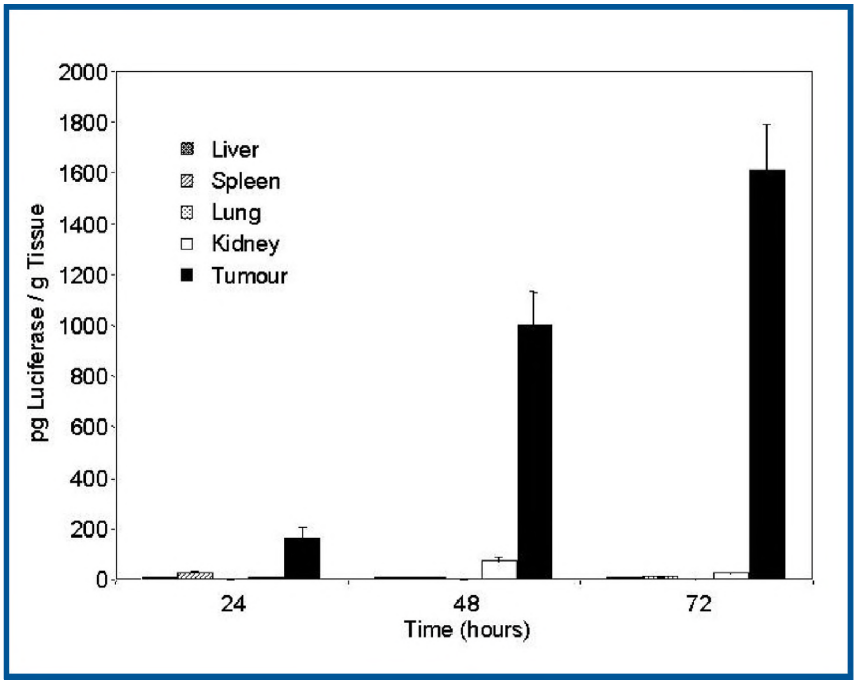

Fig. 6 **Luciferase gene expression following a single intravenous administration of SPLP in neuro-2A tumor bearing A/J mice. Reproduced from reference 45, with permission of PharmaPress Ltd.**

copyranoside. Dialysis in a HEPES-NaCl buffer (where the precise [NaCl] is critical and varies with cationic lipid content) 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 5. The SPLP are small (diameter = 70 nm), homogeneous, stable, well-defined systems containing one plasmid per particle. Further, SPLP exhibit excellent pharmacokinetic properties following i.v. injection, with long circulation lifetimes similar to those achieved for liposomal formulations of conventional drugs [44,45]. In contrast, when free plasmid DNA, or plasmid DNA in lipoplexes is injected into mice, the plasmid is cleared from the circulation almost immediately [44].

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. Tumour levels corresponding to >10% of the total injected dose of plasmid per gram of tumour tissue are observed 24 h following injection, which corresponds to more than 1000 intact plasmid molecules per tumour cell [44,45]. Administration of the same dose of plasmid DNA in the free form or in lipoplexes resulted in essentially no plasmid delivery to the tumour [44]. As shown in Figure 6, the preferential delivery of plasmid to the tumour also results in reporter gene expression [44,45], whereas naked plasmid DNA and plasmid in lipoplex form were completely ineffective [44].

INTRACELLULAR DELIVERY

One of our current research endeavours involves enhancing gene expression by increasing plasmid escape from the endosome following uptake of SPLP. These efforts were based on a series of studies examining the pH-sensitive fusion properties of LUV composed of mixtures of cationic lipid and anionic lipid, in which it was observed that equimolar mixtures yielded massive precipitates on hydration, and would not form LUV at all. Characterization of these structures by freeze-fracture electron microscopy and ^{31}P NMR revealed that these mixtures were largely composed of aggregates in the non-bilayer hexagonal H_{II} phase [8]. 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. These observations also demonstrated the value of insights derived from a research program on lipid polymorphism that was initiated in our laboratory some 25 years ago [1].

Lipids dispersed in aqueous media can adopt a variety of different 3-dimensional structures, ranging from the conventional lipid bi-layer to more exotic structures such as the hexagonal

