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Liposomes in Drug Targeting

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Introduction

Liposomes are artificial vesicles composed of one or more concentric bi-layers which enclose an aqueous space. Water-soluble drugs or biomolecules are known to be accommodated in the inner aqueous space of liposomes, whereas lipophilic substances remain associated in the hydrophilic bilayer. Initially, the use of liposomes was mainly confined to model membrane systems, but more recently their use as vehicles for the transfer of genetic and other materials into cells in culture or for drug delivery to desired cellular and subcellular sites in a living system has been the subject of much discussion (Berg and Boman, 1973; Stahl *et al.*, 1978; Poste and Papahadjopoulos, 1978; Gregoriadis, 1980a). It appears that different applications require different types of liposomes. Although the preparation of various kinds of liposomes has recently been reviewed (Szoka and Papahadjopoulos, 1981), it seems that two main types of liposomes can be distinguished, i.e. large uni-or multilamellar liposomes, which are cleared rapidly from the circulation, and small unilamellar liposomes, which remain in circulation for longer periods of time. Obviously, large multilamellar vesicles (Bangham *et al.*, 1965), which are produced by the deposition of a thin lipid film from an organic solvent on the walls of the container followed by the agitation with an aqueous solution of the material to be encapsulated, were found to be the best for drug delivery purposes. The advantage of liposomes over other delivery systems is their lack of accumulation in the body. Moreover, liposomes are poorly immunogenic and the lipids used are biodegradable. In addition, the liposome-encapsulated drugs are protected from the bioenvironment and are reported to have reduced toxicity. Because of improved rehydration and dehydration techniques (Kirby and Gregoriadis, 1984), modern drug-encapsulated liposomes can be stored as freeze-dried powder and constituted just before use.

Abbreviations: AIDS, acquired immunodeficiency syndrome; AM, alveolar macrophages; Chol, cholesterol; DCP, dicetyl phosphate; ddITP, dideoxy-inosine triphosphate; DPPE, dipalmitoyl phosphatidyl ethanolamine; HIV, human immunodeficiency virus; LDL, low-density lipoprotein; MDP, muramyl dipeptide; MLV, multilamellar vesicles; MTP-PE, muramyl tripeptide phosphatidyl ethanolamine; PC, phosphatidyl choline; PEG, polyethylene glycol; PMN, polymorphonuclear neutrophils; SM, sphingomyelin; SPC, soya phosphatidyl choline; SUV, small unilamellar vesicles.

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However, the common problem for liposome therapy is that liposomes are taken up readily by the cells of the reticuloendothelial system. Even though reversible endothelial blockade can be imposed with relative ease, it is not accompanied by any significant increase in liposome uptake by tissues outside of the mononuclear phagocyte system. The retention and uptake of circulating liposomes by reticuloendothelial cells and blood monocytes is a major obstacle to experimental efforts to 'target' liposomes to other cell types. SUV (small unilamellar vesicle) liposomes are cleared from the circulation more slowly than larger MLV (multilamellar vesicle) liposomes. However, there is no evidence that SUV liposomes or liposomes with a prolonged half-life in the circulation accumulate to any significant degree in sites other than the liver, spleen and bone marrow. Moreover, reported intravenous injection of liposomes at regular intervals is accompanied by long-term paralysis of reticulo-endothelial function. Since many of the proposed clinical applications involve multiple-dose treatment protocols, this problem represents a potentially serious drawback. Besides, there are no appreciable methods either for targeting liposomes to specific sites *in vivo* or for ensuring the delivery of liposomal contents to target cells. Although considerable amount of work has been reported on passive targeting to the reticulo-endothelial system, not much is known about active targeting to specific subsets of circulating blood cells or to vascular endothelium. Thus, efficient delivery systems suitable for site-specific targeting are being sought.

In site-specific targeting, a recognition marker (or ligand) is usually attached to the surface of liposomes. An ideal target for such liposomes would be a cell or tissue that uniquely expresses a receptor specific to the attached ligand. Surolia, Ahmed and Bachhawat (1975) reported the potential use of glycolipids as ligands in a search for receptor sites in animal tissues. Liposomes with attached sugar residues are reported to be more effective in transportation of their contents to specific organs due to the selective binding of the sugars with their specific receptors (Ghosh and Bachhawat, 1980). Recently (Das *et al.*, 1987; Medda *et al.*, 1990) plant glycosides of varying terminal sugar residues have been incorporated on to the bilayer membrane of liposomes, which have been tested for their tissue specificity or for their increased uptake to specific cellular or subcellular sites. As knowledge of the receptor status of target cells undergoing infection is a very important prerequisite for designing targeted delivery vehicles, different sugar-coated liposomes have been prepared to test the feasibility of their application not only as delivery vehicles but also as efficient ligands for the estimation of specific sugar receptor status of macrophages in normal as well as in infected states (Dutta, Bandyopadhyay and Basu, 1994).

Moreover, tetrapeptide (tuftsin)-bearing liposomes (Tuft-lip) have been shown to be quite effective in activating host mcrophages (Singh, Chabbra and Srivastava, 1992), raising the possibility of their application as targeted delivery vehicles against macrophage-associated disorders. Similarly, the increased uptake of antibody-coated liposomes by erythrocytes is suggestive of their possible use as targeted drug carriers against erythrocytes undergoing infection (Singhal and Gupta, 1986). The successful targeting of amylopectin-incorporated liposomes to lung (Takada *et al.*, 1984) also raised the possibility of their use as targeted drug carriers against lung associated disorders, although not much success was achieved in reducing the uptake of these liposomes by the cells of the reticulo-endothelial system.

Designing liposomal vehicles for site-specific delivery

The major emphasis during the past decade has been on designing and targeting liposomes to specific sites, in an attempt to show increased drug efficacy and reduced drug toxicity. Efforts were made to produce stable liposomes with larger circulation time, glycolipid/glycoside-bearing liposomes or sugar-coated liposomes, and peptide-or antibody-coated liposomes.

STABLE LIPOSOMES WITH LONGER CIRCULATION TIME

It was found that vesicle size and surface charge override the state of membrane permeability in determining rates of vesicle clearance (Senior, Crawley and Gregoriadis, 1985). Small unilamellar vesicles were cleared less rapidly than were large unilamellar ones. Moreover, the neutral and positively charged unilamellar liposomes were cleared less rapidly than were the unilamellar negatively charged ones (Juliano and Stamp, 1975).

The effect of cholesterol content on the stability of small unilamellar liposomes was examined *in vivo* and *in vitro* by Kirby, Clarke and Gregoriadis (1980). Cholesterol-rich liposomes, regardless of surface charge, remained stable in the blood of intravenously injected animals for up to at least 400 min. In addition, stability of cholesterol-rich liposomes was largely maintained *in vitro* in the presence of whole blood, plasma or serum for at least 90 min. But the stability of liposomes was found to be influenced not only by the biological environment with which they come into contact but also by their structural characteristics and those of the associated drugs. For instance, liposomes containing charged lipids retain certain drugs through electrostatic bonding (Gregoriadis, Neerunjun and Hunt, 1977), and lipophilic drugs can be anchored into the liposomal lipid phase (Gregoriadis, 1973). Liposome formulations with long circulation half-lives were reported by Allen, Hansen and Rutledge (1989). Liposomes with the longest circulation half-lives had compositions that mimicked the outer leaflet of red blood cell membranes (egg phosphatidyl choline/spingomyelin/cholesterol/ganglioside GM_1 in the molar ratio 1:1:1:0.14). Several other gangliosides and glycolipids were examined, but none could substitute for GM_1 in their ability to prolong circulation half-lives. In a recent report (Allen *et al.*, 1991), a novel synthetic lipid derivative of polyethylene glycol (PEG-PE), was found to decrease greatly liposome uptake by bone marrow macrophages in a concentration-dependent manner. Lipids which are known to increase the microviscosity of biological membraes, e.g. sphingomyelin (SM) and cholesterol (Chol), were found to decrease both *in vivo* and *in vitro* uptake of liposomes by bone-marrow macrophages. Moreover, the stability of liposomes in circulation was found to be markedly enhanced by structural modification of their phospholipid component (Bali, Dhawan and Gupta, 1983) or by appropriate tailoring of the phospholipid structure (Agarwal, Bali and Gupta, 1986b). Apart from the increased survival, liposome uptake by the liver was found to decrease by replacing natural phosphatidyl choline (PC) with carbamyl PC in liposomes (Gupta, 1983).

