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Liposomes *In Vivo:* Prospects for Liposome-Based Pharmaceuticals in the 1990s

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Introduction

The pharmaceutical industry needs new and more effective ways of using novel and established drug entities, especially those now being produced thanks to major advances in biotechnology. Drug delivery systems already provide a means of achieving slow-release therapy and can contribute to the solution of ongoing challenges, including inappropriate drug solubility, toxicity to non-target tissues and rapid inactivation of drugs in the biological milieu. These concerns are especially pressing for peptide and protein drugs now being produced in pharmaceutical grade and amounts by using recombinant DNA biotechnology.

Drug delivery strategies currently employed for products on the market or in late development include: biomechanical controlled-release devices, such

Abbreviations: BisHOP, (±)-*N*-[(2,3-dihexadecyloxy)prop-1-yl]-*N,N,N*-trimethyl-ammonium chloride; CF, carboxyfluorescein; Chol, cholesterol; DCP, dicetylphosphate; DMPC, dimyristoyl phosphatidyl-choline; DOTMA, (±)-*N*-[(2,3-dioleyloxy)prop-1-yl]-*N,N,N*-trimethylammonium chloride; DPPC, dipalmitoyl phosphatidyl-choline; DRV, dehydration-rehydration vesicles; DSPC, distearoyl phosphatidyl-choline; FATMLV, freeze and thaw multilamellar vesicles; GM_1, monosialo-galactosyl ceramide; GMP, good manufacturing practice; GUV, giant unilamellar vesicles; HDL, high-density lipoproteins,; IDL, intermediate-density lipoproteins; i.m., intramuscular; i.p., intraperitoneal; i.v., intravenous; LCAT, lecithin-cholesterol acyl transferase; LDL, low-density lipoproteins; MDP, muramyl dipeptide; MLV, multilamellar vesicles; MPEG, monovalent polyethylene glycol; MTP, muramyl tripeptide; NTA, nitrolotriacetic acid; PA, phosphatidic acid; PAC, perturbed angular correlation; PC, phosphatidyl-choline (egg); PE, phosphatidyl-ethanolamine; PEG, polyethylene glycol; PG, phosphatidyl-glycerol; PI, phosphatidyl-inositol; PS, phosphatidyl-serine; RES, reticuloendothelial system; REV, reverse-phase evaporation vesicles; SA, stearylamine; s.c., subcutaneous; SM, sphingomyelin; SRBC, sheep red blood cells; SUV, small unilamellar vesicles; Tc, liquid–crystalline phase-transition temperature; VLDL, very low-density lipoproteins.

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as transdermal patches, implantable pumps, bio-errodable polymer-based systems, liposomes, and time-release capsules for oral dosing. Such approaches increase the options available for effective drug formulation in the product development process (*see* Mackay, Chapter 8 this volume). While the cheapest and most convenient way to manufacture a new drug formulation is to compress the active compound into a tablet for oral dosing or suspend it in sterile saline for intravenous and other injectable routes, this first-line approach can fail to produce an effective drug product for a number of reasons: first, the drug may have poor solubility in conventional pharmaceutically acceptable solvents and this can result in lack of bio-availability, including adsorption to non-target sites. Secondly, powerful drugs such as hormones, growth factors and other protein mediators of cell activity, can exhibit significant toxicity in non-target tissues. Thirdly, drug inactivation and/or degradation can occur in the biological milieu, especially when relatively labile molecules such as peptides and proteins are used as drugs.

Of a variety of formulation strategies, liposomes are well characterized as a drug-delivery system. Uniquely biocompatible, being composed of non-toxic, readily metabolizable material which can be produced cheaply in kilogram amounts (Weiner, 1989), liposomes can be manufactured with remarkable flexibility thanks to recent technological advances (Klimchak and Lenk, 1988; Lichtenberg and Barenholz, 1988; Özer *et al.*, 1988; Cullis *et al.*, 1989; Weiner, 1989, 1990 a,b; Martin, 1990). Liposomes are therefore especially appropriate for parenteral drug delivery. As for any material to be administrated parenterally, a liposome-drug formulation must be sterile, pyrogen-free and stable with respect to numerous pharmaceutical parameters (Zonneveld and Crommelin, 1988). Ideally, the product must be designed so that the lipsome-based treatment does not require excessive manipulation by medical personnel prior to its administration to the patient. These specific challenges are being addressed for a number of drugs (Gregoriadis, 1988) and for some drug entities which are the subject of later discussion, many of these difficulties are being overcome. In addition to pharmaceutical challenges in manufacturing, therapeutic application of liposomes and other carriers depends upon the pharmacokinetics and biodistribution of the drug and its delivery system. Optimization of the liposome system *in vivo* by manipulating its physical characteristics is essential to realize therapeutic end-points and is discussed in detail here. Key determinants of liposome fate and behaviour *in vivo* are highlighted and strategies for future progress using liposome-based pharmaceuticals are discussed.

The term 'liposomes' was first used by Gerald Weissmann at a conference in the early seventies. Until then microscopic spheres made from lipid and enclosing an aqueous space were referred to as lipid vesicles (Bangham, Standish and Watkins, 1965) and the term 'vesicle' remains a prominent part of the nomenclature (*Table 1*). Especially when such lipid particles carry or otherwise sequester therapeutic drugs (Gregoriadis and Ryman, 1971; Gregoriadis, 1976), the general term, liposomes, is firmly established. Drug molecules can be carried in the aqueous compartment, intercalated between lipid molecules in the lipid bilayer, or can be partially embedded in the bilayer

with the remainder of the molecule extending outside the bilayer to the inside or the outside of the vesicle. Any drug that does not disrupt the lipid bilayer structure can be carried in association with liposomes. The amount of a drug sequestered will depend on the technique of manufacture, the type of lipids used, and the nature of the drug itself. In general, liposomes are formed from amphipathic lipids such as phospholipids, which have both polar and non-polar characteristics; other amphipathic molecules, such as surfactants, also form vesicles, e.g. niosomes. Such molecules spontaneously arrange themselves into bilayers in aqueous solution, to form closed structures. In the process, any substance dissolved in the aqueous phase becomes entrapped inside the vesicles. Lipid-soluble drugs can be incorporated into the lipid bilayer and rendered 'water-soluble' if the drug can subsequently become bio-available *in vivo*.

Table 1. Major liposome types in drug delivery

Liposome type	Characteristics	References
MLV; multilamellar vesicles	many layers, 100 nm–2 μm	Lichtenberg and Barenholz (1988) (review)
SUV; small unilamellar vesicles	single bilayer, <80 nm	Huang (1969)
REV; reverse-phase evaporation vesicles	1 to few bilayers 80–200 nm	Szoka and Papahadjopoulos (1978)
DRV; dehydration-rehydration vesicles	many layers, 100 nm–2 μm	Kirby and Gregoriadis (1984)
FATMLV; freeze and thaw multilamellar vesicles	many layers, 100 nm–2 μm	Lichtenberg and Barenholz (1988) (review)
GUV; giant unilamellar vesicles	cell sized >1 μm	Kim and Martin (1981)

Key variants of liposome architecture are: vesicle size and size distribution, number of lamellae, trapped volume and osmotic activity, structural and motional behaviour of the lipids in the bilayer, electrical surface potential, compositional heterogeneity and distribution of lipid components in the bilayer (Lichtenberg and Barenholtz, 1988). These characteristics are controlled by the method of manufacture of the vesicles. Liposomes are often designated according to the initial method of production, e.g. DRV, REV, detergent dialysis liposomes, etc., although further processes such as filtration, sonication and high-pressure homogenization (Microfluidizer^{TM}) technology, convert vesicles prepared by one method into smaller liposomes or a more defined size dispersion. For a comprehensive review of current liposome methodology, *see* Lichtenberg and Barenholtz (1988); for the manufacturing-scale, *see* Martin (1990). From the biological, hence therapeutic standpoint, particle size is the primary feature of liposome design which affects rapid removal of liposomes from the circulation, and also determines drug-carrying capacity. Size is controlled by the method of preparation and varies between 20 nm and several microns in diameter. Names of liposome preparation types (*Table 1*) give a useful first indication of size. The amount of drug or other marker that a liposome carries for a given amount of lipid, is

determined primarily by the diameter of the vesicles. For example, vesicles composed of phosphatidyl-choline (PC) with diameter of 30 nm (prepared by sonication) have an internal capture volume of 0·35 μl mg^{-1} phospholipid (Anzai, Yoshida and Kirino, 1990) whereas those with an average diameter of 200 ± 50 nm (prepared by detergent dialysis) have an average volume of 7·9 μl mg^{-1} phospholipid (Kojro *et al.*, 1989). This distinction is also relevant in interpretation of *in vivo* experiments where the fate of a population of vesicles of mixed size is followed. The number of lamellae or lipid bilayers present in each vesicle (*Table 1*) may contribute to the drug-carrying capacity of a stated amount of lipid, as well as to vesicle stability. However, for certain types of large vesicles formed using lyophilization techniques, the presence of relatively large numbers of lamellae does not hinder the entrapment efficiency, indeed it may contribute to the high entrapment values obtained (Kirby and Gregoriadis, 1984). If a drug has a net charge, lipid of an opposing charge can boost the overall drug incorporation. By means of these and other strategies (Cullis *et al.*, 1989; Martin, 1990), liposomes can now be generated and loaded with considerable flexibility for application to a wide range of therapeutic and pharmaceutical requirements.

Therapeutic applications of liposomes of current interest include toxicity buffering of drugs, enhancement of drug efficacy or potency, dissolution of water-insoluble drugs, sustained release and targetting to specific tissues (Weiner, Martin and Riaz, 1989). Major liposome design characteristics which control their biological properties and lead to optimized therapeutic benefits for carrier-associated drugs include: size, lipid composition and surface characteristics, drug–lipid ratio and dose (Gregoriadis, 1980; Senior, 1987). In the absence of a general understanding of the mechanisms whereby liposome delivery is of therapeutic benefit (Cullis *et al.*, 1989), it is all the more important to investigate how liposomes which vary in these design characteristics behave *in vivo*. It is relatively easy to define pharmaceutical end-points and, for liposome formulations already in the clinic, many associated problems are being overcome. However, there remains some ambiguity as to which biological end-points will optimize the therapeutic benefits of a drug. A strong predictive experimental framework for liposome behaviour *in vivo* saves time and money when assessing the suitability of new drugs in liposome formulations. However, the arrangement of drugs in relation to the membrane, drug interaction with the bilayer and surface-exposure of the drug can all result in vesicles with properties different from liposomes made in the absence of drug. Parallel studies involving model liposomes and liposomes containing specific drug entities should therefore be implemented in any parenteral formulation development programme.

