JPP Journal of Pharmacy and Pharmacology

JPP 2007, 59: 469–483

Received August 19, 2006
Accepted January 22, 2007
DOI 10.1211/jpp.59.4.0001
ISSN 0022-3573

Review Article

pH-Sensitive liposomes—principle and application in cancer therapy

H. Karanth and R. S. R. Murthy

Abstract

The purpose of this review is to provide an insight into the different aspects of pH-sensitive liposomes. The review consists of 6 parts: the first introduces different types of medications made in liposomal drug delivery to overcome several drawbacks; the second elaborates the development of pH-sensitive liposomes; the third explains diverse mechanisms associated with the endocytosis and the cytosolic delivery of the drugs through pH-sensitive liposomes; the fourth describes the role and importance of pH-sensitive lipid dioleoylphosphatidylethanolamine (DOPE) and research carried on it; the fifth explains successful strategies used so far using the mechanism of pH sensitivity for fusogenic activity; the final part is a compilation of research that has played a significant role in emphasizing the success of pH-sensitive liposomes as an efficient drug delivery system in the treatment of malignant tumours. pH-Sensitive liposomes have been extensively studied in recent years as an amicable alternative to conventional liposomes in effectively targeting and accumulating anti-cancer drugs in tumours. This research suggests that pH-sensitive liposomes are more efficient in delivering anti-cancer drugs than conventional and long-circulating liposomes due to their fusogenic property. Research focused on the clinical and therapeutic side of pH-sensitive liposomes would enable their commercial utility in cancer treatment.

Introduction

Since the revolutionary discovery of Alec Bangham roughly 40 years ago that phospholipids in aqueous systems can form closed bilayered structures, liposomes have moved a long way from being just another interesting object of biophysical research to become a pharmaceutical carrier of choice for numerous practical applications. Recent studies show that the selective delivery of the anti-cancer agent doxorubicin in polyethylene glycol (PEG) liposomes for the treatment of solid tumours in patients with breast-carcinoma metastases has resulted in a subsequent improvement in survival (Symon et al 1999; Perez et al 2002; O'Shaughnessy 2003). A combination therapy comprising liposomal doxorubicin and paclitaxel (Schwonzen et al 2000) or Caelyx (Schering-Plough) (doxorubicin in polyethylene glycol liposomes) and carboplatin (Goncalves et al 2003) has been used to target breast-carcinoma metastases. Caelyx is also in Phase II clinical trials for patients with squamous cell cancer of the head and neck (Harrington et al 2001) and for ovarian cancer (Johnston & Gore 2001). Indications, other than cancer, targeted by liposomal formulations include amphotericin B for the treatment of visceral leishmaniasis (Sundar et al 2003) and long-acting analgesia with liposomal bupivacaine in healthy subjects (Grant et al 2004). The increasing number of liposomal formulations in clinical trials, as well as on the market today, seems to show that these formulations have a very promising future.

Development in liposomal drug delivery

The major drawbacks of using liposomes are their fast elimination from the blood and the capture of liposomal preparations by the cells of the reticulo-endothelial system (RES), primarily in the liver. Several strategies have aimed to reduce or overcome these problems.

Long-circulating liposomes

Different methods have been reported to achieve long circulation of liposomes in-vivo, including coating the liposome surface with inert biocompatible polymers (such as PEG)

New Drug Delivery Systems Laboratory, Pharmacy Department, Donors' Plaza, Opp. University Main Office, M S University of Baroda, Vadodara-390 002, India

H. Karanth, R. S. R. Murthy

Correspondence: R. S. R. Murthy, New Drug Delivery Systems Laboratory, Pharmacy Department, Donors' Plaza, Opp. University Main Office, M S University of Baroda, Vadodara-390 002, India. E-mail: m_rsr@rediffmail.com

that form a protective layer over the liposome surface and delay the liposome recognition by opsonins and therefore subsequent clearance of liposomes (Klibanov et al 1990; Blume & Cevc 1993). Long-circulating liposomes are widely used in biomedical in-vitro and in-vivo studies and they have also found their way into clinical practice (Gabizon 2001). A significant feature of protective polymers is their flexibility, which allows a relatively small number of surface-grafted polymer molecules to create an impermeable layer over the liposome surface (Torchilin et al 1994; Torchilin & Trubetskoy 1995). Long-circulating liposomes exhibit dose-independent, non-saturable, log-linear kinetics and increased bioavailability (Allen & Hansen 1991).