GLYCOLIPID/GLYCOSIDE-BEARING LIPOSOMES OR SUGAR-COATED LIPOSOMES

The finding that different cells can recognize different sugar residues (Krantz *et al.*,

1976) led to the idea that by modification of liposomes, by incorporating various glycolipids or glycosides having different terminal sugars, it may be possible to direct the liposomes to various tissues. Specific galactose-binding receptors were reported to be present exclusively on the parenchymal cells of liver (Gregoriades *et al.*, 1970; Surolia and Bachhawat, 1977; Hoekstra, Tomasini and Scherphof, 1980). In addition to natural glycolipids, small molecular weight synthetic glycolipids were found to be equally effective for the introduction of saccharide determinants on to the surface of liposomes. The liposomes prepared from lactosyl-phosphatidyl ethanol amine (Ghosh, Bachhawat and Surolia, 1981) or from β-D-galactosyl-phosphatidyl ethanolamine (Ghosh and Bachhawat, 1980) were found to be taken up readily by hepatocytes. Specific receptors were demonstrated on the non-parenchymal or Kupffer cells, and were found to recognize the terminal mannose residue of glycoside-bearing liposomes (Ghosh and Bachhawat, 1980) or of biopolymers (Stahl *et al.*, 1978). Ganglioside-and other glycolipid-containing liposomes were found to bind specifically to lectins (Surolia, Bachhawat and Poddar, 1975; Juliano and Stamp, 1976; Redwood and Polefka, 1976; Boldt *et al.*, 1977), and lectin-mediated agglutination of liposomes with variable hydrophilic spacers was noted by Slama and Rando (1980).

A series of investigations on the tissue distribution of liposomes bearing different glycosides isolated from indigenous plants was carried out in our laboratory (Mahato, Sahu and Pal, 1978; Mahato, Sahu and Ganguly, 1980). The plant glycosides selected for the purpose were of variable sugar chains having different sugars at the terminal end. *Figure 1* shows two of the plant glycosides isolated and used for incorporation into liposomes. Because of their interesting structure – a hydrophilic moiety was attached to a hydrophobic aglycone – the plant glycosides were very suitable for incorporation into the liposomes. A diagram of plant-glycoside-bearing liposomes and a schematic representation of synthetic glycoside-grafted liposomes are shown in *Figure 2*. Studies with two plant glycosides (gracillin with glucose as end-sugar, and fluoribundasaponin with rhamnose as end-sugar) incorporated into liposomes encapsulated with [^{125}I]IgG demonstrated the presence of anomeric-blind glucose receptors on the non-parenchymal fraction of liver cells (Das *et al.*, 1987). The rates of uptake of [^{125}I]IgG, encapsulated in different types of liposomes, by the liver (% injected dose) are presented in *Figure 3*. The uptake of gracillin liposomes by the liver was found to be 72% of the injected dose in the case of negatively charged liposomes as against 65% for the neutral ones. The uptake of liposomes in the presence of either glycogen (α-glucose) or cellobiose (β-glucose) was 48% for both, and was almost the same as that found for the control liposomes (Das *et al.*, 1987). In another report, the hepatic uptake of liposomes made from egg lecithin, cholesterol and dicetyl phosphate and either of two plant glycosides – corchorusin D with glucose as end-sugar (Mahato and Pal, 1987), and asiaticoside (Mahato *et al.*, 1987) with rhamnose as end-sugar – was compared. The hepatic uptake of asiaticoside-bearing liposomes was reduced, whereas that of corchorusin-D-bearing liposomes was enhanced and was found to be specific for glucose (Medda *et al.*, 1993). Liver perfusion followed by cell separation showed that the uptake was mostly by the non-parenchymal fraction of liver cells (*Table 1*). When the subcellular distribution pattern was examined, the corchorusin-D-bearing liposomes were found most frequently in the lysosomal fraction of the non-parenchymal cells of liver (*Table 2*). Moreover, based on this observation, an attempt was made to deliver an enzyme of plant origin, glucose

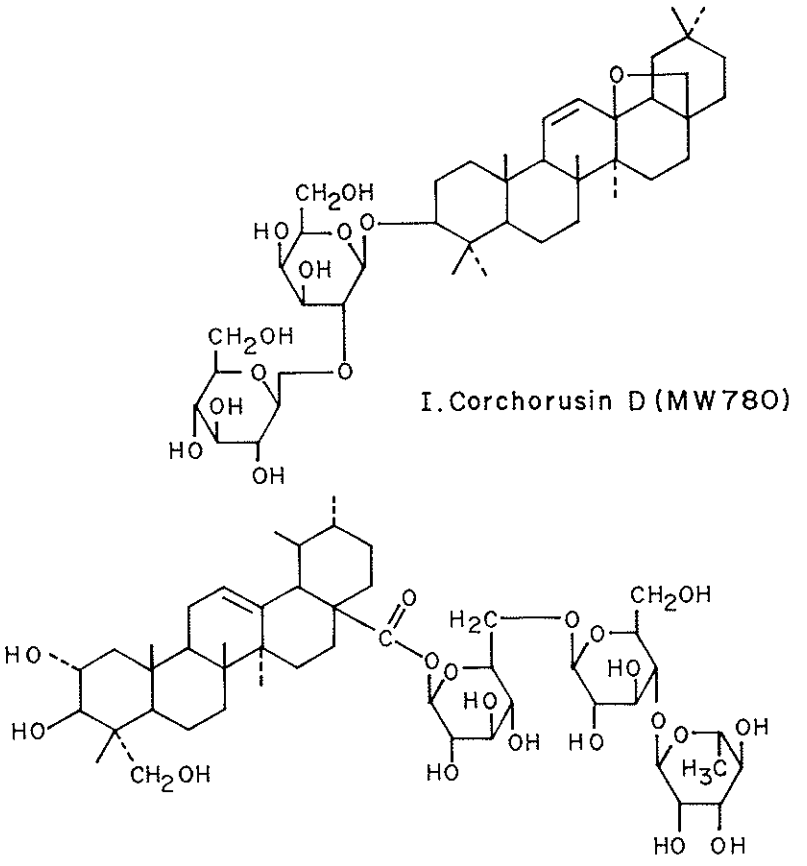

Figure 1. Structures of glycosides isolated from indigenous plants. A hydrophilic sugar moiety is seen to be attached to a hydrophobic aglycone in both of the structures.

oxidase, to lysosomes using corchorusin-D-bearing liposomes as the delivery system (Medda *et al.*, 1993). As the drug action, if any of these glycosides, when incorporated in liposomes, is expected to be effective in small doses because of enhanced cell–liposome interactions, this delivery system was designed to test the selectivity and enhancement of efficacy of some indigenous medicines containing plant glycosides. Besides the plant glycosides, the synthetic glycosides with mannose as end-sugar were also incorporated into the liposomes and were successfully used to target blood–brain barrier cells or the glial cells of the brain (Umezawa and Eto, 1988).