Success in liposome biotechnology applied to the pharmaceutical industry has progressed to date by exploitation of the natural way liposomes fit into the physiological functions of the living animal, i.e. 'passive' rather than 'active' targetting. Present therapeutic applications have been most productive where drug entrapment in liposomes increases the effectiveness of a drug by exploiting the body's degradative and removal mechanisms. Future therapeutic applications may be most productive where drug entrapment in

liposomes increases the effectiveness of a drug by circumnavigating these mechanisms. This review details what is known about the way in which liposomes are dealt with by the body's defenses and metabolism when introduced into a healthy animal.

Experimental basis for characterization of liposome behaviour *in vivo*

In order to manipulate the behaviour of a drug–liposome complex *in vivo*, and to refine the pharmacokinetic profile of the liposomal drug, it is essential to identify all factors which determine the fate of the vesicles after their introduction into the body. Experimentally, this involves locating liposomes and their contents after administration. In some past studies, the fate of a free drug is compared with that of the liposomal drug simply by analysing general drug disposition. As discussed in detail elsewhere (Senior, 1987), this approach only indicates the fate of the liposome carrier if the drug remains fully associated with the carrier. Most drugs in therapeutic liposome formulations are not convenient markers for coming to general conclusions about liposome fate and behaviour *in vivo*, just as many lipid compositions do not quantitatively retain an otherwise good marker. A systematic approach allowing clarity of interpretation of experimental findings coupled with ease of experimental design, requires reliable markers of: (1) the aqueous compartment, such that any leakage of marker is readily measurable; and (2) the lipid bilayer itself, such that the marker does not exchange with or transfer to other molecules or cells *in vivo*. In the case of aqueous space marker, distinction has to be made between how much drug is inside the liposomes *in vivo* and how much has leaked into or exchanged with, other compartments. This is facilitated experimentally using 'designer' model compounds which have readily measurable self-indicating means of characterizing marker retention.

Fluorescent markers, such as carboxyfluorescein (CF) a fluorescein derivative less sensitive to pH changes, 'calcein', and sodium fluorescein, have been used extensively *in vitro* and *in vivo* to investigate the susceptibility of different types of liposomes to the induction of leakiness under defined conditions (Gregoriadis and Davis, 1979; Allen and Cleland, 1980; Kirby, Clarke and Gregoriadis, 1980a,b; Damen, Regts and Scherphof, 1981; Senior and Gregoriadis, 1982a,b, 1984), and remain one of the most convenient molecules for evaluating the structural integrity of particular liposome systems (Senior, 1987). This is because dye entrapped inside the liposome is self-quenched at concentrations of 0·1 M and above, whereas dye which has leaked out of the liposomes and which has become diluted into the surrounding plasma, serum or buffer, will fluoresce. The percentage of total CF present in a given sample of liposomes is the latency of the system. Thus a vesicle preparation which is very stable with respect to dye retention will maintain high latency values on incubation in plasma or blood at 37°C for several days (Senior, Crawley and Gregoriadis, 1985). *In vivo*, free dye is eliminated from the circulation within minutes, implying that clearance of quenched CF will parallel clearance of intact liposomes (Gregoriadis and Davis, 1979). Vesicle clearance is most faithfully measured using this marker,

when liposomes fully retain the dye on dilution into plasma (Senior, Crawley and Gregoriadis, 1985).

Another well-established technique for establishing liposome behaviour *in vivo* is the use of radioactive indium ions ($^{111}In^{3+}$) complexed with a chelating agent, nitrolotriacetic acid (NTA) (Hwang and Mauk, 1977). The chelated indium complex is encapsulated in the aqueous compartment of the liposomes. Conventional γ-radioisotopic counting can be used to analyse the quantity of marker present in blood and tissues. The great advantage of this marker system is that the structural integrity of the liposomes can be monitored even when the liposomes have been incorporated into tissues (Hwang, Luk and Beaumier, 1980; Roerdink *et al.*, 1989). Measurements are carried out using γ-ray perturbed angular correlation (PAC). PAC values will be about four times higher if the chelated indium complex is inside the vesicles rather than for marker which has leaked out, allowing a time-course for liposome disintegration in a particular tissue to be determined (Hwang, 1984). Other radiolabelled markers which are well retained inside liposomes of suitable lipid composition and have been usefully employed as models in liposome studies include: inulin (Tall, 1980; Mimms *et al.*, 1981), bleomycin (Segal *et al.*, 1976; Senior, Crawley and Gregoriadis, 1985) and sucrose (Kirby and Gregoriadis, 1981; Allen and Everest, 1983).

Systematic studies of drugs currently in clinical trials, which aim to evaluate liposome *in vivo* behavioural characteristics, utilize doxorubicin (Balazsovits *et al.*, 1989; Mayer *et al.*, 1989; Storm *et al.*, 1989) and polyene antibiotics, such as amphotericin B (Mehta, 1989).

Factors influencing liposome fate *in vivo*

The main factors that control the fate and behaviour of liposomes *in vivo* are:

1. The biological milieu (environment) encountered by the vesicles on their introduction into the animal, obviously dependent on the route of administration;
2. The physical parameters of liposome design, such as their size, surface characteristics and other aspects of the manufacturing process;
3. The nature, accessibility and surface characteristics of the target.

These factors are inevitably interrelated; hence, for example, liposome design will determine vesicle resistance to destruction in the blood and the extent of their access to tissues.

BIOLOGICAL ENVIRONMENT—ASSOCIATED FATE OF LIPOSOMES

Liposomes administered to animals, including humans, will be subjected to the usual degradative and removal processes for particles entering the body by the chosen route. The healthy gut, for example, will treat liposomes as a lipid-rich meal. Conditions after administration by other routes may be less immediately destructive, but liposomes are still subjected to the usual degradative and absorptive processes accompanying the introduction of any

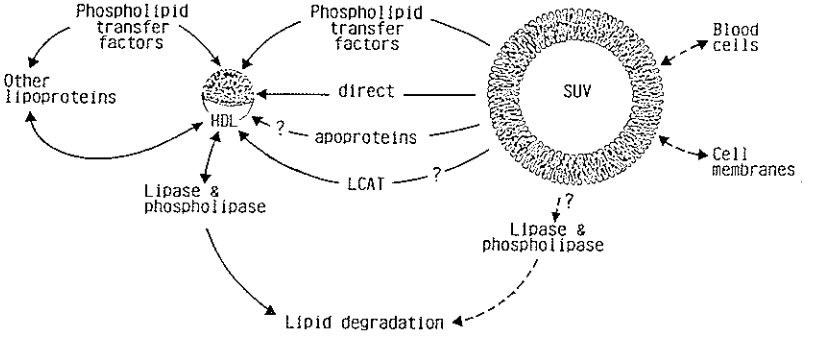

Figure 1. Movement of phospholipid between liposomes and HDL in normal lipid metabolism. Arrows indicate net transfer of phospholipid (usually PC). Broken lines indicate minor transfer or exchange only. (Reprinted with permission from Senior, 1987; copyright CRC Press, Inc., Boca Raton, Florida.)

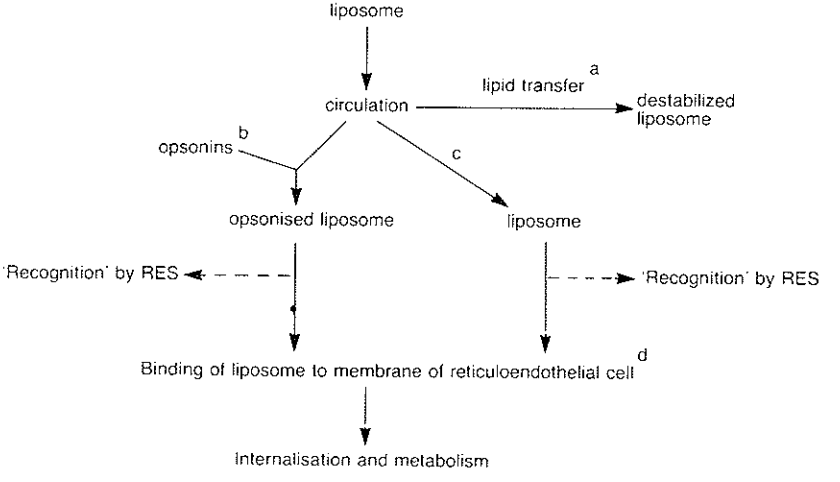

Figure 2. Physiological stages in liposome removal from circulating blood. Liposomes in the absence of specific targetting groups are treated as a lipid-rich particle (destabilization) or as a foreign particle (RES recognition). (a) See *Figure 1*; (b) See text; (c) putative pathway for liposome uptake by cells of the reticuloendothelial system (RES) in the absence of opsonins. It is speculated that liposomes could bind directly to RE cells mediated by a specific lipid, or by a charge-sensitive or other receptor; (d) cells other than phagocytic macrophages of the reticuloendothelial system, e.g. hepatocytes, may process small liposomes via pinocytosis.

exogenously derived material. The extent to which particles succumb to these processes depends, at least in part, upon their design characteristics.

Removal and breakdown of liposomes with the accompanying release of contents can be a factor-mediated (humoral) process (*Figure 1*), and/or a cellular-uptake process which, controversially, may also be factor-mediated. Liposomes administered parenterally [intravenous (i.v.), intramuscular (i.m.), subcutaneous (s.c.), intraperitoneal (i.p.), etc.] will encounter similar elimination processes, although the relative importance of individual factors is route-dependent. Liposome fate after entering the circulation (*Figure 2*) is central to any consideration of *in vivo* clearance mechanisms, since some portion of liposomes administered parenterally usually reaches the circula-

tion, and because factors present in the blood circulation (e.g. lipoproteins) can also be present in other tissues. Degradative and cellular uptake processes are summarized here as humoral and/or cell-mediated, and have been extensively reviewed elsewhere (e.g. Senior, 1987; Gregoriadis, 1988; Juliano, 1989). Liposomes can, theoretically, interact with a number of humoral factors (i.e. factors present in the blood and other body fluids). For example, liposomes can be substrates for lipid metabolism; the role of plasma lipoproteins, e.g. high-density lipoproteins (HDL), low-density lipoproteins (LDL), very low-density lipoproteins (VLDL), and other factors which interact with plasma lipoproteins to effect lipid transfer in the circulation, e.g. lecithin-cholesterol acyl transferase (LCAT) and lipoprotein transfer factors (*Figure 1*), is discussed. Liposomes also present a foreign surface to blood components; the role of clotting and complement enzymes and opsonins is discussed. Finally in this section the role of cells of the reticuloendothelial system (RES) and other cells in liposome uptake, is discussed.