Modern research on PEGylated liposomes focuses on attaching PEG in a removable manner to facilitate liposome capture by cells. After PEG-liposomes collect at the target site, through the enhanced permeability and retention (EPR) effect (Maeda et al 2001), the PEG coating is detached under the action of local pathological conditions (acidic pH in tumours).

Although PEG remains the gold standard for the steric protection of liposomes, attempts were made to prepare long-circulating liposomes using poly[*N*-(2-hydroxypropyl) methacrylamide)] (Whiteman et al 2001), poly-*N*-vinylpyrrolidones (Torchilin et al 2001b), L-amino-acid-based biodegradable polymer–lipid conjugates (Metselaar et al 2003) and polyvinyl alcohol (Takeuchi et al 2001). Studies of the relative roles of the liposome charge and protective polymer molecular mass revealed that opsonins with different molecular masses might be involved in the clearance of liposomes containing differently charged lipids (Levchenko et al 2002).

Immunoliposomes

To increase liposomal drug accumulation in the preferred tissues and organs, the use of targeted liposomes with surface-attached ligands capable of recognizing and binding to cells of interest has been tried. Immunoglobulins (Ig) of the IgG class, and their fragments, are the most widely used targeting moieties for liposomes, which can be attached to liposomes without affecting liposomal integrity or the antibody properties, by covalent binding to the liposome surface or by hydrophobic inclusion into the liposomal membrane after alteration with hydrophobic residues (Torchilin 1985).

Despite improvements in targeting efficacy, the greater part of immunoliposomes accumulate in the liver as an outcome of insufficient time for the interaction between the target and targeted liposome; better target accumulation can be anticipated if liposomes can be made to remain in the circulation for a long time. Combination of the properties of long-circulating liposomes and immunoliposomes in one preparation to overcome certain drawbacks of immunoliposomes have been reported (Torchilin et al 1992; Blume et al 1993; Abra et al 2002). Experiments have been initially performed by simple co-immobilization of an antibody and PEG on the surface of the same liposome, although the protective polymer can create steric hindrances for target recognition with the targeting moiety (Torchilin et al 1992). To attain better selectivity of PEG-coated liposomes, it is advantageous to attach the targeting ligand using a PEG spacer arm so that the ligand is extended outside of the dense PEG brush, which reduces steric hindrance of binding to the target. At present, various advanced technologies are used, and the targeting moiety is usually attached above the protecting polymer layer by coupling it with the distal water-exposed terminus of the activated liposome-grafted polymer molecule (Blume et al 1993; Torchilin et al 2001a).

Antibody-mediated liposome targeting. The greater part of research in this area relates to cancer targeting, which utilizes a variety of antibodies. Internalizing antibodies are required to achieve a much-improved therapeutic efficacy with antibody-targeted liposomal drugs, as shown with B-lymphoma cells and internalizable epitopes (CD19) (Sapra & Allen 2002). An attractive idea was developed to target HER2-overexpressing tumours using anti-HER2 liposomes (Park et al 2001). The antibody CC52, which is directed against rat colon adenocarcinoma CC531 lines, was tagged to PEGylated liposomes and resulted in the restricted accumulation of liposomes in a rat model of metastatic CC531 (Kamps et al 2000). Nucleosome-specific antibodies capable of recognizing various tumour cells via tumour-cell-surface-bound nucleosomes enhanced doxorubicin (Alza) targeting to tumour cells and improved its cytotoxicity (Lukyanov et al 2004). Immunoliposomes containing the novel antineoplastic drug fenretinide, and targeting the ganglioside GD2, induced apoptosis in neuroblastoma and melanoma cell lines, and demonstrated strong anti-neuroblastoma activity both in-vitro and in-vivo in mice (Raffaghello et al 2003). The grouping of immunoliposome and endosome-disruptive peptide enhances the cytosolic delivery of the liposomal drug, increases cytotoxicity and opens up new avenues for developing targeted liposomal systems. This was revealed with the diphtheria toxin A chain, which was pooled with pH-dependent fusogenic peptide diINF-7 into integrated liposomes specifically targeted to ovarian carcinoma (Mastrobattista et al 2002).