PEPTIDE- OR ANTIBODY-COATED LIPOSOMES

Antibody-mediated targeting was done with actively endocytotic cells. Gregoriadis and Neerunjun (1975) formed large multilamellar liposomes in the presence of IgG specific for surface antigens on the target cells. Antibodies were assumed to associate

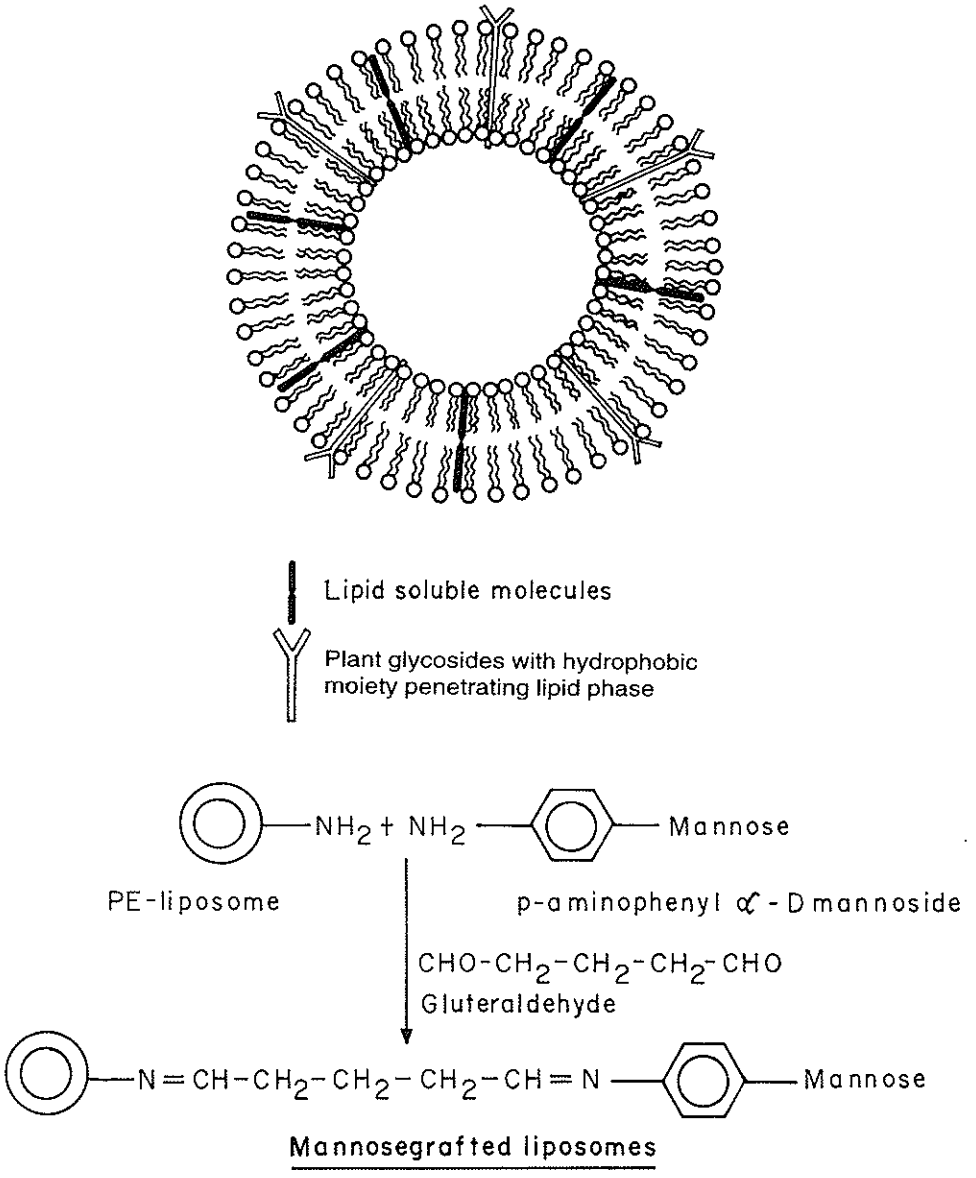

Figure 2. Diagrammatic representation of a liposome incorporating plant glycosides (top), and a schematic representation of the formation of synthetic glycoside-grafted liposomes (bottom).

with liposomes by their F_c portions, and the resulting liposomes showed preferential binding to the corresponding cells. When the cells were fractionated, most of the liposome-entrapped drug was recovered from the lysosomal fraction, consistent with the predominance of endocytosis. Weissman *et al.* (1975) formed multilamellar liposomes in the presence of heat-aggregated immunoglobulin and stimulated phagocytosis via the F_c receptor. Again, uptake was largely into the lysosomal fraction

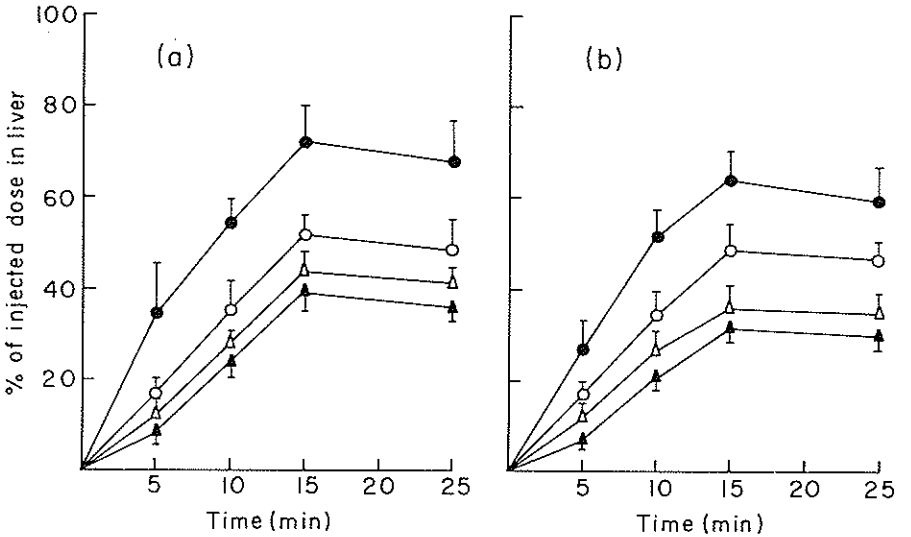

Figure 3. Rate of uptake by liver of [^{125}I]human IgG entrapped in (a) negatively charged and (b) neutral liposomes. The different liposomes used were: ●, gracillin; O, fluoribundasaponin; Δ, control with aglycone; and ▲, control without aglycone. The differences between the uptake of the gracillin liposomes, both negatively charged and neutral, and their corresponding controls are significant at $p < 0.001$.

Table 1. Differential uptake of [^{125}I]human-IgG-entrapped liposomes by liver cell types

Liposomes	Parenchymal cells (% uptake)	Non-parenchymal cells (% uptake)
Egg PC + Chol + DCP + corchorusin D (A)	20.0 ± 4.7 (3)	69.3 ± 2.3 (3)
(A) + Glycogen	41.8 ± 0.3 (2)	42.1 ± 2.2 (2)
(A) + Mannan	19.7 ± 0.3 (2)	62.9 ± 2.5 (2)
(A) + Asialofetuin	14.5 ± 4.5 (2)	76.0 ± 3.1 (2)

The livers of experimental rats were perfused by collagenase treatment. The parenchymal and non-parenchymal cells were separated by differential centrifugation. The uptake of liposomes in both these fractions presented as a percentage of the total radioactivity in the liver cell suspension before the separation of the cells. Results are expressed as mean ± SD. For each experiment duplicate analyses were made and the number of independent experiments conducted is indicated in parentheses.