Humoral factors in liposome elimination in vivo

Humoral factors are circulating molecules, e.g. plasma proteins, which act either to destabilize liposomes or to accelerate their uptake by cells. Although the parallel with humoral and cell-mediated immunity is apparent, there is no firm evidence that factors which mediate the humoral immune response are the same as those involved in non-specific particle clearance. Additionally, liposomal lipid can become a substrate for lipid metabolism. The relative role of plasma factors and cellular uptake is described.

Liposomes as substrates for lipid metabolism. When liposomes composed of unsaturated PC with tracer radiolabelled lipid incorporated into the bilayer, are incubated at 37°C with plasma, radiolabel is recovered with HDL plasma lipoproteins occurring within 10 minutes of injection (mice or rats) for sonicated liposomes *in vivo* (Kirby, Clarke and Gregoriadis, 1980a,b; Tall, 1980). Lipid transfer to HDL results in loss of solutes associated with liposomes, both *in vivo* and *in vitro*, e.g. ^{125}I-albumin (Scherphof *et al.*, 1978) and CF (Kirby, Clarke and Gregoriadis, 1980a,b; Kirby and Gregoriadis, 1980). Liposomes which interact most readily with plasma HDL are those composed of phospholipid in a relatively fluid state at 37°C, e.g. dimyristoyl phosphatidyl-choline (DMPC) (Tall and Small, 1977) and egg PC (Krupp, Chobanian and Brecher, 1976). The link between bilayer fluidity and liposome susceptibility to HDL attack was shown by incorporating increasing amounts of cholesterol into liposomes with fluid bilayers (Gregoriadis and Davis, 1979; Kirby, Clarke and Gregoriadis, 1980a,b; Tall, 1980). SUV composed of egg PC and cholesterol (Chol) in molar ratios of 2:1 and 1:1 (PC:Chol), with tracer radiolabelled PC and cholesterol oleate, were incubated in plasma at 37°C and transfer of lipid to HDL was analysed. Thirty minutes later, 43·9% of recovered radioactivity was associated with HDL for SUV without cholesterol, whereas for vesicles with equimolar cholesterol only 21·2% of [^{3}H]PC was HDL-associated. A similar reduction in plasma-

induced transfer of radiolabelled lipid markers was shown subsequently for lipid compositions incorporating saturated phospholipids or the sphingolipid, sphingomyelin (SM), which also form relatively non-fluid bilayers. Thus ease of lipid transfer is clearly related to the permeability of liposomes to aqueous markers and drugs.

Regardless of phospholipid, inclusion of cholesterol at a high enough molar ratio (up to equimolar) will considerably reduce liposome permeability to aqueous solutes compared with phospholipid in the absence of cholesterol (Gregoriadis and Davis, 1979; Kirby, Clarke and Gregoriadis, 1980a,b; Senior and Gregoriadis, 1982a,b; Senior, Crawley and Gregoriadis, 1985). It has been suggested that this is because plasma lipoproteins can no longer work their way into the bilayer to acquire phospholipid, the process in which the liposomal bilayer is disrupted and entrapped material escapes (Gregoriadis, 1980). It is debatable whether liposomes with appropriately fluid bilayers will disintegrate in plasma, or whether the HDL molecules 'punch holes' in the vesicles, allowing the contents to escape. There is evidence that HDL accumulates liposome-derived phospholipid by a net transfer of lipid *in vivo* (Damen, *et al.*, 1980; Tall 1980; Damen, Regts and Scherphof, 1981); 10 min after i.v. injection of rats with cholesterol-free liposomes, HDL isolated from rat plasma was enriched with liposome-derived [^{14}C]PC (60 ± 4% of injected dose), the other plasma lipids remaining unchanged. However, leakage could also occur via effective pores without the need for liposomes to disintegrate completely (Kirby and Gregoriadis, 1981).

In another approach to define the role of HDL *in vivo*, plasma-derived lipoproteins were mixed in turn at physiological concentrations *in vitro* with plasma from mice made lipoprotein-free *in vitro* (Damen *et al.*, 1980; Tall, 1980; Tall and Green, 1981) and *in vivo* (Senior, Gregoriadis and Mitropoulos, 1983). Plasma lipoproteins VLDL, LDL and intermediate-density lipoproteins (IDL) failed to disrupt liposome permeability, for neutral liposomes composed of PC, whereas HDL was highly effective. However, other plasma factors may also be involved in loss of phospholipid to HDL *in vivo*: moderate heating of plasma (56°C for 30 min) can affect liposome permeability (Finklestein and Weissmann, 1979; Senior, Gregoriadis and Mitropoulos, 1983). While such heating of plasma is classically associated with inactivation of complement components, other protein activities will also be inactivated. Indeed, a number of lipid transfer factors, which function in association with plasma lipoproteins, are heat labile (Brewster *et al.*, 1978).

The search for heat-labile plasma constituents which destabilize liposomes has included investigation of lecithin-cholesterol acyl transferase (LCAT), an acyl-group transferring enzyme closely associated with HDL, lipid transfer factors (which act in association with lipoproteins) and apolipoproteins (protein components of lipoproteins). Phospholipases, clotting and complement enzymes do not appear to be involved in lipsome destabilization in the plasma (Senior, 1987). LCAT catalyses the conversion of free cholesterol to cholesterol ester, using phospholipid as a source of fatty acids, and is believed to play a part in removing cholesterol from peripheral tissue to the liver via lipoproteins (Cooper, 1985). When sonicated liposomes composed of PC are

incubated in plasma at 37°C in the presence of LCAT inhibitors (5,5'-dithionitrobenzoic acid or *p*-chlormercuribenzoic acid), transfer of PC to HDL is not affected. Although inhibiting LCAT activity *in vitro* with sulphydryl-blocking agents does not alter the movement of phospholipid from small liposomes composed of PC *in vitro*, the situation *in vivo* may be more complex; LCAT may be indirectly involved by causing shifts in the equilibria of lipoprotein metabolism that favour liposome breakdown. It is likely that lipoprotein-mediated destruction of liposomes also involves lipid transfer proteins as part of normal plasma lipoprotein metabolism (*Figure 1*).

In summary, liposomes with a relatively fluid bilayer, such as those composed of a phospholipid with a liquid–crystalline phase-transition temperature (Tc) below 37°C (e.g. egg PC or DMPC), are susceptible to HDL attack *in vivo*. Those composed of lipids with higher Tc values, such as SM, dipalmitoyl phosphatidyl-choline (DPPC), and distearoyl phosphatidyl-choline (DSPC), are less susceptible because their bilayers are less fluid at body temperature. However, in all cases, addition of equimolar cholesterol imparts rigidity to the lipid bilayer and hence resistance to HDL attack; for example, very little if any liposomally derived radiolabelled phospholipid can be recovered in the HDL fraction of plasma after *in vitro* incubation at 37°C of liposomes composed of high-Tc phospholipid and equimolar cholesterol (Senior and Gregoriadis, 1984). Thus HDL induces liposomal solute permeability when liposome bilayers have a fluidity similar to that of the commonly transported, naturally occurring phospholipids. PC molecules incorporated into HDL are either consumed or exchanged with other lipoproteins (Cooper, 1985). Exchange is greatly facilitated by plasma phospholipid exchange protein (Eisenberg, 1978; Albers *et al.*, 1984).

Liposomes as foreign surfaces in the blood. Liposomes may also be recognized as foreign surfaces in the blood by plasma components such as clotting factors, complement and opsonins. Plasma factors are known to coat particles introduced into the circulation so that the particles more readily become prey to phagocytic cells (Saba, 1970). The term 'opsonin' (Greek *opsonion*, victuals; Latin *opsonium*, relish) used in an immunological context applies to immunoglobulin and complement components, but can also be applied in a non-immunological context. It is uncertain whether immunoglobulin and complement can themselves influence particle clearance *in vivo* non-specifically, rather than as part of the immune response in an animal with circulating antibodies (Saba, 1970; Alving and Richards, 1983). 'Non-immune' molecules proposed as opsonins include fibronectin (van Oss *et al.*, 1974a; Blumenstock *et al.*, 1977; Molnar *et al.*, 1983), fibrin (Knisely, Bloch and Warner, 1948), as well as a non-specific role for immunoglobulins (van Oss and Stinson, 1970) and complement (Saba, Filkins and Di Luzio, 1966). α_2-Macroglobulin is known to bind to liposomes (Black and Gregoriadis, 1976) and can promote phagocytosis (Molnar *et al.*, 1977). A decrease in phagocytosis is brought about by a 'dysopsonin' the α_1-acid glycoprotein, orosomucoid (van Oss *et al.*, 1974b).

If opsonization is important in liposome clearance, it should be possible to demonstrate plasma components adsorbed to liposomes, and to demonstrate that the reisolated factors possess opsonic activity. This goal has so far proved elusive. A negatively charged plasma component of human plasma which interacts with the liposome surface regardless of size, has been identified as α_2-macroglobulin (Black and Gregoriadis, 1976). This protein and other proteins may be associated with MLV of different surface charge (Juliano and Lin, 1980; Bonté *et al.*, 1987). Recent findings (Moghimi and Patel, 1988) suggest that there is more than one type of opsonin at work in accelerating liposomal removal from the circulation, and that the type of opsonin determines the tissue destination of the vesicles, i.e. liver or spleen (Moghimi and Patel, 1989a).

Some types of negatively charged liposomes interact with clotting factors (Juliano and Lin, 1980), but no change in clotting factors was observed for a patient treated with neutral and negatively charged vesicles for more than one year (Gregoriadis *et al.*, 1980). Although complement components bind to surfaces, serum previously exposed to liposomes can still cause lysis of sheep red cells, with C_3 levels unaffected (Juliano and Lin, 1980). This suggests that complement components do not bind to liposomes in appreciable amounts, but does not rule out a possibility that they could act at very low levels.

