Model Membrane Systems as Drug Delivery Vehicles

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Abstract

Model membrane systems have been developed primarily to provide simplified versions of biological membranes, which allow the properties of individual components to be studied in detail. Model systems have provided a great deal of information on the conformation of lipids in membranes, the rates and type of motions undergone by individual lipid molecules, and the types of polymorphic phases preferred by lipids in isolation and in mixtures. This has led to many insights concerning the roles of lipids in membranes, such as the relationship between non-bilayer phases and membrane fusion (Cullis and De Kruijff, 1979; Bloom et al., 1991; Siegel, 1993). However, model membranes and the techniques used to generate them have considerable utility in their own right for drug delivery applications. This utility is straightforward to illustrate. An ideal drug delivery system will exhibit several characteristics: (i) a small size (<100 nm diameter) combined with reasonably long circulation lifetimes to be able to access disease sites such as tumours, (ii) efficient loading with a biologically active agent, and (iii) fusogenic properties that allow the contents of the carrier to be delivered into target cells. All of these properties can be satisfied by utilizing our knowledge of model membrane systems.

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Size and Circulation Lifetime

Many model membrane studies have involved multilamellar vesicles (MLVs), large multilamellar lipid dispersions with diameters on the order of microns (Cullis and De Kruijff, 1979; Bloom et al., 1991). While excellent systems for biophysical studies involving magnetic resonance and fluorescence techniques, MLVs are unsuitable for examining many fundamental membrane properties, such as lipid asymmetry and membrane permeability. These types of studies require unilamellar vesicles, which can be formed in sizes ranging from 20 nm to several microns by a variety of techniques (Hope et al., 1993; Bloom et al., 1991). The extrusion technique, a procedure developed to make large unilamellar vesicle (LUV) model membrane systems (Hope et al., 1993), requires only hydra-

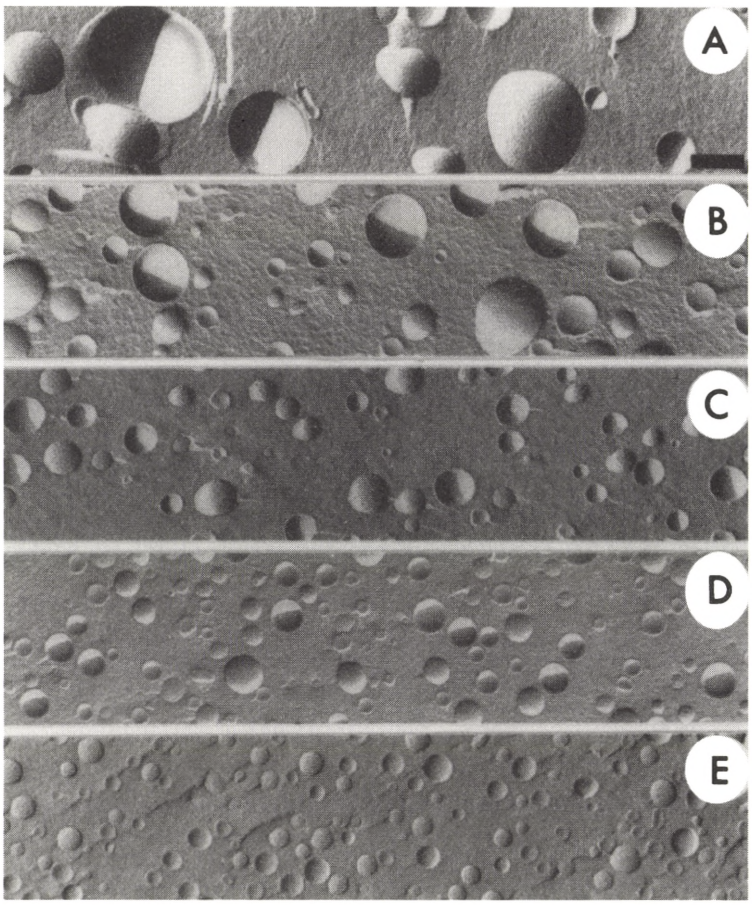

Figure 1. Freeze-fracture electron micrographs of egg phosphatidylcholine LUVs prepared from MLVs (freeze-and-thawed) passed 20 times through polycarbonate filters of various pore sizes. The pore sizes of the filters employed were (A) 400 nm, (B) 200 nm, (C) 100 nm, (D) 50 nm, and (E) 30 nm. The bar in panel A represents 150 nm and all panels represent the same magnification. Reproduced from Hope et al. (1986) with permission.