Table 2. Subcellular distribution of [^{125}I]human-IgG-entrapped liposomes by liver cell types

Liposomes	Parenchymal cells[a]			Non-parenchymal cells[a]		
	Nuclear fraction (P_1)	Mitochondrial/ lysosomal fraction (P_2)	Plasma and nuclear membrane fraction (P_3)	Nuclear fraction (P_1)	Mitochondrial/ lysomal fraction (P_2)	Plasma and nuclear membrane fraction (P_3)
Egg PC + Chol + DCP+Corchorusin	18.0 ± 2.0	32.0 ± 2.0	28.0 ± 3.0	12.0 ± 5.0	50.2 ± 4.0	18.1 ± 2.1

[a] Percentage of the total count in isolated cell fractions.
Results are shown as mean ± SD ($N = 3$).
P_2 fractions of both the parenchymal and non-parenchymal cells were analysed for the marker enzyme aryl sulphatase. The activity of the enzyme (*p*-nitrocatechol liberated/45 min/mg protein) was found to be around 0.156 ± 0.006 μmoles.

(Cohen *et al.*, 1976). The binding to lymphocytes was found to be three times greater when the liposomes were prepared with $F(ab')_2$ fragments (*Figure 4*) than with the whole antibody, and, as expected, was almost absent with the monovalent F(ab') fragments (Weinstein *et al.*, 1978). Heath, Fraley and Papahadjopoulos (1980) reported the cell specificity obtained by conjugation of $F(ab')_2$ to the vesicle surface. The binding of liposomes to erythrocytes in whole blood or *in vivo* was found to increase considerably (by at least 20 times) by covalently attaching anti-erythrocyte $F(ab')_2$ to their surface (Singhal and Gupta, 1986; Singhal, Bali and Gupta, 1986). It was interesting to note that the integrity of the liposomes did not alter after the binding process and a significant fraction of the cell-bound liposomes could deliver their content to the target cells presumably via membrane–membrane fusion. As the specificity and efficacy of the targeted liposome binding to cell surfaces depend on the specificity of antibody molecules, it would be preferable to use the monoclonal antibodies. Huang, Huang and Kennel (1980) covalently coupled monoclonal antibodies with fatty acids for *in vitro* liposome targeting. Cell-specific drug transfer from liposomes bearing monoclonal antibodies has already been reported (Lesserman and Barbet, 1981).

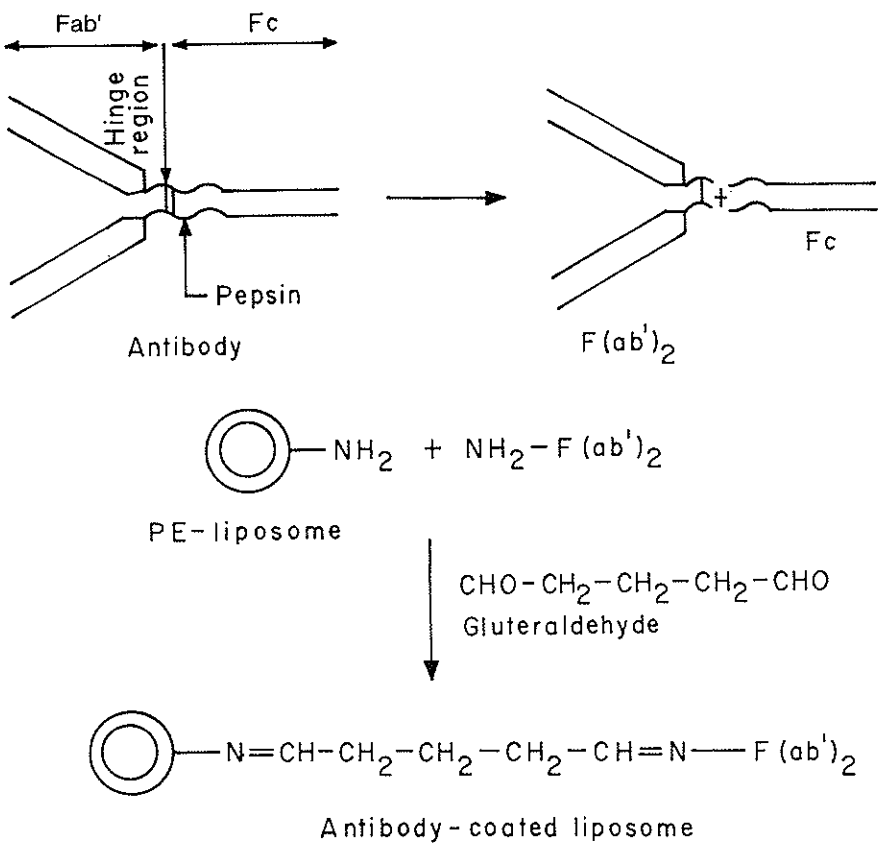

Figure 4. Schematic representation of the formation of antibody-coated, or more specifically $F(ab')_2$-fragment-coated, liposomes.

Synthetic peptides (e.g. *N*-fMet–Leu–Phe, *N*-fMet–Leu–Phe–Phe, *N*-fMet–Nle–Leu–Phe) that are reported to be chemotactic to macrophages and monocytes (Schiffmann, Corcoran and Wahl, 1975; Niedel, Wilkinson and Cuatrecasas, 1979; Snyderman and Fudman, 1980; Snyderman and Goetzel, 1981) are being utilized in our laboratory for coupling to the liposomes. The peptide-coated liposomes have already been tested against both normal and *Leishmania donovani* infected macrophages *in vitro* (Banerjee and Basu, unpublished observation). The possibility of using peptide-coated liposomes *in vivo* encapsulated with antileishmanial drug is now being explored. Tuftsin, a tetrapeptide (Thr–Lys–Pro–Arg) known to bid specifically to macrophages, monocytes and polymorphonuclear neutrophils (PMN) leucocytes and also to potentiate the natural killer activity of these cells, has successfully been used for grafting on the liposome surface (Singhal *et al.*, 1984). Subsequent studies with tuftsin-bearing liposomes revealed that the pretreatment of animals could render them resistant to malaria and leishmania infections (Gupta *et al.*, 1986; Guru *et al.*, 1989). Tuftsin-bearing liposome-intercalated Amp B has been reported to be used against experimental aspergillosis (Owais *et al.*, 1993). Liposomes bearing surface-attached antibody (L-Ab) were prepared to deliver dideoxy-inosine triphosphate (ddITP) to human monocyte/macrophages in an attempt to suppress the replication of human immunodeficiency virus (HIV), which causes the acquired immunodeficiency syndrome (AIDS), by inhibiting the reverse transcriptase of viral RNA (Betageri, Jenkins and Ravis, 1993).