Role of cellular uptake in liposome elimination in vivo

Liposomes are treated as foreign particles in the circulation. It is now very well known that much larger standard particles, such as sheep red blood cells (SRBC), injected intravenously into mice, are about 90% removed by the sinus-lining Kupffer cells of the liver, 5% by macrophages of the spleen, and less than 5% by bone marrow macrophages. Clearance of these exogenously derived cells from the circulation is confined to macrophages which line blood vessels, i.e. cells of the RES. A wide range of colloidal particles, e.g. carbon particles, aggregated albumin, lipid emulsion and latex particles, show similar clearance kinetics after intravenous administration, the major variable being colloid concentration (Normann, 1974). While liver uptake appears to be the major clearance route, Kupffer cell phagocytic function can be supplemented by clearance into spleen and bone marrow when liver clearance is saturated (Bradfield, 1984).

Liposomes entering the circulation can be trapped initially in the pulmonary capillaries, especially if the particle size is relatively large and lipid dose high. However, over 1–2 days, liposomes, or their released marker, can redistribute back into the circulation (Poste, 1983). For most liposome preparations and other particles in the size-range 0·1–2 μm, liver, spleen and bone-marrow macrophages will provide the main sites for particle absorption. Passive uptake of liposomes by liver, spleen and bone-marrow is determined not only by the number of macrophages lining the blood channels, but also by the relative blood flow, local kinetics of uptake and capacity at each site. Study of individual organs shows that the liver and spleen are parallel with other clearance sites. This means that if the predominant clearance into liver

is depressed, e.g. during i.v. blockade, total spleen and bone marrow uptake is greatly increased.

Reports (summarized in Senior, 1987) that red cells can be made 'liver-seeking' or 'spleen-seeking', using different types of antibodies (Arend and Mannik, 1971) or by altering surface sugars (Kolb-Bachofen *et al.*, 1982), have an interesting parallel in recent work using liposomes (Moghimi and Patel, 1988, 1989a). These authors suggest that serum contains opsonins which are specific for hepatocytes or splenocytes, and that these opsonins are sensitive to bilayer fluidity (Moghimi and Patel, 1989b). Preliminary experiments suggest that the liver-specific opsonin is a heat-stable protein and that a dialysable heat-stable factor (apparently Ca^{2+}) inhibits its opsonic activity; whereas the spleen-specific opsonin is heat labile and a dialysable cofactor is required for optimum opsonic activity (Moghimi and Patel, 1989a).

The surface charge of particles may also affect which organ's phagocytes will predominate. For example, polystyrene latex made negatively charged with gum arabic, or positively charged with polylysine, at pH 7·5, [as assessed by microelectrophoresis (Wilkins and Myers, 1966)] distribute to different phagocytic cell populations on injection into rats. The negatively charged particles are taken up mostly by the liver, whereas the positively charged particles initially accumulate in the lungs, but later redistribute to the spleen. Particles, including liposomes, regardless of initial charge, appear to acquire the same (negative) charge on contact with blood (Wilkins and Myers, 1966; Black and Gregoriadis, 1976). It is therefore possible that positively charged liposomes will acquire more coating to achieve a net negative charge, and this renders the coated particle large enough to be trapped in the lung capillaries. For further discussion *see* pp. 294–296.

Cellular uptake of particles can be active (presumably receptor-mediated) or passive, where particles of a small enough size are taken up by a general pinocytosis that is particle-independent (Praaning-van Dalen, Brouwer and Knook, 1981). The latter mechanism is probably the way in which particles small enough to pass through the fenestrations in the liver (i.e. around 100 nm in diameter or less) are taken up by hepatocytes (Beaumier and Hwang, 1983). Receptor-mediated uptake by phagocytes and endocytes has been described for a number of particles and individual proteins, including glycoproteins, lipoproteins, enzymes, denatured albumin and altered LDL (summarized in Senior, 1987). Liposome removal from the circulation could be receptor-mediated if a protein or other factor coating the vesicles after contact with plasma, is recognized. Alternatively, a receptor which usually recognizes other circulating particles or molecules, could incidentally recognize and internalize liposomes. Such a receptor would recognize a surface feature of the liposome, or lipid in general. If liposomes indeed acquire a surface coating on contact with plasma, then the latter seems unlikely, and cellular recognition of the plasma factor(s) associated with liposomes becomes the crucial step in vesicle clearance from the blood. Thus a distinction can be made between factors which bind and lead to particle breakdown, and those which bind and render the particle more susceptible to cell uptake. In the case of liposomes, the former are generally factors of lipid

metabolism, and the latter would be described as opsonins. An interesting overlap in these two categories was recently suggested by Heath; *in vitro* work suggesting that LDL binds specifically to small amounts of negatively charged liposomes, leads to the interesting speculation that LDL-coated liposomes could be removed via the LDL or scavenger receptor (Comiskey and Heath, 1990).

LIPOSOME DESIGN FEATURES WHICH CONTROL THEIR FATE *IN VIVO*

Aspects of liposome design and administration dramatically affect their clearance from the site of injection, from the circulation, after topical administration, e.g. to the eye, skin or in aerosol form to the nose or lung. Variables include vesicle size, dose, number of doses and vesicle surface characteristics, including charge, hydrophilicity/hydrophobicity and the presence of surface ligands. As discussed in the previous section, removal of liposomes from the circulation by the RES relies on the perception of exogenously derived particles as 'foreign' by phagocytic cells. Little is known in general about clearance mechanisms for particle-removal other than that mediated by an immune response (Saba, 1970; Bradfield, 1980). However, there is a body of information based on studies with model liposome systems which define the physical parameters controlling clearance after i.v. administration, and comparable data for other routes is being accumulated gradually (Senior, 1987; Weiner, 1989). Systematic studies using liposome-entrapped drugs in development for clinical and commercial use also are in progress (Bakker-Woudenberg, Roerdink and Lokerse, 1989; Druckmann, Gabizon and Barenholz, 1989; Mayer *et al.*, 1989; Meisner, Pringle and Mezei, 1989; Price, Horton and Baxter, 1989; Caride, 1990). While the role of each liposome characteristic is discussed in turn here, overlap between parameters is inevitable. Practical approaches to liposome engineering, such as manipulating the lipid composition, allows a scheme for strategies to give the vesicles the best chance of avoiding premature removal or disintegration, and will be highlighted in this section.

Effect of size

As described in the introduction, liposome size is determined by the method of preparation. For constant amounts of lipid, vesicle size is the overriding factor determining plasma half-life of a liposome population after i.v. injection (Juliano and Stamp, 1975; Senior, Crawley and Gregoriadis, 1985). Vesicle diameter controls the accessibility of the vesicles to hepatocytes after i.v. administration (Abra and Hunt, 1981; Roerdink *et al.*, 1981; Rahman *et al.*, 1982; Beaumier and Hwang, 1983; Roerdink *et al.*, 1984) and also determines the ease with which liposomes will gain access to the circulating blood when liposomes are administered s.c. or i.m. (see below).

For i.v. injection, liposomes of defined size and composed of neutral phospholipids with a rigid bilayer structure, need to be extensively sonicated

until 90% of the vesicles are less than 100 nm in diameter, in order to obtain slow, linear, clearance profiles (Juliano and Stamp, 1975; Gregoriadis and Senior, 1980; Allen and Everest, 1983; Beaumier and Hwang, 1983). *In vitro* and *in vivo* studies to determine the cell types involved in clearance suggest that the cell type responsible for liposome uptake depends upon the size of the liposome population. For example, phagocytic and non-phagocytic cell types were isolated from the livers of rats after administration of 0·4 μ-filtered liposomes of various lipid compositions and incorporating [^{125}I]polyvinyl pyrrolidone as an aqueous space marker (Roerdink *et al.*, 1981). Less than 3·4% of the total dose of radioactivity was recovered in the non-phagocytic endothelial cells and hepatocytes, the remainder of liver cell uptake being associated with the phagocytic Kupffer cells which line the liver sinusoids. When liver uptake is compared for SUV and MLV (Beaumier and Hwang, 1983) using a marker which is fully retained in liposomes up to 24 h after administration *in vivo* ($^{111}In^{3+}$ chelated with NTA), liposomes composed of SM:Chol (2:1 molar ratio) gave a constant high PAC value of 0·59 ± 0·02, so were intact. PAC values in livers isolated at time intervals after liposome administration suggest that SUV were broken down more slowly than MLV. Since MLV were loaded with $^{111}In^{3+}$ only in the outermost layer, marker release can be readily compared with that from unilamellar liposomes. The observation that MLV breakdown was slow *in vitro* but fast *in vivo*, whereas that of the SUV was slow in both, suggested that different pathways were involved for each size of vesicle. This was ascribed to the more rapid disintegration of particles captured by the Kupffer cells, i.e. the larger liposomes. SUV were subsequently shown to reach liver hepatocytes in significant numbers (Roerdink *et al.*, 1984); similar amounts of tritiated inulin marker was recovered in parenchymal cells and Kupffer cells on a per-cell basis, when administered i.v. in liposomes 25–80 nm in diameter composed of SM:Chol:phosphatidyl-serine (PS) (molar ratio 4:5:1). Inclusion of negatively or positively charged lipids does not appear to prevent uptake of small liposomes by hepatocytes (Rahman *et al.*, 1982; Roerdink *et al.*, 1984).