New ligands for targeting liposomes

Folate-mediated liposome targeting. Folate-modified liposomes targeting to tumours represents a widespread approach, as folate receptors (FRs) are recurrently over-expressed in a range of tumour cells. Initial studies established the possibility of delivering macromolecules (Leamon et al 1991) and then liposomes (Lee & Low 1994) into living cells using FR endocytosis, which could avoid multidrug resistance; interest in folate-targeted drug delivery by liposomes grew rapidly (Lu & Low 2002a, b; Gabizon et al 2004). Delivery of liposomal daunorubicin (Ni et al 2002), as well as doxorubicin (Pan et al 2003), to various tumour cells through FRs demonstrated increased cytotoxicity.

Recently, folate-modified doxorubicin-loaded liposomes combined with the induction of FRs using all-*trans* retinoic acid was applied for the treatment of acute myelogenous leukaemia (Pan et al 2002). Folate-targeted liposomes have been projected as delivery vehicles for boron neutron capture therapy (Stephenson et al 2003) and also used for targeting tumours with haptens for tumour immunotherapy (Lu & Low 2002a). In the field of gene therapy, folate-targeted liposomes have been used for gene targeting to tumour cells (Reddy et al

2002) as well as for targeting tumours with antisense oligonucleotides (Leamon et al 2003).

Transferrin-mediated liposome targeting. Transferrin (Tf) receptors (TfRs) are over-expressed on the surface of many tumour cells. Consequently, antibodies against TfRs, as well as Tf itself, are suitable ligands for liposome targeting to tumours and entry into tumour cells (Hatakeyama et al 2004). Modern studies have focused on the coupling of Tf to PEG on PEGylated liposomes to unite longevity and targetability for drug delivery into solid tumours (Ishida et al 2001a). An identical approach was effective in the delivery of agents for photo-dynamic therapy, including hypericin, into tumours (Derycke & De Witte 2002; Gijsens et al 2002), and for intracellular delivery of cisplatin into gastric cancer (Iinuma et al 2002).

Increased binding and toxicity were demonstrated against C6 glioma cells (Eavarone et al 2000) by Tf-coupled doxorubicin-loaded liposomes. The increase in the expression of TfRs was also revealed in post-ischaemic cerebral endothelium, which was used to deliver Tf-modified PEG liposomes to post-ischaemic brain in rats (Omori et al 2003). Tf (Joshee et al 2002), as well as anti-TfR antibodies (Xu et al 2002; Tan et al 2003), has also been used to support gene delivery into cells by cationic liposomes. Tf-mediated liposome delivery was also used for brain targeting effectively.

Immunoliposomes containing the OX26 monoclonal antibody, which is aimed to rat TfR, were found to assemble on brain microvascular endothelium (Huwyler et al 1996).

pH-Sensitive liposomes

Of late, the focus of research in the area of liposomes has been the development of strategies to increase the ability of liposomes to mediate intracellular delivery of biologically active molecules. This resulted in the emergence of a modified form of liposomes called pH-sensitive liposomes. These liposomes are stable at physiological pH (pH 7.4) but undergo destabilization and acquire fusogenic properties under acidic conditions, thus leading to the release of their aqueous contents.

The theory of pH-sensitive liposomes emerged from the reality that certain enveloped viruses developed strategies to benefit from the acidification of the endosomal lumen to infect cells, as well as from the observation that some pathological tissues (tumours, inflamed and infected areas) exhibit an acidic environment as compared with normal tissues (Torchilin et al 1993). Different classes of pH-sensitive liposomes have been proposed in the literature, based on the mechanism of triggering pH sensitivity (Torchilin et al 1993; Drummond et al 2000). The most commonly established hypothesis involves the blend of phosphatidylethanolamine (PE) or its derivatives with compounds containing an acidic group (e.g. carboxylic group) that acts as a stabilizer at neutral pH (Ellens et al 1985; Liu & Huang 1989; Duzgunes et al 2001; Torchilin 2005). Contemporary studies describe the use of novel pH-sensitive lipids, synthetic fusogenic peptides/proteins either encapsulated (Mastrobattista et al 2002; Provoda et al 2003) or included in the lipid bilayer (Parente et al 1988; Ishiguro et al 1996; Bailey et al 1997; Nir et al 1999; Turk et al 2002), and attachment of pH-sensitive polymers with liposomes (Leroux et al 2001; Mizoue et al 2002: Roux et al 2002a, 2004; Simoes et al 2004).