Application of liposomal delivery systems in chemotherapy

AS A CARRIER OF ENZYME AND PROTEIN

The use of liposomes as enzyme carriers was reported for the first time in the treatment of lysosomal storage diseases (Gregoriades, Leathwood and Ryman, 1971; Gregoriadis and Ryman, 1972a). After administration of liposome-entrapped β-fructofuranosidases the enzyme activity was found to concentrate mainly in the lysosome-enriched fractions of liver and spleen, indicating the uptake by endocytosis (Gregoriadis and Ryman, 1972b). Gregoriadis and Neerunjun (1974) incorporated gangliosides into liposomes and showed the hepatic uptake and clearance from the circulation by measuring radioactivity of the entrapped [^{125}I]albumin or invertase. The uptake by hepatic cells of monosialo-ganglioside-bearing liposomes encapsulated with invertase was studied by Surolia and Bachhawat (1977). It was observed that model hepatitis, induced by D-galactosamine, could be prevented by administration of asialo-GM_1 liposomes encapsulated with uridine, prior to the administration of galactosamine (Mathias, Dorai and Bachhawat, 1977).

The use of liposomes for enzyme replacement therapy in Gaucher's disease has already been discussed, the missing enzyme being glucocerebrosidase. Mannose-grafted liposome-entrapped [^{125}I]γ-globulin was reported to be found in Kupffer and endothelial cells, and a potential decrease in the uptake of those liposomes was noticed when mannan was used as inhibitor (Ghosh and Bacchawat, 1980). A number of reports appeared in the literature confirming the fact that β-galactosidase liposomes are specifically taken up by the liver (Spanger and Scherphof, 1983; Szoka and Mayhew, 1983). Incorporation of D-glucose oxidase through the blood–brain barrier

into the brain by means of liposomes was achieved by Naoi and Yagi (1980). Using liposomes bearing plant glycosides, our laboratory succeeded in delivering [^{125}I]glucose oxidase to the lysosomal fraction of non-parenchymal cells (Medda *et al.*, 1990).

AS A CARRIER OF CYTOTOXIC AND ANTICANCER DRUGS

Cytotoxic drugs are known for their indiscriminate action on both diseased and normal cells. Attempts have been made to direct the cytotoxicity specially to the diseased area of the body. The mechanism by which liposomal cytotoxic agents act *in vivo* was studied in partially hepatechtomized rats injected intravenously with liposomes containing actinomycin D in their lipid phase. Upon injection of actinomycin-D-or 5-fluorouracil-entrapped liposomes, it was found that cancerous tissues preferentially take up high amounts of these drugs, and there was little uptake of the drugs by bone marrow, which showed no side-effects (Gregoriadis, 1976). Several anticancer agents were incorporated in liposomes and their therapeutic efficacy was evaluated (Rahman *et al.*, 1974a; Mayhew *et al.*, 1976). The liposome-encapsulated anticancer drugs (e.g. methotrexate or 1-β-D-arabinofuranosyl-cytosine) were found to be more potent than the free drug in mice (Kosloski *et al.*, 1978; Rustum *et al.*, 1979; Kimbelberg, 1980). Many studies on the use of liposomes containing antitumour drugs for probing experimental tumours in animal models have already been reported (Kaye, 1981). There have been reports about the increased tumoricidal property of muramyl dipeptide (MDP) or its lipophilic derivatives after their incorporation into liposomes (Sone and Fidler, 1980; Kleinerman *et al.*, 1983). It was thought possible that MDP or its derivatives are protected from extracellular degradation before reaching the target cells, resulting in enhanced tumoricidal activity. Enhancement of the activity of bleomycin on Ehrlich ascites carcinoma cells by liposomal encapsulation has been reported (Sur, Hazra and Roy, 1984). Moreover induction or suppression of the immune response was found to be effected by incorporating drugs into liposomes with surface-coupled antigen (Shek, Lopez and Heath, 1986). However, no definite case was shown for which liposomes could be looked upon as one of the best drug delivery systems in cancer treatment. In solid tumours the malignant cells are known to be situated in the extravascular compartment. The inability of liposomes to extravasate from the microcirculation is obviously relevant to this problem. This must be considered as a possible factor limiting the effectiveness of liposomes in treating metastatic disease. A second major factor limiting the effectiveness of liposome-encapsulated antitumour drugs in cancer treatment originates from the problem of phenotypic heterogeneity among tumour cells (Poste, Krish and Koestler, 1984). It has been pointed out that malignant tumours are not uniform entities populated by cells with identical phenotypes. They would contain multiple subpopulations of tumour cells which could possibly exhibit notable phenotypic diversity for a wide range of cellular properties (Poste and Fidler, 1980; Poste, 1982). It was argued that the heterogeneous response of different tumour cell subpopulations to cytostatic and cytotoxic drugs seems responsible for the majority of failures in cancer chemotherapy (Poste, Krish and Koestler, 1984). Very recently, liposomal monencin has been tried in an attempt to eliminate selectively the cancer cells, and was found to potentiate the cytotoxicity of immunotoxins or hormonotoxins (Madan, Vasandani and Ghosh, 1993; Vasandani, Madan and Ghosh, 1993). However,

considering all the limitations of liposomes to treat certain disorders, they have been exploited successfully in the treatment of several diseases. Also, 5-fluorouracil in combination with liposome-encapsulated MDP has been used for the detection of chemo-immunotherapy of murine liver metastases (Daemen *et al.*, 1993). Muramyl tripeptide phosphatidyl ethanolamine (MTP-PE) liposomes have been reported to act as biological response modifiers in the treatment of cancer current status (Frost, 1992). An antitumour effect of liposomes containing taxol has been reported in a taxol-resistant murine tumour model system (Sharma, Mayhew and Straubinger, 1993). Based on recent reports (Kubo *et al.*, 1993), indium III-labelled liposomes have been used successfully for dosimetry and tumour detection in patients with cancer. On the other hand, lung cancer has been shown to be eradicated by antibody-targeted delivery of doxorubicin entrapped in sterically stabilized liposomes (Ahmad *et al.*, 1993). Recent human trials in the USA have reached their second phase for the treatment of infections in cancer patients, using liposomes containing antibiotics.

AS A CARRIER OF ANTILEISHMANIAL DRUGS

The therapy of leishmaniasis poses some problems. The drugs that are often used are known to be very toxic. In experimental infections in hamsters, the toxic dose of the standard pentavalent antimonial drug was found to be equivalent to the therapeutically effective dose (Alving *et al.*, 1978). However, the liposome-encapsulated drugs were found to be much safer and more efficacious in the treatment of experimental leishmaniasis (Alving and Steck, 1979). The therapeutic dose was found to be 200–800 times higher for the unencapsulated compared to the encapsulated drug (Black, Watson and Ward, 1977; Alving *et al.*, 1978; New *et al.*, 1978). But, the requirement of dose, even for liposome-encapsulated drug, could be higher, depending on the degree of infection or the stage of the disease (Alving, 1982). The therapeutic effects of antimonial drugs, e.g. sodium stibogluconate and meglumine antimoniate, were compared with those of lepidines, e.g. 8-aminoquinolines (Kinnamon *et al.*, 1978) and tetracycline (Alving *et al.*, 1980). The efficacies of a series of liposome-entrapped drugs in the treatment of both experimental cutaneous and experimental visceral leishmaniasis were critically analysed and reported by Chance and New (1980). In addition to the visceral leishmaniasis, New, Chance and Heath (1981a) extended their observations to show that liposomes also enhance the activity of sodium stibogluconate against experimental cutaneous leishmaiasis, where the parasite is located in macrophages in peripheral tissues, rather than in the liver. The scope of their investigation was further widened to cover other compounds, e.g. griseofulvin, 5-fluorocytosine and amphotericin B, all of which were known to exhibit antifungal activity (New, Chance and Heath, 1981b). A new approach to the problem of localizing intracellular liposomes and their entrapped agents utilizing the heavy metal properties of the entrapped antileishmanial compound, sodium antimony gluconate (Pentostam), and the non-therapeutically active intracellular marker, chromium, were reported by Heath, Chance and New (1984). Besides the critical evaluation of various antileishmanial drugs, the critical evaluation of liposomes and other drug delivery systems in the treatment of leishmaniasis was also published (Croft, Neal and Rao, 1989).