The effect of liposome size on distribution to tissues other than the liver after i.v. administration has mostly been studied for small (sonicated) liposomes, in order to avoid the otherwise overwhelming contribution of the liver in particle clearance. Interpretation of such biodistribution studies needs to account for the following factors; marker can remain in the circulating blood at the time of death, thus elevating perceived tissue levels in non-perfused organs; marker can leak from vesicles before uptake by tissues; marker can leak out of liposomes, and even the tissues themselves, after vesicle uptake. With these reservations in mind, several studies using relatively well-defined marker systems, liposomes or associated marker, have detected marker in kidney, spleen, heart, lung, intestine, fat, skin, tail, legs, carcass, brain, stomach, urine and faeces, as well as in liver and blood (Hwang, Luk and Beaumier, 1982; Senior, Crawley and Gregoriadis, 1985; Hwang *et al.*, 1987). For the smallest liposomes, the biodistribution of SUV (20 ± 5 nm average diameter) composed of SM:Chol (molar ratio 2:1), was recently reinvestigated for different doses up to 24 h after i.v. injection

(Hwang *et al.*, 1987). In summary, up to 24 h after administration, liposomes of this composition remained intact; 15·7% of the dose was found in the blood, 44·3% in the liver, with the rest distributed to the carcass (10·7%), skin (9·3%), intestine (5·7%), kidney (2·1%) and spleen (1·8%). Analysis of radioactivity in urine and faeces was not included for the liposomal marker, but a proportion of labelled material in the intestine may reflect marker excreted from the liver via the bile. For SUV with a larger average diameter (64 ± 6 nm) and with a lipid composition which enables liposomes to quantitatively retain aqueous markers for days in plasma at 37°C (DSPC :Chol molar ratio 1:1), biodistribution of [^{111}In]bleomycin 72 h after i.v. injection of SUV (50 mg phospholipid per kg in mice) was measured when the proportion of radiolabel in the blood was less than 5% of dose (Senior, Crawley and Gregoriadis, 1985). At this timepoint, the amount of radioactivity recovered in the liver and spleen was less than 30%. Around 35% of radioactivity ([^{111}In]bleomycin) was recovered in the carcass after organs were removed and significant amounts of label were recovered in the urine and faeces (22%), possibly derived from liposome degradation in the liver and eliminated via biliary excretion. Thus liposomes which are not removed by liver and spleen apparently reach the bone marrow, but those that are small enough to penetrate discontinuous blood capillaries, may be detectable (Hwang *et al.*, 1987) in non-reticuloendothelial tissue other than hepatocytes, such as skin and intestine, and be taken up by pinocytosis (fluid-phase endocytosis).

Particle size also appears to be a key factor in determining the length of time liposomes remain at the injection site for vesicles administered intramuscularly and subcutaneously. Elimination of drug administered intramuscularly occurs by absorption into the blood capillaries and the lymphatic vessels, probably by diffusion and phagocytosis (Ballard, 1968). The effect of particle size and diffusivity of liposome-encapsulated drug has been investigated for large liposomes prepared by a freeze-thaw method and composed of egg yolk phospholipid and administered intramuscularly in rats (Ohsawa *et al.*, 1985). Using markers for both lipid ([^{3}H]DPPC) and drug ([^{14}C]inulin) suggests that lipid and drug do not disperse simultaneously from the injection site. Indeed, the ratio of markers at the injection site changes with time; liposomes are preferentially recovered in lymph nodes whereas the drug is recovered in blood and urine. The liposomes themselves appear to pass into the lymph nodes which drain the muscle before reaching the efferent lymphatics (Ohsawa *et al.*, 1985). Although encapsulated inulin probably leaks out, at least partially, at the injection site, the drug could also be released after liposomes reach the regional lymph nodes or after phagocytosis of liposomes by histiocytes in the muscle. Recovery of lipid label in the liver and spleen suggests that intact liposomes can reach the circulation. Other studies indicate that small, unilamellar liposomes drain into regional lymphatics more readily than larger, multilamellar vesicles (Jackson, 1980; Mauk, Gamble and Baldeschwieler, 1980; Jackson, 1981; Khato, Del Campo and Sieber, 1983; Naeff, Plinska and Weder, 1990) and that neutral or positively charged liposomes are more readily located in the lymphatic system than negatively

charged vesicles (Ryman *et al.*, 1978). Since liposomes and drugs are not cleared at the same rate or by the same route (Ohsawa *et al.*, 1985), studies should be interpreted with caution with respect to liposome leakiness (*see* p. 283–284). Depending upon the size and lipid composition of vesicles, liposomes will break down at the injection site, releasing the drug, including peptide drugs, over extended periods. These findings have important implications for i.m. peptide drug delivery (Weiner, 1989).

Effect of surface charge

Appropriately charged lipids incorporated into the liposomal bilayer result in vesicles with an overall neutral, negative or positive charge at physiological pH. The ratio of charged lipid to other lipid will control the charge density. Reasons for providing liposome bilayers with net charge include:

1. Improvement of entrapment efficiency for drugs, e.g. proteins, by using a phospholipid with a complementary charge to that of the protein or peptide;
2. Targetting; for example, PS-containing liposomes can increase the uptake of liposomes in the lung after *in vivo* administration (Kimelberg, 1976; Fidler *et al.*, 1980; Poste *et al.*, 1982; Abra, Hunt and Lau, 1984);
3. To prevent vesicle aggregation on long-term storage (Martin, 1990);
4. To facilitate targetting to cell populations *in vivo*, especially to liver macrophages (see below).

Lipids commonly used to confer net negative or positive charge are shown in *Table 2*.

Table 2. Lipids which confer net surface charge to liposomes

Lipid component	References
Negative charge	
PA; phosphatidic acid	Gregoriadis and Neerunjun (1974); Senior, Crawley and Gregoriadis (1985)
PS; phosphatidyl-serine	Fidler *et al.* (1980); Poste (1983)
PG; phosphatidyl-glycerol	Lopez-Berestein *et al.* (1983)
PI; phosphatidyl-inositol	Gabizon and Papahadjopoulos (1988)
DCP; dicetyl phosphate	Gregoriadis and Neerunjun (1974)
gangliosides	Gregoriadis and Neerunjun, (1974);
GM_1; monosialo-galactosyl ceramide	Allen, Ryan and Papahadjopoulos (1985)
cardiolipin	Rahman *et al.* (1985); Seltzer *et al.* (1984)
sulphatides	Allen, Hansen and Rutledge (1989); Yagi (1989)
Positive charge	
SA; stearylamine	Gregoriadis and Neerunjun (1974)
DOTMA; dioleyloxypropyltrimethylammonium	Felgner *et al.* (1987)
BisHOP; dihexadecyloxypropyltrimethylammonium	Tan and Gregoriadis (1989)

Relatively little is known about the use of positively charged liposomes *in vivo*. Previous *in vitro* studies have suggested that liposomes containing stearylamine (SA) may be cytolytic (Yoshihara and Nakae, 1986), irritant

(Taniguchi *et al.*, 1988), and after intracranial administration *in vivo*, toxic to the central nervous system (Adams *et al.*, 1977). However, it is uncertain whether such toxic effects are a generalized phenomenon for positively charged lipids or whether they are a feature of the single-chain fatty acid-type lipid due to the relative ease with which such a single-chain derivative may leave the lipid bilayer. i.v. Studies in which mice were given a single dose of SA-containing SUV (approximately 2 μmole SA) indicated no obvious toxic effects (Kirby, Clarke and Gregoriadis, 1980a; Tan and Gregoriadis, 1989). Furthermore, a liposomal formulation of cyclosporine has recently been demonstrated to give equivalent concentrations of the drug after intravenous administration to mice, without accompanying nephrotoxicity (Vadiei *et al.*, 1989). PC and phosphatidyl-ethanolamine (PE) are zwitterionic and provide a neutral charge at physiological pH, although chemical modification of the amine group of PE or the choline of PC may result in a net negative charge due to exposure of the phosphoryl group.

Negatively or positively charged lipids can dramatically alter the clearance pattern of liposomes after i.v. injection, as demonstrated for small liposomes with otherwise long plasma half-lives (*Table 3*). Experiments with small liposomes composed of DSPC and equimolar cholesterol, where markers are fully retained, suggest that when 1 in 3 phospholipid molecules are negatively charged, vesicles are cleared from the circulation much more rapidly than comparable neutral liposomes (Senior, Crawley and Gregoriadis, 1985). Regarding positive charge, it was previously reported (Kirby, Clarke and Gregoriadis, 1980a) that SUV composed of PC, stearylamine and cholesterol (molar ratio 7:1:7) were cleared at a rate similar to neutral SUV. However, preferential uptake of positively charged small liposomes composed of DSPC, cholesterol and 6-amino-mannose-cholesterol (molar ratio 4:1:1) by the liver (Profitt *et al.*, 1983) suggested that neutral and positively charged SUV are not cleared by the same mechanism (Senior 1987).

Recent evidence may explain these observations (Tan and Gregoriadis,

Table 3. Influence of surface charge on plasma half-life of small unilamellar liposomes

Lipid components (molar ratio)	CF latency *in vitro**	Plasma half-life *in vivo* (h)†	References
Neutral			
DSPC	98·9	14–20 h	Senior, Crawley and Gregoriadis (1985)
Negative			
DSPC : DSPA (0.75 : 0.25)	98·1	1–2 h	Senior, Crawley and Gregoriadis (1985)
DSPC : PA (0.9 : 0.1)	88·1	40 min	Senior, Crawley and Gregoriadis (1985)
DSPC : GM_1 (0.9 : 0.1)	99·4	10 h‡	Senior and Gregoriadis, unpublished
Positive			
DSPC : SA (0.9 : 0.1)	nd	1–1·5 h	Tan and Gregoriadis (1989)
DSPC : BisHOP (0.9 : 0.1)	nd	40 min to 1 h	Tan and Gregoriadis (1989)

* Incubation in five volumes of mouse plasma for 24 h at 37°C.
† Cholesterol-rich (equimolar with phospholipid) small, unilamellar liposomes, av. diameter 60 nm, administered (approx. 1 mg phospholipid per 25 g body weight) intravenously in a single dose to mice.
‡ Control liposomes in the absence of GM_1 were cleared with a half-life of 7 h in this experiment (*in vitro* latency at 24 h was 99·5%).

1989); clearance of SUV was assessed using entrapped CF as the aqueous phase marker in cholesterol-rich (equimolar with phospholipid) liposomes composed of DSPC, with 10 mol% of phospholipid replaced with stearylamine. Liposomes of this composition should quantitatively retain aqueous solutes and present a rigid bilayer surface, to plasma components. SUV containing SA demonstrated accelerated clearance (half-life 1–1·5 h) compared with neutral liposomes (half-life 15 h) (*Table 3*). These findings suggest that positive charge imposes accelerated clearance from the circulation upon liposomes. Although cholesterol-rich liposomes composed of PC exhibit similar plasma clearance profiles in the presence or absence of stearylamine in the lipid bilayer, this may be because the single-chain, positively charged lipid is not fully retained in the bilayer during contact with blood plasma. This has been investigated (Tan and Gregoriadis, 1989) using a saturated acyl-chain, positively charged synthetic lipid known as BisHOP ((±)-*N*-[(2,3-dihexadecyloxy)prop-1-yl]-*N,N,N*-trimethyl-ammonium chloride). This lipid is somewhat similar in structure to DPPC except that the phosphoryl group, which would provide net negative charge at physiological pH, is absent, leaving the trimethyl ammonium group, to provide only net positive charge. BisHOP appears to be retained slightly more readily in the liposomal bilayer than stearylamine (*Table 3*).