tion of the lipid and extrusion under medium pressures through polycarbonate filters with pore size of 200 nm or smaller. Vesicles as large as 400 nm diameter can be made, but some of these are not unilamellar. The procedure is quick (15 minutes) and yields a homogeneous population of LUVs in the size regime ideal for drug delivery applications (100 nm diameter). Vesicles larger than this are cleared more rapidly by the reticuloendothelial system, and are less readily taken up into cells by endocytosis, whereas vesicles much smaller have an internal volume that is too small to transport sufficient quantity of drug. Freeze-fracture electron micrographs of LUVs ranging in size from 30 to 400 nm are shown in Figure 1.

The requirement for reasonably long circulation lifetimes can be satisfied in two additional ways. The first involves the use of lipid compositions such as saturated phosphatidylcholines or sphingomyelins, in combination with cholesterol in (nearly) equimolar concentration. This lipid composition, which corresponds to the lipids found on the outer monolayer of eukaryotic membranes such as the erythrocyte membrane (Op Den Kamp, 1979), binds relatively small amounts of serum proteins, the presence of which triggers uptake

by fixed and free macrophages in the circulation (Chonn et al., 1992). The second method of increasing circulation times is to attach hydrophilic polymers, such as polyethylene glycol (PEG), to the liposome surface. As little as 5 mol% of a phosphatidylethanolamine-PEG construct incorporated into an LUV provides a steric barrier to plasma proteins which can greatly increase circulation time (Gabizon and Papahadjopoulos, 1988).

Loading of Drugs into LUVs in Response to Transmembrane pH Gradients

The requirement for efficient loading of the drug delivery system can also be satisfied using techniques developed for model membrane systems. The first method for loading LUVs followed logically from the development of a model membrane system exhibiting asymmetric transbilayer distributions of lipids, such as is observed in biological membranes. It was reasoned that transmembrane pH gradients (ΔpH) should result in asymmetric transbilayer distributions of lipids that are weak acids or weak bases, a prediction that was borne out for lipids such as fatty acids, stearylamine, and some phospholipids (phosphatidic acid and phosphatidylglycerol) (Hope et al., 1989). This suggested that other lipophilic weak bases, a class which includes a large proportion of commonly used drugs, would also migrate across lipid bilayers in the presence of a ΔpH (Cullis et al., 1997). The mechanism for uptake is illustrated in Figure 2, which represents the addition of a drug such as doxorubicin (a weak base with a $pK_a = 8.6$) to LUVs exhibiting a ΔpH (inside acidic). For external pH values near neutrality, the drug will exist in a mixture of neutral and charged forms. The neutral forms are membrane permeable and can diffuse into the vesicle interior where they are protonated and trapped (due to the impermeability of the charged form). It is straightforward to show that at equilibrium $[\text{Drug}]_{in}/[\text{Drug}]_{out}=[H^+]_{in}/[H^+]_{out}$, and thus for a pH gradient of 3 units the internal drug concentration will be 1000 times higher than the external. Conditions can be achieved for LUVs with an acidic interior where essentially 100% uptake and retention of drugs (such as the major anticancer drugs doxorubicin and vincristine) can be attained at drug-to-lipid ratios which are much higher than can be achieved by other techniques (Mayer et al., 1993; Cullis et al., 1997). As shown in Figure 3, essentially complete uptake of doxorubicin into 200 nm egg phosphatidylcholine/cholesterol LUVs can be achieved in less than 5 minutes.

Other methods for drug loading have been described, which are also based on the generation of a transmembrane pH gradient. For example, LUVs exhibiting a transmembrane ammonium sulfate gradient develop secondary pH gradients, which form as a quantity of neutral ammonia leaks out of the vesicle, leaving its protons behind, acidifying the interior. This pH gradient can then drive drug uptake (Haran et al., 1993). An alternative loading strategy is based on drug-uptake driven by pH gradients generated by the addition of a suitable ionophore to LUVs containing an appropriate entrapped metal ion (Fenske et al., 1998). For example, the addition of nigericin to LUVs containing K_2SO_4 leads to generation of a ΔpH and rapid uptake of anticancer drugs such as vincristine, or of antibiotics such as ciprofloxacin. Similar results are obtained for the ionophore A23187 in conjunction with LUVs containing entrapped $MnSO_4$. These applications follow from early studies by Deamer et al. (1972), who demonstrated the ionophore-mediated generation of a pH gradient in small unilamellar vesicles.