Antileishmanial activity of liposome-encapsulated amphotericin B in hamsters and

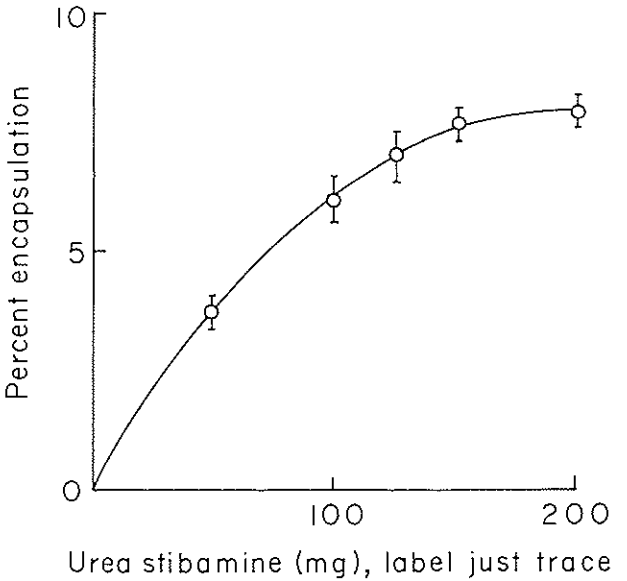

Figure 5. The relationship between percentage encapsulation and varying amounts of urea stibamine, with a trace of label used for entrapment within the liposomes. The composition and lipid concentration of the liposomes were constant throughout. The results are plotted as means ± SD ($N = 3$).

monkeys was reported by Berman *et al.* (1986). Successful use of liposomal amphotericin B in the treatment of drug-related visceral leishmaniasis has also been reported (Ahmad *et al.*, 1991; Davidson *et al.*, 1991). Croft, Davidson and Thornton (1991) used liposomal amphotericin B (AmBisome) in the treatment of patients suffering from multidrug-resistant visceral leishmaniasis. As part of a non-empirical approach to antileishmanial drug design and delivery, the lipoprotein-mediated antileishmanial chemotherapy was reported by Hart (1987). The idea for utilization of a specific receptor for low-density lipoprotein (LDL) and acetylated LDL on leishmania-infected macrophages was exploited *in vitro* to deliver the antileishmanial drug, Adriamycin, selectively (Hart, 1987). Tuftsin-coated Pentostam-encapsulated liposomes were reported to be used for antileishmanial activity (Guru *et al.*, 1989).

Our laboratory has used the other classical antimonial drug urea stibamine to probe experimental leishmaniasis in the hamster model (Das *et al.*, 1990). Taking advantage of the presence of mannosyl fucosyl receptors on the macrophage surface, mannose-bearing liposomal delivery was designed and planned to be used with entrapped urea stibamine for site-specific delivery (Das *et al.*, 1990). *Figure 5* shows the percentage encapsulation of urea stibamine as a function of drug concentration. The efficacies of urea stibamine – free, liposome-encapsulated and mannose-bearing liposome-encapsulated – have been compared using a 30-day infected hamster model (*Table 3*). The results demonstrate that liposome encapsulation of urea stibamine enhanced its effectiveness, an effect that was greater when mannose-bearing liposomes were used.

Very recently, different sugar-coated liposomal delivery systems were designed, either by incorporating plant glycosides or by grafting synthetic glycosides, and their specificity towards macrophages was tested *in vitro* using appropriate inhibitors (Medda *et al.*, 1993). The neoglycosylated liposomes so formed were also used as

Table 3. Effect of urea stibamine on a 30-day infected hamster model of *Leishmania donovani*

Treatment	Liver weight (g) (mean ± SD)	Spleen weight (mg) (mean ± SD)	Parasite load in the spleen (mean ± SD × 10^7)	% Suppression of parasite load in the spleen
No drug (control)	4.02 ± 0.32	267 ± 14	3.41 ± 0.34	–
Free drug	3.62 ± 0.28	238 ± 10	2.53 ± 0.56	26.0
Empty liposomes	3.24 ± 0.20	219 ± 16	2.89 ± 0.15	15.0
Empty mannose-bearing liposomes	3.22 ± 0.22	215 ± 19	2.67 ± 0.15	22.0
Liposome-encapsulated drug	2.46 ± 0.24	150 ± 12	1.54 ± 0.18[a]	55.0
Mannose-bearing liposome-encapsulated drug	2.09 ± 0.26	108 ± 12	0.97 ± 0.11[a]	72.0

Results are shown as mean ± SD ($N = 4$).
[a] $p < 0.001$ compared to no drug (control); the drug used was urea stibamine.

efficient ligands for the estimation of specific sugar receptor status of macrophages in health and in experimental leishmaniasis (Dutta, Bandyopadhyay and Basu, 1994). The efficacy and the toxicity of the drug, urea stabamine, were critically compared in a 60-day infected hamster model, using both glucose-bearing and mannose-bearing delivery systems (Medda *et al.*, 1993). The drug encapsulated in mannose-bearing delivery systems was found to be more efficient and less toxic than that encapsulated in the glucose-bearing systems. The non-antimonial classical drugs, e.g. pentamidine isothionate, and their analogues were also examined *in vitro* for antileishmanial activities (Mahato *et al.*, 1993; Nandi *et al.*, 1993) and their therapeutic efficacy and the resultant toxicity were critically analysed when encapsulated in mannose-bearing liposomes (Banerjee *et al.*, submitted). Moreover, hamycin, a polyene antibiotic, was examined in our laboratory for antileishmanial activity. When tested *in vivo* against artificial leishmaniasis in a hamster model, the liposomal hamycin and mannose-bearing liposomal hamycin were found to be more potent than the regular hamycin; the mannose-bearing liposomal hamycin being the most effective in reducing the spleen parasite load. Toxicity of the drug was reduced in the liposomal forms as judged from haemoglobin level and the level of specific enzymes related to normal liver function (*Table 4*) but was reduced further when sterol-rich liposomes were used as a delivery system (Banerjee, Bhaduri and Basu, 1994).

Table 4. Specific enzyme levels in sera of hamsters undergoing experimental leishmaniasis

Groups	Alkaline phosphatase[a]	Serum glutamate pyruvate transaminase (SGPT)[b]
Normal	8.2 ± 2.5	42.0 ± 6.0
Infected	10.2 ± 1.4	64.5 ± 18.9
Infected + free drug treated	17.6 ± 3.4	116.7 ± 21.8
Infected + liposome-intercalated drug treated	15.1 ± 3.3	103.5 ± 15.0
Infected + mannose-bearing liposome-intercalated drug treated	6.7 ± 3.0	72.5 ± 15.0

[a] μmol of *p*-nitrophenol released min^{-1} dl^{-1} serum.
[b] μmol of sodium pyruvate released min^{-1} l^{-1} serum.
Results are shown as mean ± SD ($N = 6$). Three animals per group were examined in two separate sets of experiments. The drug used was hamycin.