Surface charge may influence plasma half-life of liposomes directly by enabling interaction of vesicles with charge-sensitive receptors on cells, or indirectly, where charged groups may alter the nature of liposome interaction with plasma components. For lipid compositions where the lipid bilayer is relatively fluid, sufficient amounts of the charged lipid itself can alter the permeability properties of the liposomes (Senior, Crawley and Gregoriadis, 1985), and may enable plasma components involved in plasma clearance to bind more readily (Senior and Gregoriadis, 1982b).

In summary, for liposomes with a rigid bilayer, positive charge appears to be as effective as negative in accelerating liposome clearance after intravenous administration. It is not known whether the charge density is important in determining clearance, although there is probably a threshold of surface charge required before accelerated clearance is triggered. Pharmaceutical applications of charged liposomes administered topically, indicate that positively charged liposomes adhere more readily to the surface of the eye (Shek and Barber, 1987; Meisner, Pringle and Mezei, 1989) although ocular irritability has been observed with some formulations (Taniguchi *et al.*, 1988). For parenteral administration, neutral or positively charged liposomes are more readily located in the lymphatic system than negatively charged vesicles after i.m. or s.c. injection (Ryman *et al.*, 1978).

Effect of bilayer fluidity

After size and net surface charge, the factor most likely to determine the rate at which liposomes are cleared from the circulation in the absence of targetting ligands, is bilayer fluidity (*see Table 3*). Bilayer fluidity is controlled primarily by lipid composition. The simplest means of reducing bilayer

fluidity is to include up to equimolar cholesterol in the lipid composition (Kirby, Clarke and Gregoriadis, 1980a,b). Increased bilayer rigidity can also be obtained using sphingomyelin (Senior and Gregoriadis, 1982a,b) or other sterols in place of cholesterol (Demel, Bruckdorfer and van Deenen, 1972). The nature of the acyl chains of the phospholipid used contributes substantially to the overall fluidity of the bilayer at body temperature; saturated fatty-acyl chains such as DSPC and DPPC give a more rigid bilayer than unsaturated ones with the same number of hydrocarbon units. Liposomes made from phospholipids which form relatively fluid bilayers at body temperature appear to be cleared from the circulation more rapidly than those with less-fluid bilayers (Senior, 1987). Increased surface hydrophobicity may contribute to the number and type of plasma factors which bind to surfaces *in vivo*, hence to prolonged half-lives observed for vesicles for these compositions (Senior, 1987).

Lipid composition may also determine the rate of clearance of large and small liposomes from the injection site after i.m. or s.c. injection (Arakawa *et al.*, 1975; Jackson, 1981; Tümer *et al.*, 1983; Arrowsmith, Hadgraft and Kellaway, 1984; Schreier, Levy and Mihalko, 1987). However, it is not always possible to unravel entirely the relative contributions of size, charge, bilayer fluidity and dose in the different animal models used in these studies to distinguish between clearance of drug, vehicle and vehicle-associated drug from the injection site. After intramuscular administration of large (3–5 μm average diameter) liposomes composed of PC, carrying gentamicin sulphate and with tracer [^{125}I]hydroxybenzamidine-PE or [^{3}H]DPPC as markers, lipid is retained at the injection site from 1 to 6 days (Schreier, Levy and Mihalko, 1987). Whereas large liposomes with reduced membrane fluidity, composed of DPPC, exhibit a longer clearance half-life of 8·5 days from the muscle of rabbits given 1·5 mg kg^{-1} liposomes, with total clearance of lipid from the muscle over a period of at least 14 days (Arrowsmith, Hadgraft and Kellaway, 1984).

Effect of surface-associated ligands

Ligands present at the surface of liposomes are molecules or parts of molecules which are either attached to preformed liposomes, or are incorporated during liposome formation and become surface-expressed in the final liposome dispersion under thermodynamic or steric constraints. These ligands can be incorporated for two, essentially opposing, purposes:

1. To act as a targetting device: the ligand expressed on the vesicle surface interacts with the corresponding target cell receptor;
2. To reduce recognition of liposomes by phagocytes.

It is not known to what extent the well-characterized immune-mediated recognition processes apply to general non-specific particle recognition, leading to clearance from the circulation of parenterally introduced material. However, it is clear that different surface characteristics, including the

presence of ligands, can influence the pharmacokinetics of liposomes and of other particles, as discussed previously (Senior, 1987).

Ligands used for targetting *in vivo* include antibodies (for reviews *see*: Gregoriadis *et al.*, 1985; Machy and Leserman, 1987; Peeters, Storm and Crommelin, 1987), and carbohydrates (e.g. Ghosh and Bachhawat, 1980; Profitt *et al.*, 1983; Gregoriadis and Senior, 1984; Ahmad, Sarkar and Bachhawat, 1989; Atsuta and Kondo, 1989). Successful targetting requires accessibility of target cells and target cell receptor, effective surface presentation of ligand on liposomes that have sufficient plasma stability to reach the target with a substantial payload of drug on board, and internalization and release of active drug within the appropriate cellular compartment. These stringent requirements mean that receptor-mediated targetting is an ambitious goal for drug delivery. However, recent work elegantly demonstrates the effectiveness with which *in vivo* antibody-mediated targetting within the vasculature can be achieved (Hughes *et al.*, 1989), and carbohydrate-mediated targetting to the mannose receptor of liver cells can increase efficacy in amphotericin B treatment of aspergillosis in mice (Ahmad, Sarkar and Bachhawat, 1989).

Liposomes which can at least partially evade phagocytic removal from the circulation may expand the range of therapeutic applications for such liposomes, compared with liposomes that are readily acquired by phagocytic cells. Lipids such as gangliosides can facilitate this evasion when incorporated into the liposomal bilayer, and these liposomes have been called 'Stealth™', after the US Air Force bomber which is able to evade radar detection. In evaluating the contribution of surface ligands to liposome pharmacokinetics, as pointed out above, it is often difficult to establish exactly which parameter is causing changes in the plasma half-life of liposomes when introduction of even one new variable can affect more than one liposome characteristic that determines clearance. For example, when 10 mol% neutral phospholipid is replaced by negatively charged GM_1 or PI, each with a hydrophilic headgroup, delayed uptake by liver and spleen results (Gabizon and Papahadjopoulos, 1988; Allen, Hansen and Rutledge, 1989). To what extent is the delay due to the presence of negative charge, due to the hydrophilic nature of the lipids, or specific to these lipids? The effect of charge has been discussed above; in model studies where liposomes quantitatively retain their contents, liposomes with 10 mol% negatively charged lipid are cleared more rapidly from the circulation than liposomes with identical *in vitro* stability properties, without negative charge. With regard to the possible role of hydrophilic coatings of liposomes in increasing plasma half-lives, results using other delivery systems (Illum *et al.*, 1987) suggest that coating polystyrene microspheres with hydrophilic polymer, e.g. polyoxyethylene, can prolong their plasma half-life. Using a technique for increasing the hydrophilicity of liposomes which already exhibit long plasma half-lives, further increases in half-life were obtained; liposomes that quantitatively retain aqueous markers were covalently coupled (Senior *et al.*, 1990) to a hydrophilic polymer [a monovalent derivative of polyethylene glycol (PEG) 5000]. When administered intravenously to mice (Senior *et al.*, 1990), MPEG-coupled liposomes

were cleared up to 30% more slowly than liposomes without monovalent polyethylene glycol (MPEG). Preliminary studies with MPEG-bearing liposomes suggest that particle size is only marginally increased by the attachment of the polymer (Senior *et al.*, 1990). It therefore seems that negative charge is not a factor in prolonging the half-life of GM_1- and PI-containing liposomes, whereas the hydrophilic nature of the head-group may be playing the major role, possibly by acting sterically to prevent interaction of liposomes with clearance-accelerating cell receptors and/or plasma factors (Senior *et al.*, 1990). This is shown schematically in *Figure 3*. PEG has been coupled to protein molecules to prolong their circulation half-life (Abuchowski *et al.*,

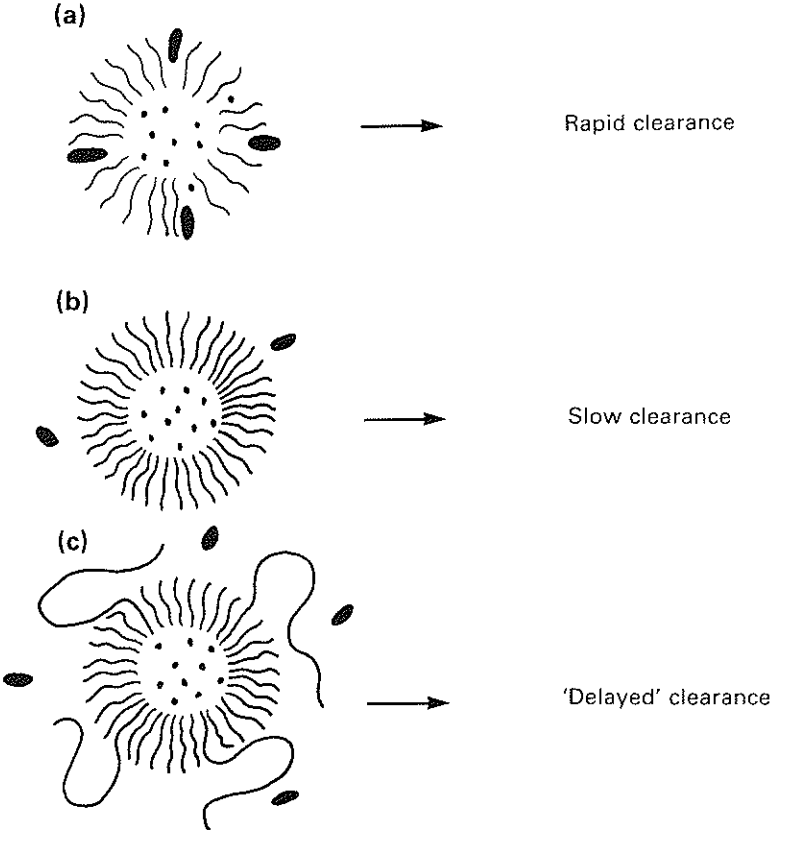

Figure 3. Opsonization and liposome clearance *in vivo*. (a) Relatively loosely packed lipid bilayers (lines) are penetrated by plasma components (elipses), allowing entrapped solute to escape (dots). Rapid clearance follows. (b) Tightly packed lipid bilayers are not readily penetrated by plasma components and entrapped solutes are retained. However, plasma components can still adsorb to the liposome surface. Slow clearance results. (c) Tightly packed bilayers which also have a steric constraint at their surface avoid contact with plasma components, promoting a further delay in clearance. Opsonization is proposed to play a role in (b) and (c) and may be involved, in addition, to proteins of lipid metabolism, in (a).