Liposomes need to be stable in biological fluids and establish long circulation times when administered intravenously, enabling them to reach target cells (such as tumor cells) and mediate cytoplasmic delivery (Simoes et al 2004). Usage of lipids with high transition temperatures, such as distearoylphosphatidylcholine (DSPC), hydrogenated soya PC (HSPC), the incorporation of cholesterol (Chol) and lipid conjugates, such as phosphatidylethanolamine–poly(ethylene glycol) (PE–PEG), has led to a considerable decrease in leakage of the encapsulated drugs throughout the circulation or in the extracellular environment. These lipids also diminish non-specific interactions between the liposomes and serum proteins (opsonins), thus avoiding liposome clearance by the cells of the RES. Furthermore, the use of liposomes of size < 150 nm can contribute to the increase of the circulation time (Gabizon & Papahadjopoulos 1988; Woodle 1995; Zalipsky 1995). Antisense oligonucleotides can be delivered into cells by anionic pH-sensitive phosphatidylethanolamine (PE)-containing liposomes that are stable in blood but undergo phase transition at endosomal acidic pH (Fattal et al 2004). The combination of liposome pH sensitivity and specificity of ligand targeting for cytosolic drug delivery using decreased endosomal pH values has been described for both folate and Tf-targeted liposomes (Turk et al 2002; Shi et al 2002; Kakudo et al 2004).

Mechanisms of intracellular delivery mediated by pH-sensitive liposomes

Biophysical properties underlying the pH sensitivity of liposomes

In contrast to the majority of phospholipids, PE presents a minimally hydrated and small head group that occupies a lower volume as compared with the respective hydrocarbon chains, exhibiting a cone shape (as opposed to the cylinder shape of bilayer stabilizing phospholipids), thus obstructing the formation of a lamellar phase (Cullis & de Kruijff 1979; Seddon et al 1983). The cone shape of PE molecules favours the formation of strong intermolecular interactions between the amine and phosphate groups of the polar head groups, illustrating the strong tendency of these molecules to acquire the inverted hexagonal phase above the phase transition temperature (T_H) (for dioleoylphosphatidylethanolamine (DOPE) the T_H is 10°C) (Figure 1).

Intercalation of amphiphilic molecules containing a protonatable acidic group (negatively charged at physiological pH) between PE molecules favours electrostatic repulsion and allows the formation of bilayer structures, which leads to liposome formation at physiological pH and temperature (Lai et al 1985; Duzgunes et al 1985). This successive approach constitutes the basis for the biophysical mechanisms underlying the pH sensitivity exhibited by PE-containing liposomes. Although stable liposomes are formed at physiological pH, acidification triggers protonation of the carboxylic groups of the amphiphiles, reducing their stabilizing effect, leading to destabilization of liposomes, as under these conditions PE molecules revert into their inverted hexagonal phase