Besides liposomes, use of the non-ionic surfactant vesicles, 'niosomes', was proposed (Baillie *et al.*, 1986) and the therapeutic efficacy of the antimonial drug, sodium stibogluconate, encapsulated either in 'niosomes' or in liposomes, was compared *in vivo* against artificial leishmaniasis in hamsters and found to be equiactive (Hunter *et al.*, 1988). In our laboratory, 'niosomes' were prepared using Tween-20 and the therapeutic efficacy as well as the toxicity of the other antimonial drug, urea stibamine, were compared *in vivo* against artificial leishmanial infection (Medda and Basu, 1994). Although the drug toxicity at equivalent drug concentration was comparable in both the vesicular systems, the therapeutic efficacy of hamycin in the 'niosomal' form was found to be better than that in the liposomal form (*Table 5*). The non-ionic surfactants being cheaper, less toxic, biodegradable and non-immunogenic, the 'niosomes' might replace liposomes as drug carriers in the future.

Table 5. Effect of hamycin on a 60-day infected hamster model of *Leishmania donovani*

Treatment	10^{-7} x Parasite load in the spleen	Suppression of parasite load in the spleen (%)
No drug (control)	7.2 ± 0.8	–
Free drug	4.9 ± 0.4	32
Empty liposomes	6.5 ± 0.8	10
Liposomal drug	3.3 ± 0.3	54
Empty niosomes	6.2 ± 0.8	14
Niosomal drug	2.8 ± 0.2	65

Results are means ± SD ($N = 3$).
The same equivalent drug concentration was used for free, liposomal and niosomal treatments.

AS A CARRIER OF ANTIFUNGAL DRUGS

The fungal infections, e.g. aspergillosis and candidiasis, are very common among immunosuppressed patients. Such infections can be severe, sometimes causing death. Some polyene antibiotics, e.g. amphotericin B, hamycin and nystatin, are very toxic but are the only available drugs for combating the diseases related to fungal infections. Because of their highly toxic nature, the use of such drugs in high dosage, necessary for therapeutic efficacy, is restricted. Attempts to reduce drug toxicity but simultaneously to preserve therapeutic efficacy were made without success until liposomes were used as drug carriers for fungal disease treatment. Liposomes were reported to give a reduction of the toxicity of amphotericin B with complete retention of therapeutic efficacy, in a case of candidiasis (Lopez-Berestein *et al.*, 1983). Although some reports (Szoka, Milholland and Barza, 1987) on the toxicity and *in vitro* fungicidal activity of liposome-intercalated amphotericin B were published, until 1988 no systematic study on the toxicity and therapeutic efficacy of liposome-intercalated amphotericin B in an animal model was published. In 1989, while comparing the toxicity of free amphotericin B and liposomal amphotericin B in experimental aspergillosis in BALB/c mice, Ahmad, Sarkar and Bachhawat (1989) found a several-fold increase in LD_{50} of amphotericin B when associated in liposomes. Although inclusion of cholesterol in neutral liposomes drastically reduced the drug toxicity, no effect on the toxicity was shown when both positively and negatively charged liposomes were used as delivery systems (Ahmad, Sarkar and Bachhawat,

1990). Tuftsin-bearing liposomes with cholesterol were reported as a vehicle for amphotericin B in the treatment of experimental aspergillosis in mice. The drug efficacy was significantly increased by pretreating the animals with drug-free tuftsin-bearing liposomes, demonstrating that macrophage activation (possibly by tuftsin) can considerably enhance the therapeutic efficacy of antifungal drugs such as amphotericin B (Owais *et al.*, 1993). The relative toxicity and the therapeutic efficacy of the other polyene antibiotic, hamycin, intercalated in liposomes, were also tested against experimental aspergillosis in mice, although the toxicity study was concentrated only on the determination of LD_{50} of the drug intercalated in various liposomal formulations (Moonis, Ahmad and Bachhawat, 1992). The toxicity was found to be reduced when negatively charged liposomes, with increasing cholesterol composition, were used as the delivery systems (Moonis, Ahmad and Bachhawat, 1993b). Moreover, when hamycin was intercalated in mannose-bearing liposomes, the toxicity of the drug was further reduced, showing enhanced antifungal activity. The LD_{50} increased by almost twice with the inclusion of sterol, even in the neutral liposomal composition, from 1.4 (mg kg^{-1}) for SPC/DPPE-Man (molar ratio 9:1) liposomes to 2.8 (mg kg^{-1}) for SPC/Chol/DPPE-Man (molar ratio 4:5:1) liposomes (Moonis, Ahmad and Bachhawat, 1993a). Liposomal amphotericin B (AmBisome) was found to have a role in the treatment of patients with systemic fungal infections (Lopez-Berestein and Rosenblum, 1992) and in successful prophylactic use against fungal infections in bone marrow transplant recipients (Tollemar *et al.*, 1993).

AS A CARRIER OF DRUGS FOR VARIOUS OTHER DISORDERS

Alving *et al.* (1979) reported that liposomes containing neutral glycolipids with a terminal glucose or galactose, when injected intravenously, prevented the appearance of erythrocytic forms of malaria (*Plasmodium berghei*) in mice, even in the absence of antimalarial drugs. Very recently, chloroquine delivery to erythrocytes in *Plasmodium berghei* infected mice, using antibody-bearing liposomes as drug vehicles (Chandra, Agarwal and Gupta, 1991), has been reported.

In a number of storage diseases there is an inherited enzyme deficiency that leads to the accumulation of the relevant substrates. Thus treatment of such diseases with liposomes containing the appropriate enzymes seems feasible. Macrophages or fibroblasts, previously loaded with sucrose in their lysosomes, were exposed to invertase encapsulated in liposomes. Hydrolysis of sucrose to glucose and fructose occurred, and the sugars diffused out through the lysosomal membranes (Gregoriadis and Buckland, 1977). When dextran-loaded rats were treated with liposome-entrapped dextranase, a considerable loss of dextran was noted from the liver.

Attempts have been made to deliver uridine to the liver, using liposomes as carriers in model hepatitis (Mathias, Dorai and Bachhawat, 1977). The effect of modification of liposomal lipid composition on the regeneration of hepatitic liver revealed that the entrapped uridine was effective in the following order: asialoganglioside liposome > phosphatidic acid liposome > dicetylphosphate liposome.

Liposomes raise new possibilities for the oral therapeutic use of proteins, and probably other substances, which are otherwise not suitable for oral administration. Attempts to introduce liposome-encapsulated insulin into the blood circulation by the oral route have met with considerable success (Patel and Ryman, 1976). Insulin

entrapped in egg phosphatidyl choline/cholesterol liposomes was less effective than in liposomes containing dipalmitoyl lecithin/cholesterol.

Application of liposomes has been extended to the treatment of rheumatoid arthritis, and it was found that cortisol palmitate-containing liposomes are stable in rheumatoid synovial fluid at 37°C. The level of the liposomal steroid in the tissue was inversely related to the chronicity of inflammation; the degree and the duration of its anti-inflammatory activity was greatest in the initial acute phase of the inflammation. These promising results have led to a preliminary clinical trial in patients with rheumatoid arthritis (de Silva *et al.*, 1979); intra-articular treatment with liposomal cortisol palmitate resulted in an improvement in both subjective and objective indices of inflammation in the affected joint. Very recently, liposome-entrapped methotrexate, injected intra-articularly, has been reported to be used in the treatment of antigen-induced arthritis in rabbits (Foong and Green, 1993). For treatment of various brain diseases, attempts have been made to target liposomes to rat brain via the internal carotid artery (Sakamoto and Ido, 1993).