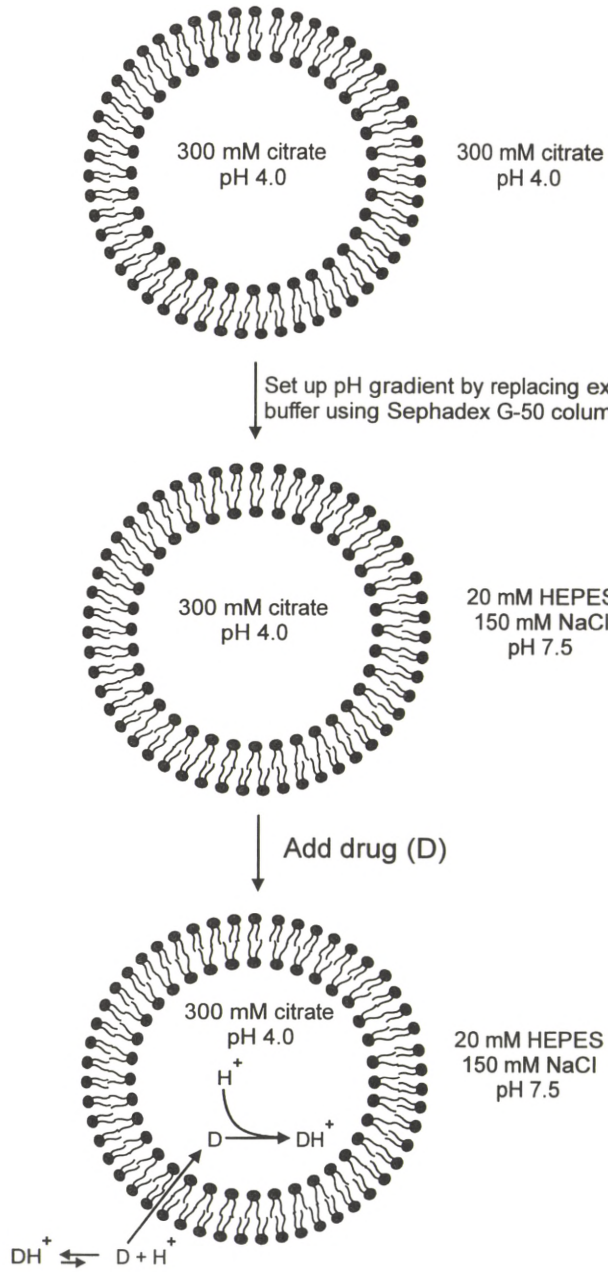

Figure 2. Diagrammatic representation of drug loading in response to a transmembrane pH gradient. The lipid mixture is hydrated and subsequently extruded in 300 mM citrate pH 4.0 (top). A pH gradient is established by passage of the LUVs down a column of Sephadex G-50 hydrated in 20 mM HEPES 150 mM NaCl pH 7.5 (center). The remote loading process involves addition of drug to the LUVs exhibiting the pH gradient. The external drug exists in neutral and protonated forms, and it is the former which can diffuse across the membrane (down its concentration gradient). Once inside the vesicle, the drug is protonated and trapped, resulting in an extremely low internal concentration of neutral drug, which therefore continues to drive uptake.