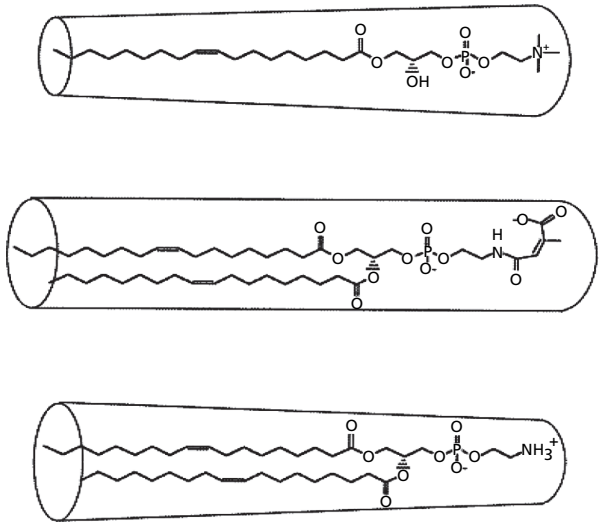

Figure 1 Molecular shapes of lipids. Lipids with a single acyl or alkyl chain, such as lysolipids and detergents, have a small molecular cross-sectional area in their hydrophobic acyl chains relative to their hydrophilic head groups, giving the lipid a conical shape (top). Most naturally occurring phospholipids, such as phosphatidylcholine or sphingomyelin, or N-acylated phosphatidylethanolamines (middle) have an approximately equivalent cross sectional area in the head group and acyl chains, giving a cylindrical shape. Lipids such as unsaturated phosphatidylethanolamines (bottom) have a weakly hydrated head group and thus a small molecular area in the head group relative to the acyl chains. Lipids will form structures so as to maximize the interaction of water with both itself and hydrophilic groups on the lipids while excluding water from hydrophobic areas (the hydrophobic effect). The types of structures formed by lipids are greatly influenced by their molecular shapes. (This figure was reproduced with permission from Leroux (2000) and Elsevier).

(Torchilin et al 1993; Lasic 1998). The selection of the amphiphilic stabilizers, as well as its molar percentage with respect to the PE content, is enforced by the desired properties of the liposomes, together with the extent of cellular internalization, the fusogenic ability, pH sensitivity and stability in biological fluids. The aforementioned properties determine the liposome efficacy to mediate cytoplasmic delivery of the encapsulated molecules (Torchilin et al 1993; Chu & Szoka 1994; Drummond et al 2000).

Binding and cell internalization

It has been reported that pH-sensitive liposomes are internalized more effectively than non-pH-sensitive formulations (Schroit et al 1986; Chu et al 1990). This internalization has been attributed to the tendency of PE-containing liposomes to form aggregates, owing to the poor hydration of its head group, which can explain their high affinity to adhere to cell membranes (Chu & Szoka 1994; Collins 1995).

Different strategies have been explored based on receptor-mediated targeting through specific ligands coupled to the liposome surface, in an attempt to further improve their binding and cellular internalization. pH-Sensitive liposomes composed of DOPE/oleic acid (OA) (4:2) or DOPE/OA/Chol (4:2:4) and targeted by the anti-H-2K^k antibody, showed a higher efficacy in mediating cytoplasmic delivery of their aqueous contents than the same formulations missing the antibody (Connor & Huang 1986; Collins & Huang 1987; Wang & Huang 1989). These results showed that the extent of liposome internalization is a vital step in the process of intracellular delivery and that receptor-mediated endocytosis is more effective than non-specific endocytosis. These observations suggest that fusion or destabilization of liposomes induced by acidification of the endosomal lumen represents the most important stage in the process of intracellular delivery.

Figure 2 demonstrates the main steps involved in the internalization and intracellular delivery mediated by pH-sensitive liposomes. After binding to cells, the liposomes are internalized by means of the endocytotic pathway, with or without the involvement of clathrin-coated vesicles. Liposomes will be retained in early endosomes, independent of the internalization process, which mature into late endosomes. The capability of pH-sensitive liposomes lies in their potential to undergo destabilization at this stage, preventing their degradation at the lysosomal level, and therefore increasing entry to the cytosolic or nuclear targets (Collins 1995; Yoshimura et al 1995).

Destabilization of liposomes at the endosomal level

Investigations concerning the incubation of cells with lysosomotropic agents (e.g. ammonium chloride or chloroquine, which prevent endosome acidification) reveal that the efficacy of pH-sensitive liposomes depends on the drop in pH upon endosome maturation. Besides, kinetic studies have shown that liposomes composed of DOPE/OA, DOPE/palmitoylhomocysteine (PHC) or DOPE/dipalmitoylsuccinylglycerol (DSPG) (Collins et al 1989) or of DOPE/cholesteryl hemisuccinate (CHEMS) (Collins et al 1992) release their contents into the cytoplasm over a period of time that ranges from 5 to 15 min upon their incubation with the cells, thus signifying that cytoplasmic delivery occurs from early and late endosomes.