Liposomes bearing asiaticoside, a plant glycoside, have been reported as used successfully *in vitro* against *Mycobacterium leprae* and *M. tuberculosis* (Medda *et al.*, submitted). Liposomes bearing tuftsin as rifampicin vehicles have been used *in vivo* in the treatment of tuberculosis in mice (Agarwal *et al.*, 1994). There have been reports on the modification of reticuloendothelial functions by muramyl dipeptide-encapsulated liposomes in jaundiced rats treated with biliary decomposition (Ding *et al.*, 1993). On the other hand, liposome-encapsulated muramyl tripeptide phosphatidyl ethanolamide was used successfully in the treatment of experimental *Klebsiella* pneumonial infection (Melissen *et al.*, 1992). Liposomes seem particularly appropriate for delivery of drugs to the lungs as they can be prepared from materials endogenous to the lung as components of lung surfactant (Schreier, Gonzalez-Rothi and Stecenko, 1993). In fact, liposomes were administered to the lung both as a means of delivering phospholipids to the alveolar surface (Gonzalez-Rothi *et al.*, 1991) and to modulate the duration of active or pulmonary absorption of pharmacologically active agents (Taylor and Newton, 1992; Taylor and Farr, 1993). In the earliest studies of pulmonary administration of liposomes for therapeutic purposes, liposomes were given as an exogeneous lung surfactant in the treatment of the neonatal respiratory distress syndrome (Ivey, Roth and Kattwinkel, 1977). Subsequently a range of liposome-associated drugs were administered to the lungs of humans and animals. These include cytotoxic drugs (McCullough and Juliano, 1979), antiasthma drugs (McCalden, Abra and Mihalko, 1989; Taylor *et al.*, 1989), antimicrobial compounds (Debs *et al.*, 1987; Gilbert *et al.*, 1988; Mihalko, Schrier and Abra, 1988) and drugs delivered for systemic action (Mihalko, Schrier and Abra, 1988; Shek *et al.*, 1988; Meisner, Pringle and Mezei, 1989).

Toxicity of liposomes

The idea of using liposomes as a convenient drug carrier might be jeopardized by their toxic effects, if any. Toxic effects could originate from liposomes themselves, from the active agents they contain or by synergistic action of the carrier and its contents. Because of the versatile nature of the use of liposomes in a drug delivery mode, acceptance of a liposomal preparation for clinical use will eventually depend on

whether the overall effect is favourable to the patient. Adverse reaction may occur as a result of certain structural features; the lipid component of liposomes (Gregoriadis, 1980b); altered liposomal drug distribution; and the patient's reaction to the liposomal carrier. There have been reports that liposomes above a certain size may block lung capillaries (Rahman *et al.*, 1974) and that a positively charged surface may bind circulating α_2-macroglobulin (Black and Gregoriadis, 1976) upsetting coagulation processes. But, a patient when injected intravenously with about 100 mg lipid in the form of negatively charged liposomes showed no decrease in α_2-macroglobulin levels for up to 3 days after treatment. Besides several other minor reports, another potential liposomal toxicity was observed by Chen and Keenan (1979) who noticed that egg phosphatidyl choline liposomes inhibit lymphocyte activation by attracting, and thus reducing, cellular cholesterol.

Among the reports on toxicity as a result of lipid components, a direct *in vivo* toxicity in the form of epileptic seizures or cerebral tissue necrosis has been noticed after the intracerebral injection of large amounts of liposomes containing either dicetyl phosphate or stearylamine. But, at comparable doses, the liposomes containing stearylamine were found to be non-toxic by other groups (Mayhew *et al.*, 1976; Kimbelberg and Mayhew, 1978) for long-term intraperitoneal treatment. Systematic studies on liposomal toxicity were carried out in rats and beagle dogs who received intravenously, on a daily basis, sonicated soya phosphatidyl choline for several weeks. The results in behavioural changes, urine biochemistry and histology were negative (Gregoriadis, 1978). Repeatedly, clinical experience with a small number of cancer patients injected with liposomes, composed of egg lecithin, cholesterol and phosphatidic acid (molar ratio 7:2:1), has been uneventful (Gregoriadis *et al.*, 1974). Thus, the toxicity of liposomes should not be a great obstacle in opening new avenues for their chemotherapeutic use.

The chronic inhalation of soya phosphatidyl choline (SPC) liposome in an attempt to target drugs to the lung, specifically to pulmonary alveolar macrophages (AM), showed no histological changes in the lung or untoward effects on the general health or survival of animals. The properties of alveolar macrophages such as phagocytic function, intracellular killing and fatty acid composition were not affected. Transmission electron microscopy and morphometry (computerized image analysis) of alveolar macrophages showed no alterations as a result of the treatment. This finding validates the concept of alveolar macrophage-directed delivery of liposome-encapsulated agents to the lung via inhalation (Myers *et al.*, 1993). These data confirm and extend prior findings regarding the functional and morphological interactions of liposomes with alveolar macrophages *in vitro* (Gonzalez-Rothi *et al.*, 1991).

Summary and perspectives

The popularity of liposome research is based on two attractive features of the system, namely its versatility and its similarity to natural membranes. Liposomes can vary widely in size, composition and surface characteristics, and can be made to accommodate a remarkable array of pharmacologically active substances, including antitumour and antimicrobial drugs, enzymes, hormones and vaccines. For site-specific delivery, specialized liposomes are prepared nowadays, and this has led to considerable interest in the possibility of therapeutic use of liposomes. But

for liposomes, crossing barriers to the extravascular space still remains a problem. The liposomes can cross the membrane, but because of their limitations in minimal size, it is obvious that not all membranes will be crossed by even the smallest liposomes. Until drug specificity is achieved, liposomes, in spite of their limitations, should play an important role in optimizing drug action (Gregoriadis, 1980a). However, reported studies of liposome-mediated therapy are as yet too fragmentary to determine whether current investigations will lead to useful methods of treatment. Probably our incubation period for taking a positive approach towards their application in chemotherapy is still not over.

However, at present, with a much clearer understanding of liposome technology's limitations (as well as a better technical understanding of design and manufacture of these phospholipid-based microspheres) companies are putting an impressive number of liposome-encapsulated drugs into human trials, initiating a technology move from the laboratory into the clinic. In fact, more than 15 liposome products were scheduled to be in US clinics during 1988 (*Table 6*). The companies (e.g. The Liposome Company, Princeton, NJ and Vestar, Pasadana, CA) believe that there is potential in the use of liposomes for critical-care patients with cancer and infectious diseases. Vester has been able to increase the shelf-life of its liposomes from weeks to years and can now manufacture 'targeted' liposomes that congregate at tumours without having to rely on the body's macrophages to escort them. Vester's targeted daunorubicin formulation is already scheduled to go into clinical trials for the treatment of cancer, fungal infections and post-surgical healing. Technology Unlimited, Wooster, OH, had two topical liposome-based preparations in the clinic already in 1988. They have been able to coat the exterior of liposomes with sialic acid residues, thereby allowing the microspheres to avoid the reticuloendothelial system, thus raising the possibility of using these liposomes, perhaps with the help of covalently attached monoclonal antibodies, to target areas outside this system (Klausner, 1988).

Table 6. Liposome products likely to be used in human chemotherapy (after Klausner, 1988)

Liposome products	Producers	Applications
Doxorubicin	The Liposome Co.	Cancer
Daunorubicin	Vester	Cancer
Amphotericin B	Squibb/The Liposome Co.	Fungal infections
Amphotericin B	Lypho Med/Vester	Fungal infections
Insulin	Technology Unlimited	Diabetes
Miconazole	Ortho/The Liposome Co.	Vaginal infections
Gentamicin	The Liposome Co.	Gram-negative infections
Anti-inflammatory drug (non-steroidal)	Ethicon/Vester	Post-surgical healing

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