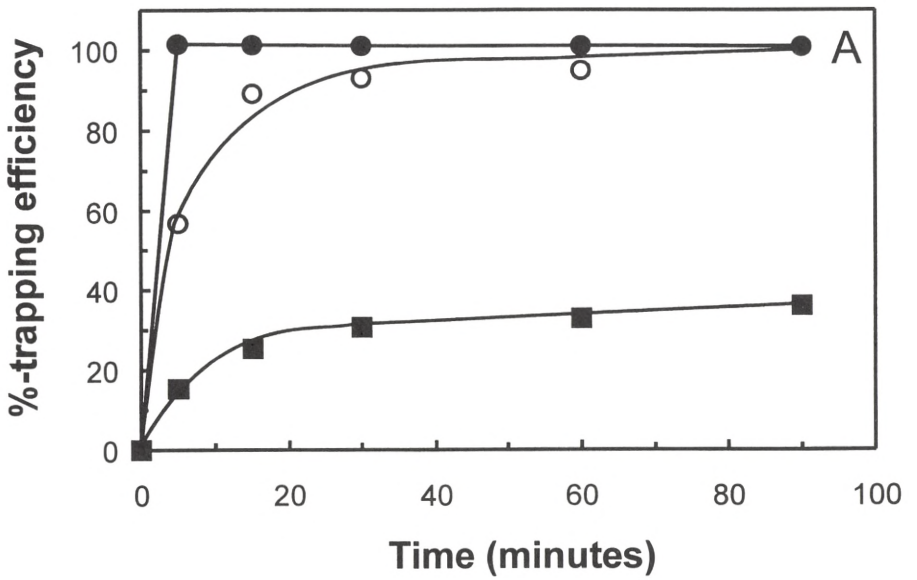

Figure 3. Uptake of doxorubicin into 200 nm EPC/cholesterol (55:45 mol/mol) LUVs exhibiting a transmembrane pH gradient (pH 4 inside, 7.8 outside). Doxorubicin was added to LUVs (drug-to-lipid ratio = 0.3 wt:wt) equilibrated at 21°C (closed squares), 37 °C (open circles), and 60°C (closed circles). Note that rapid and complete uptake only occurs in fluid membranes. The trapping efficiency was calculated as the percentage of the drug initially in the exterior medium which was accumulated into the LUVs. Reproduced from Mayer et al. (1989) with permission.

In the case of large "biopharmaceutical" drugs such as plasmids for gene therapy, loading techniques developed for model membrane systems can also be applied. For example, recent work has shown that incubation of plasmids with cationic lipids results in hydrophobic entities which are soluble in organic solvent. In order to entrap this construct within a lipid based system it was found that a variant of the detergent dialysis procedure used for reconstituting membrane proteins in vesicles (Kagawa and Racker, 1971) can be employed. The resulting DNA delivery systems are small (<100 nm diameter) and stable in biological fluids (J.J. Wheeler, unpublished results).

Fusogenic Properties of Drug Delivery Systems

The requirement for fusogenicity for intracellular delivery of carrier contents can be approached in two ways. First, it is well known from model membrane studies that the presence of "non-bilayer" lipids such as unsaturated phosphatidylethanolamines (PE) can dramatically enhance the ability of lipid bilayers to fuse (Cullis and De Kruijff, 1979; Cullis et al., 1990; Siegel, 1993). Second, as can also be shown employing model membrane systems, fusion between vesicles is enhanced by electrostatic interactions. Vesicles containing negatively charged lipids such as fatty acids or phosphatidic acid can be induced to fuse by the addition of Ca^{2+} (Cullis et al., 1997), and vesicles containing positive charge can be induced to fuse