1977); such PEG-modified enzymes were shown to be safe and to have therapeutic value in clinical trials (Fuertges and Abuchowski, 1990).

An important contribution of such surface coating would be if clearance is delayed for large liposomes, which can potentially carry a bigger payload of drug and should have simpler manufacturing technology requirements. There is evidence that this is indeed the case (Allen and Chonn, 1987; Hughes *et al.*, 1989) with application in cancer chemotherapy using doxorubicin (Gabizon and Papahadjopoulos, 1988; Gabizon, Shiota and Papahadjopoulos, 1989).

TARGET-ASSOCIATED FACTORS THAT DETERMINE LIPOSOME FATE

The overwhelming role of the RES in determining the fate of injected liposomes and other particles can undoubtedly be exploited to reduce toxicity of certain drugs by directing vesicle-associated material away from vulnerable organs such as kidney and heart. Blood-borne macrophages which acquire a liposome–drug load in the circulation, may be attracted to a site of infection and/or inflammation in the course of their normal function. What is the scope for achieving liposome-based drug delivery to sites other than the RES? Access to non-RES target tissue can be facilitated by using a route of administration which maximizes the opportunity for vesicles to interact with a target and, by manipulating liposome characteristics, which favours the escape of vesicles from sites of RES uptake.

Site-directed approaches through choice of appropriate route of administration

Liposomal drugs delivered parenterally other than via the intravenous route may have therapeutic benefits in targetting the lymphatics. For example, direct delivery of immunoregulatory peptides to the lymph nodes offers an intriguing therapeutic opportunity, and liposomal marker has been located in regional lymph nodes after s.c. administration (Tümer *et al.*, 1983). Stable multilamellar liposomal formulations of peptides show a gradual increase in lipid/peptide ratio over extended periods at the injection site after i.m. injection, supporting the contention that large liposomes gradually disintegrate and release peptide at the injection site (Weiner, 1989). Similar findings have been obtained for other drugs (Schreier, Levy and Mihalko, 1987).

Topical administration of drug in liposome-based formulations applied directly to the required site of drug-action, or in transdermal or aerosol-delivery, is an on-going option in new-drug formulation. For example, in pulmonary drug-delivery, lung capillaries are rich in macrophages which lie on the air interface and are therefore accessible to particles delivered in aerosols to the lung via inhalation. These macrophages may be able to cross the lung capillaries, since liposomes or their markers can be detected in the blood after pulmonary administration of liposomal drug in aerosol form (Wyde *et al.*, 1988; Taylor *et al.*, 1989). Topical administration of protein has been successfully evaluated *in vitro* (Egbaria *et al.*, 1990).

Manipulation of liposome characteristics to reduce their removal by sites of classical RES uptake

In order to maximize the opportunity for liposomes to interact with non-phagocytic cells and/or to slowly release their contents, liposome structural characteristics may be manipulated to prolong the survival of liposomes in circulating blood. However, liposome characteristics which prolong the survival of liposomes in the circulation may not necessarily be appropriate for speedy release of therapeutic agents into the target site within the cell. Liposomes with the longest plasma half-lives are also those vesicles with the lowest entrapment efficiency, i.e. small liposomes, and large vesicles with the greatest capacity for carrying drug, have the fastest clearance. Ideally, the most effective way to use liposomes with a higher encapsulation efficiency would be to circumvent the natural clearance processes for large liposomes. Approaches where the surface of both large and small liposomes is camouflaged with groups which disguise its foreign appearance are being developed (*see* pp. 297–300).

Significance of findings for future use of liposomes in drug delivery

Challenges for drug development in a liposomal formulation can be broken down into two main (overlapping) areas: pharmaceutical and biological.

PHARMACEUTICAL CONSIDERATIONS

The commercial justification for selecting liposomes to formulate new drugs, including peptides or proteins, depends upon issues crucial to successful development of any pharmaceutical product. These include:

Unique efficacy

Liposome formulation of a drug must show an improved therapeutic ratio (effective dose/toxic dose) over other formulations of the same drug. In addition to existing formulations where this has already been demonstrated (*see* pp. 282, 306), one area in particular where liposomes may provide unique therapeutic advantages over other formulations is in facilitating and maintaining drug solubilization. Mixed solvent systems currently used to solubilize drugs with poor aqueous solubility in the physiological range, include surfactants and other excipients which can themselves have some associated toxicity and which may not maintain solubility on dilution *in vivo*. Non-toxic lipids used in liposome formulation give a much-needed, biocompatible alternative. Effective solubility of the drug in a liposome-based drug formulation can then provide an improvement in bio-availability. For parenteral formulations requiring sustained release of a drug, liposomes offer *in vivo* protection for relatively labile molecules, such as proteins and peptides.

Reproducibility of efficacy

Once efficacy of a particular liposome-based drug formulation has been demonstrated, the effect must be shown to be reproducible within a number of set pharmaceutical criteria. For example, during clinical studies, intersubject variability in terms of pharmacodynamics and pharmacokinetics of the drug will need to be comparable with that of more conventional dosage forms, and there should be no batch-to-batch variability *in vivo*. Furthermore, batch-to-batch variability in terms of physicochemical properties (product specifications), including particle size distribution, % drug entrapment, lipid ratios and net charge, should be comparable with conventional parenteral products, i.e. ± 10% max. for the inactive components (lipids), and ± 5% for the drug itself.

Biocompatibility

Lipids themselves are a highly diverse group of molecules, some forming emulsions (*Table 4*) and others with amphiphilic properties, forming thermodynamically stable, closed vesicles (liposomes). Of the many combinations and types of lipids which can form vesicles and emulsions, only a relatively small number have been clinically evaluated in association with drugs. It is possible that alternative synthetic lipids, e.g. those that can be polymerized, or with modified surface properties such as hydrophilic coating or targetting moieties, will provide further therapeutic advantages. Lipids already approved and used in commercial formulations as excipients (vehicle) or constituents (lipid emulsions for parenteral feeding) include various oils and PC ('lecithin') derived from natural sources (*Table 4*). Toxicological screening and biocompatibility of a formulation prior to clinical testing is performed routinely to ensure a safe and efficacious end-product, and in clinical studies so far, no lipid-associated toxicity has been observed for parenteral liposome-based drug formulations. Extensive toxicological studies comparable with

Table 4. Approved parenteral excipients for water-immiscible drugs

Excipient	Drug	Route of admin.	Dosage form	Manufacturer	% w/v
Oils					
Cottonseed	Testosterone cypionate	i.m.	Solution	Upjohn	56·0–87·4
Peanut	Dimercaprol	i.m.	Solution	H. W & D	70·0
Peanut	Vasopressin tannate	i.m.	Suspension	P-D	q.s.
Safflower	Fat emulsion	i.v.	Emulsion	Abbott	10–20
Sesame	Nandrolone derivatives	i.m.	Solution	Organon	q.s.
Sesame	Testosterone, oestradiol	i.m.	Solution	Savage	q.s.
Sesame	Aurothioglucose	i.m.	Suspension	Schering	q.s.
Sesame	Fluphenazine decanoate	i.m./s.c.	Solution	Squibb	q.s.
Soybean	Fat emulsion	i.v.	Emulsion	Cutter	10–20
Phospholipids					
Lecithin	Penicillin G derivatives	i.m.	Suspension	Wyeth	0·3–0·6
				Squibb	0·5–2·3
				Lilly	1·0
				Pfipharmecs	0·6–1·0

those required for new drug substances will screen more exotic liposomal formulations. For all formulations, pyrogenicity testing and specifications for lipid raw material and residual solvents in the final product will need to be pharmaceutically acceptable. In addition, residues of chemicals used in processing should be minimized and lipid raw material will have to be manufactured under good manufacturing practice (GMP) procedures, the code of practice by which drug safety in manufacturing is controlled.

Stability of liposome-based formulations with respect to drug retention, and vesicle-size distribution, sterility and efficacy: retention of chemical and physical properties of drug and lipid components

While research efforts for liposome formulations of drugs currently in development have understandably concentrated on establishing therapeutic efficacy of the formulations, pharmaceutical issues such as drug stability, vesicle shelf-life and drug retention also need comprehensive attention. For example, lipids in the formulation are being evaluated in terms of their stability and storage properties with respect to hydrolysis and oxidation (if appropriate) of the lipid components (Grit *et al.*, 1989). Since it is conceivable that drug and lipid will behave differently in one-another's presence, stability analyses for clinical formulations of liposomal drugs need to be performed with this possibility in mind.

For long-term shelf-life, the continued performance of the liposome formulation according to the product specification will be the ultimate test of the effectiveness of product composition, manufacturing technology, storage conditions and packaging design. Stored liposome formulations of any drug must remain within specifications with respect to potency, drug entrapment, sterility, pyrogen level, size profile and many other characteristics (*see Table 5*). This means, for example, that preservatives required for products intended for parenteral administration, need to retain their effectiveness in the presence of liposomes, as well as avoiding interference with liposome and drug function. If liposomes are surface-modified, then the stability of liposomes in terms of surface properties will need to be established. Specific methodology is being developed to study the pharmacokinetics of drug formulations in clinical trails, e.g. doxorubicin (Balazovits *et al.*, 1989). For injectable formulations, manufacturing technology is being developed which incorporates such features as efficient, reproducible encapsulation, uniform size and the option to sterile-filter the product immediately prior to filling (Martin, 1990). Other technology to simplify stability considerations includes lyophilization processes, which allow a drug to be stored and distributed in a dry form. Immediately before use, the product is rehydrated, and for some products, especially for vesicles in the size-range 50–200 nm, it is possible that sugars, especially the disaccharides trehalose and sucrose, can act as cryoprotectants (Crowe *et al.*, 1986). Finally, stability concerns can also be addressed with respect to the use of lipophilic prodrugs designed to anchor the drug in the lipid bilayer and give very high encapsulation efficiencies without subsequently requiring extensive unencapsulated-drug removal.