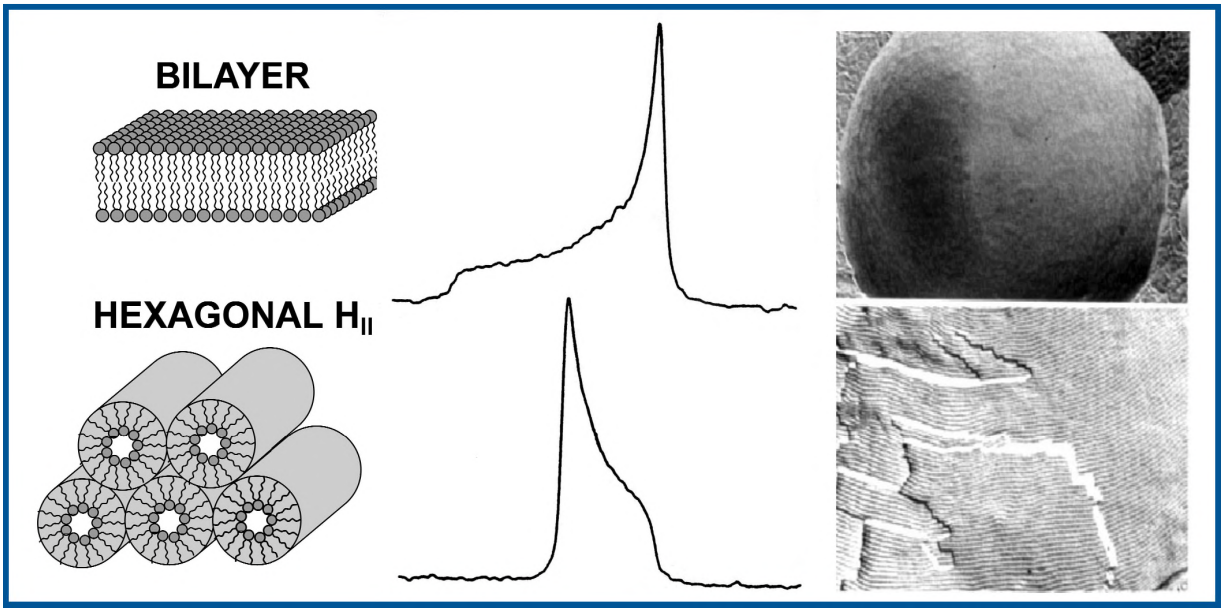

Fig. 7 **Characterization of bilayer and hexagonal phase lipid by 31P NMR and freeze-fracture electron microscopy. The 31P NMR spectra of lipids in the fluid bilayer phase exhibit a low-field shoulder and high field peak, whereas lipids in the hexagonal HII phase exhibit spectra that have reversed asymmetry and that are a factor of two narrower. Freeze-fracture micrographs obtained from lipids in the bilayer organization display smooth fracture faces, while lipids in the HII phase exhibit a corrugated pattern.**

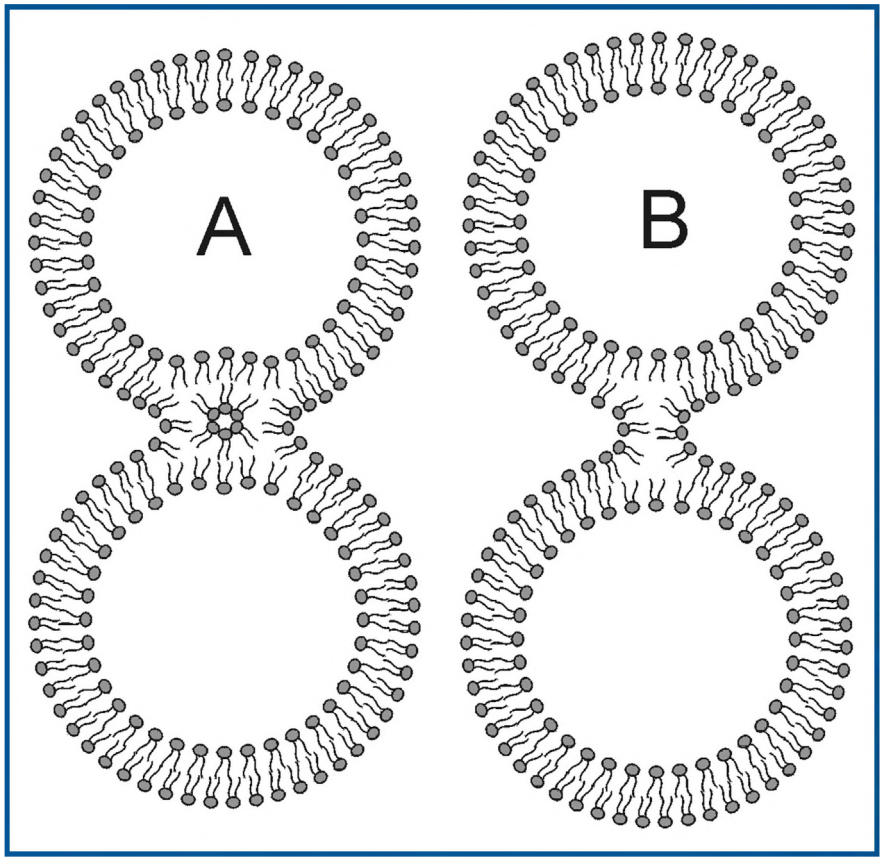

Fig. 8 **Model of membrane fusion relying on the formation of non-bilayer intermediates. The inverted micellar intermediate (IMI) pictured in (A) was the initial intermediate suggested. Current models indicate that the most likely intermediate is a stalk structure (B). See text for details.**

and cubic phases [46]. The polymorphic phase preferences of lipid dispersions have been studied in detail using 31P NMR, a technique that is sensitive to the type and rate of molecular reorientations [1,13,46]. These studies revealed that a large proportion of lipids in biological membranes prefer the non-bilayer hexagonal H_{II} phase in isolation [1]. 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 electron microscopy to identify lipid polymorphism is illustrated in Figure 7.

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 an obvious candidate as it is a topological impossibility for two membranes 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 [47,48] that membrane fusion required a non-bilayer intermediate structure as shown in Figure 8. While the actual type of intermediate may differ from the inverted micellar intermediate pictured (current models favour the 'stalk' structure proposed by Chernomordik [49,50] and later developed by Siegel [51]), 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 [52] 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 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, "helper" lipids such as DOPE and cholesterol enhance cell transfection using cationic lipids, and also promote H_{II} phase organization in mixed lipid systems [53,54,55]. Similarly, Ca^{2+} can trigger bilayer-to-hexagonal phase transitions in lipid mixtures containing negatively charged lipids [56]. In agreement with the hypothesis, it was found that Ca^{2+} increases the transfection potency of lipoplex by 5-20 fold [57], and of SPLP in BHK cells by some 600-fold [A.M.I. Lam & P.R. Cullis, unpublished results]. Related effects were observed for SPLP that contain a cationic PEG-lipid (CPL) to enhance cell uptake [9]. In the absence of Ca^{2+}, very little gene expression was observed, whereas in the presence of Ca^{2+}, increases up to 105-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 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. It is anticipated that increased understanding of the biochemistry and biophysics of membrane lipids will also lead to the next major breakthroughs in liposome drug delivery.

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