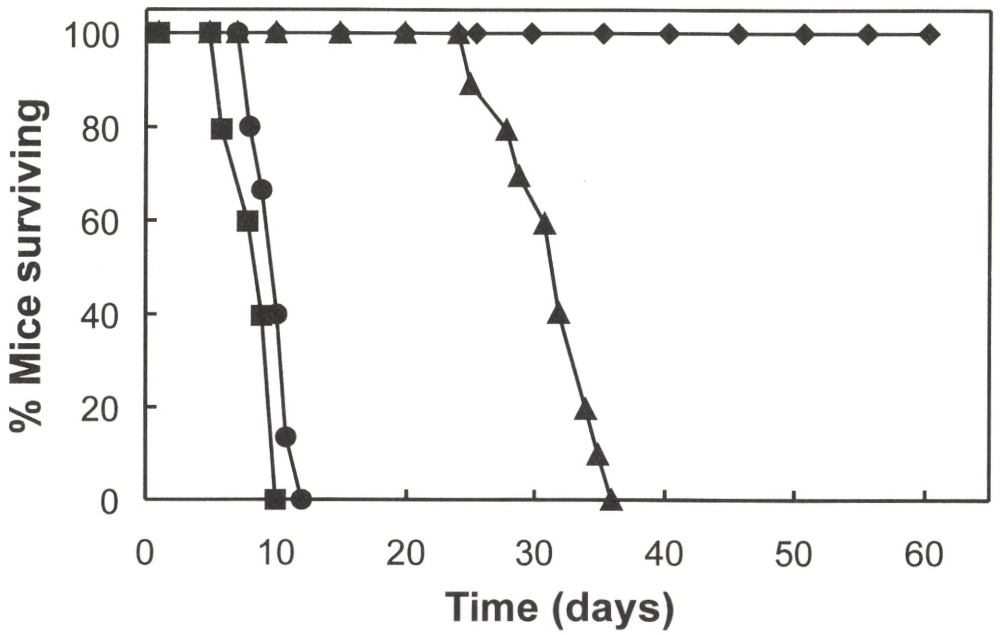

Figure 4. Influence of drug encapsulation and intravesicular pH on the efficacy of vincristine encapsulated in DSPC/Chol LUVs against P388 tumors. BDF1 mice bearing peritoneal ascitic P388 tumors were untreated (squares) or were treated with free vincristine (circles) or LUVs composed of DSPC/Chol containing encapsulated vincristine and with an intravesicular pH of either 4.0 (triangles) or 2.0 (diamonds). Reproduced from Boman et al. (1995) with permission.

with those possessing negative charge (Bailey and Cullis, 1997). As the plasma membrane of cells exhibits a negative charge, including a positive charge in the delivery vesicle can enhance fusion. For example, vesicles composed of dioleoylphosphatidylethanolamine (DOPE) and N,N-dimethyl-N,N-di-9-cis-octadecenylammonium chloride (DODAC) (the latter possessing a permanent positive charge) in a 1:1 molar ratio will fuse with erythrocyte membranes (Bailey and Cullis, 1997). A problem with these and other tactics to improve intracellular delivery, however, is that these sytems will tend to fuse with the first cells that they encounter, rather than after arrival at a disease site. This property can be corrected, at least in part, by coating the vesicle with a polyethylene glycol (PEG) coating, originally developed to engender longer circulation lifetimes for LUV systems (Gabizon and Papahadjopoulos, 1988). However, this also has the effect of inhibiting fusion with cells after arrival in target tissue. In order to render these systems fusogenic at these later times, we have developed PEG coatings that dissociate from the carrier at well defined rates, thus inhibiting fusion immediately after injection, but rendering them more fusogenic at later times. This is accomplished by varying the length of the fatty acyl chains in the lipid anchor of the PEG-lipid construct (Holland et al., 1996).

Two applications of these lipid-based delivery systems concern delivery of anticancer drugs such as vincristine and delivery of plasmids containing therapeutic genes for gene therapy applications. With regard to vincristine, delivery systems with appropriate retention and drug payout characteristics provide a remarkable improvement in efficacy in animal tumour models (Boman et al., 1995; Chonn and Cullis, 1995). A particularly striking example of this is shown in Figure 4, where the influence of drug encapsulation and intravesicular pH on vincristine efficacy is illustrated for BDF1 mice bearing ascitic peritoneal P388 tumours. A

lower internal pH results in improved vincristine retention in the LUV. The survival time of mice treated with free vincristine was only slightly better than for those receiving no treatment (10-12 days). Encapsulation of vincristine in LUVs with an internal pH of 4 more than tripled the survival time, and a further reduction of the internal pH to 2 resulted in 100% survival over a period of 60 days. These survival results correlate directly with the retention characteristics of the drug delivery systems.

While still in the early phases of research, plasmid delivery systems have now been developed which lead to expression of marker genes in a variety of tissues *in vivo*, suggesting their utility for gene therapy applications.

Summary

In summary, the techniques used for the generation of model membranes, and the results derived from model membrane studies, have played a direct role in the development of drug delivery systems. The extrusion technique provided a quick and reliable method for obtaining stable populations of vesicles with diameters ideal for drug delivery applications. The accumulation of lipophilic weak bases within LUVs in response to transmembrane pH gradients permitted the development of loading techniques that exhibited high levels of uptake and excellent retention in vivo. The use of highly ordered lipid compositions (saturated phospholipids and cholesterol) and/or inclusion of PEG-lipids greatly increased LUV circulation lifetimes, allowing accumulation of vesicles at sites of disease. Finally, the inclusion of fusogenic lipids increased the intracellular delivery of liposome contents to target cell populations. Future advances in the development of drug delivery systems for both conventional anticancer drugs and genetic drugs (e.g., plasmid DNA and antisense oligonucleotides) will undoubtedly depend on continued research using model membrane systems.

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