Ease of large-scale sterile processing

Any liposome formulation which demonstrates good therapeutic efficacy and pharmaceutical acceptibility on a laboratory research scale will require manufacture on a larger scale to support clinical trials and, once successfully licensed, further scale-up will be required for production. The manufacturing process must result in a sterile, pyrogen- and particle-free product, allow a minimum of 100 litre batches of product to be made, and be amenable to straightforward validation in terms of regulatory and manufacturing requirements. Process equipment must be acceptable for use in a sterile manufacturing environment and be compatible with, and minimize use of, organic solvents. An approach appropriate for topical applications involves manufacture of lipid mixtures in the form of a flowable powder produced by spray-drying, which is then hydrated and homogenized under controlled conditions. For other approaches, the feasibility and cost of scale-up will depend on the equipment and methology available in-house, and the extent to which formulations of liposome-based drug products will differ from existing manufacturing practices. Concerns that apply to liposome manufacturing can also apply to drug-delivery formulations, such as emulsions, suspensions and systems such as microspheres. Thus the key to a successful liposome-based drug product, is excellent therapeutic indications to justify the expense and utilization of resources.

Overall cost

Any formulation of a drug that goes beyond a simple tablet or saline solution of the drug must be fully justified by the therapeutic advantage achieved by the more sophisticated approach. In the case of liposomes, the cost of manufacturing technology and the drug development process, including costs of high-purity GMP lipids, must be balanced against the therapeutic gains in terms of market-based improved product performance or a new clinical indication to be achieved by the high-tech presentation of the drug.

The cost of raw material for liposomes depends on the type of lipid required, and ranges from 0·2 to 0·35 US$ per g for production-scale (20–100 US$ per g for research-scale) quantities of hydrogenated or natural PC to 1200 US$ per g for research-scale amounts of more exotic lipids such as gangliosides (Weiner, 1989). Manufacture and testing of lipids under GMP procedures can add to raw-material costs. However, prices should still be competitive, especially if the cost of other excipients, such as cyclodextrins, remains high.

A more significant cost consideration is in the manufacturing technology (*see* previous discussion). Manufacturing concerns include sterile processing, solvent incompatibility with process equipment, solvent-related safety issues, particle-size control, separation of free drug and elimination of residual solvents and/or detergent, if appropriate (Klimchak and Lenk, 1988). A number of these issues have already been addressed in a commercially successful manner by the cosmetic industry; thanks to the introduction of

spray-drying technology, ton quantities of liposome-based products are made by using hydrogenated soya PC and other ingredients. The level of sterility is bound to be less rigorous than that required for injectable pharmaceuticals, the technology is patent-protected, and profit margins are high. However, success in this market indicates that a technology relevant to parenteral formulations is in place. Approaches applicable to large-scale manufacturing include: lyophilization (Kirby and Gregoriadis, 1984; Crommelin and van Bommel, 1984; Crowe *et al.*, 1985; Özer *et al*, 1988; Cullis *et al.*, 1989) 'Microfluidiser®' technology (Talsma *et al.*, 1989) and detergent dialysis (Thoma and Schmid, 1989). Combinations of these processes are being developed, especially by the independent liposome companies, and issues of stability, reproducibility, high encapsulation efficiency, particle-size control, sterility assurance and scale-up, are being successfully addressed (Martin, 1990).

Regulatory approval

Liposome formulations of drugs which are already approved and available on the market will require resubmission for approval by the appropriate regulatory agencies (the Food and Drug Administration in the USA). For parenteral administration, extensive toxicological and clinical reinvestigation of a liposome-based drug product is reasonable because the therapeutic intention is that liposome encapsulation or presentation will favourably alter the effectiveness, toxicity, pharmacokinetics and pharmacodynamics of the drug. If the liposome or lipid emulsion is primarily acting as a matrix to solubilize the drug, more limited toxicological and clinical work will be required. For topical, transdermal, nasal and oral applications, extensive studies may be required. Examples of lipid-type excipients already in use include oils for

Table 5. Regulatory aspects of liposome-based drug formulation development

Aspect	Parameter
Preclinical	toxicology
Characterization	structure
	particle size
	lamellarity
	free v. liposome-associated drug stability as a function of time and storage conditions
	residual solvents
	in vitro drug-release rates
Manufacturing procedures	sterile particle-free product
	validation of process
Specifications of final product	potency
	sterility
	pyrogenicity
	heavy metal content
	residual solvents
	size distribution
	pH
	osmolarity

i.v., i.m. and s.c. dosing, and PC for i.m. formulations of penicillin (*Table 4*). A summary of formulation-related activities which require regulatory review are given in *Table 5*.

Synthesis of therapeutically effective lipophilic derivatives of established drugs and their subsequent efficient incorporation into liposomes has been achieved (e.g. Fidler *et al.*, 1982; Perez-Soler *et al.*, 1989). For synthesis of lipophilic derivatives of previously approved drugs, complete biological, pharmacological, toxicological and clinical reinvestigation will be required. However, a lipophilic derivative of a drug which has otherwise been clinically unsuccessful could provide significant therapeutic benefit if liposome association improves bio-availability of the drug and/or incorporation into target tissue, thereby warranting the extensive regulatory review.

BIOLOGICAL CONSIDERATIONS

An elegant pharmaceutical delivery system requires a strong biological, i.e. therapeutic, advantage in using it. The biological rationale for developing liposome-based drug formulations depends upon: (1) an increase in the therapeutic index of a drug being 'liposome-dependent' (Heath, 1988); and (2) further refinements in the manipulation of the physiological response to liposomes *in vivo*. Possibilities are open to the ingenuity and imagination of liposome technologists, chemists, pharmacologists, biologists, biochemists and physicians.

Three liposomal drug preparations currently showing increased efficacy over free drug in clinical trials are: liposomal amphotericin B (a lipid-soluble drug with high toxicity, especially to the kidney); liposomal MTP-PE (the lipid-soluble form of muramyl dipeptide (MDP) which is effective in activating macrophages); liposomal doxorubicin (Adriamycin®, UK), a partly lipid-soluble drug which is most widely used in cancer chemotherapy in the US but which causes acute toxicities such as myelosuppression, gastrointestinal toxicity and cardiotoxicity in cumulative high doses. These drugs are showing increased clinical efficacy on the basis of an overall reduction in toxic effects, using relatively simple liposome formulations of each drug (Cullis *et al.*, 1989). If this much can be achieved with what amounts to a simple, passive targetting, there is a huge untapped resource in liposome formulation technology, not only through the use of more sophisticated liposome formulations, but also for drugs in the early stages of development.

Mechanism of action of liposome-base pharmaceuticals

Liposomes can aid parenteral drug delivery on three different levels: at the simplest level, liposomes can improve the therapeutic effectiveness of a drug by increasing drug bio-availability in the circulation. The mechanism for this increase in effectiveness is probably twofold: first in terms of bio-availability, the liposome emulsion solubilizes the drug, and, secondly, prevents the immediate and rapid precipitation of a water-insoluble drug compound upon dilution in the circulation. Clearance of the drug from the blood or other injection site can also be delayed.

At the next level of sophistication a more plasma-resistant liposome formulation redirects the drug to tissue macrophages in liver, spleen and bone marrow. In terms of toxic effects, a drug which in its free form causes toxicity to the kidneys, as the main organ of drug elimination, in liposome-form is redirected to the liver, spleen and bone marrow, organs better equipped to deal with toxic compounds. Liposomes also enhance diagnostic imaging *in vivo*; liposome-based imaging agents, such as radiotracers, radiopaque agents, paramagnetic compounds or sonoreflective agents, can remain in the circulation after parenteral administration much longer than the free imaging agents and are particularly suitable for sensitive imaging of RES tissue sites (Caride, 1990). The relative proportions and specific cells accessed depend upon the details of liposome composition, as described previously. Circulating macrophages and those capable of migration to sites of inflammation due to infection or other causes, may also take up liposomal drugs. Macrophage migration could explain liposomal efficacy in extravascular sites, and such macrophage uptake may be facilitated by the use of liposomes with prolonged clearance half-lives. Whatever the mechanism, there is increasingly compelling evidence supporting the preferential accumulation of liposome-encapsulated molecules at sites of inflammation (Axelsson, 1989), infection (Morgan, Williams and Howard, 1985) and certain solid tumours (Profitt *et al.*, 1983).

The most heroic level of liposome–drug administration is that in which vesicles cross endothelial, or indeed epithelial, barriers, or at least facilitate the passage of vesicle contents across membranes. Some of the most challenging targets for access parenterally include tumours, non-phagocytic cells bearing infections, and perhaps the ultimate challenge, the blood–brain barrier.

Realistically, immediate goals for improving the biological efficacy of liposomal drug formulations must be based on innovative and systematic *in vivo* studies. Such studies provide a framework for optimizing current design and manufacturing processes, for solving problems and anticipating them, and for finding new ways of exploiting and circumventing the body's own physiological response to the delivery system. Optimization of biological aspects of liposome design aim to produce major developments in liposome-based drug delivery, including selective RES targetting, delayed removal of liposomes by the RES, preferential accessing of non-RES target sites, improved formulations for parenteral administration of labile proteins and peptides, case-specific topical drug delivery and many other areas.

Conclusions

Recent clinical successes with liposome-based drug formulations rely on the significant therapeutic benefits achieved by virtue of favourable pharmacokinetics of a liposomal formulation compared with the free drug, the primary benefit being a reduction in toxicity (while maintaining or increasing efficacy). Enhanced drug solubility and passive targetting of liposomally encapsulated bio-active molecules are also areas of active pharmaceutical research.

In an injectable form, liposomes are highly biocompatible, non-toxic and versatile in terms of their physical characteristics, such as size and surface properties. In order to take advantage of recent and potential developments in biological and therapeutic applications, a strong liposome formulation and manufacturing base needs to be developed. It is essential that production-scale technology is cost effective, in compliance with current good manufacturing practices, and the finished product highly reproducible. Major manufacturing and stability issues are being tackled realistically, especially for liposome–drug formulations currently in the clinic. A developing area of interest is in the formulation of proteins and peptides, and drug-delivery systems including liposomes have an important role to play in resolving formulation issues for protein-based pharmaceuticals. Recent advances in procedures for rapid and reproducible generation of liposomes and new techniques for the efficient and stable entrapment of drugs at high drug/lipid ratios are being applied to a number of conventional drugs and those which are the products of advances in biotechnology, such as cytokines, growth factors, interferons and hormones. With the current rate of progress, and given the inherent biocompatibility of liposomes, especially for parenteral applications, liposomes could become the preferred formulation technique for biopharmaceuticals and other drugs with unique effects and problems in development.

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