Three hypothetical mechanisms have been proposed (Figure 2): firstly, destabilization of pH-sensitive liposomes triggers the destabilization of the endosomal membrane, presumably through pore formation, leading to cytoplasmic delivery of their contents; secondly, upon liposome destabilization, the encapsulated molecules diffuse to the cytoplasm through the endosomal membrane; thirdly, fusion between the liposome and the endosomal membranes leads to cytoplasmic delivery of their contents (Collins 1995; Ropert et al 1995). The fusogenic properties of PE associated with its trend to form an inverted hexagonal phase under certain conditions suggest that the first and second hypotheses are the most acceptable.

At low pH (5.0) aggregation, release of contents and lipid intermixing are observed with DOPE/CHEMS liposomes, while no intermixing of aqueous contents takes place (Ellens et al 1985). However, these liposomes are efficient in delivering their encapsulated contents into cultured cells (Chu et al 1990). Out of the involved mechanisms, the efficacy of cytoplasmic delivery mediated by pH-sensitive liposomes is drastically reduced upon increase of the molecular weight of the encapsulated molecules. Investigations performed with

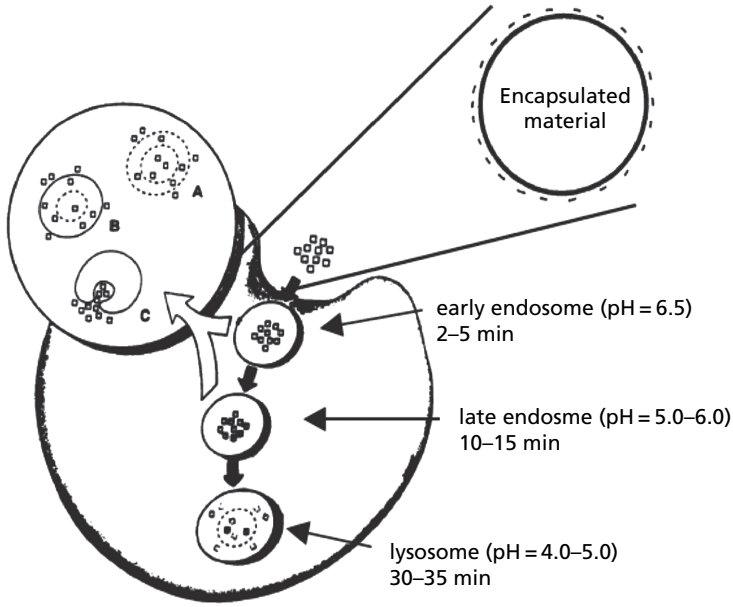

Figure 2 Hypothetical mechanisms of internalization and intracellular delivery of pH-sensitive liposomes. (This figure was reproduced with permission from Simoes et al (2004) and Elsevier).

high-molecular-weight proteins (e.g. DTA and BSA) show that only 0.01–10% of the molecules are released into the cytoplasm, in contrast to essentially 100% release observed with low-molecular-weight fluorescent probes like calcein (Chu et al 1990).

Role of DOPE in pH-sensitive liposomes

DOPE liposomes and mechanisms of cytosolic delivery

Liposomes composed of DOPE/phosphatidylglycerol (PG), DOPE/phosphatidylserine (PS), DOPE/PC and DOPE/CHEMS were incubated under very acidic conditions. Among these formulations, DOPE/CHEMS liposomes were those with the highest extent of cell association, while not exhibiting any pH sensitivity (Simoes et al 2001). These results suggest that the processes underlying the intracellular efficacy of the different DOPE-containing liposomes involve more complicated mechanisms than the mere decrease of the endosomal pH. The combination of these results with those reported formerly (Slepushkin et al 1997) indicates that the presence of DOPE is the vital factor determining the ability of such liposomes to undergo destabilization upon acidification of the endosomes. Moreover, in those studies non-pH-sensitive liposomes composed of DSPC/CHEMS/DSPE–PEG were the only ones where a correlation between a lack of pH sensitivity in buffer and failure to mediate intracellular delivery was observed. Interestingly, studies on fusion between endocytotic vesicles (isolated from reticulocytes) and liposomes with different compositions showed that the presence of PE, when compared with other phospholipids, namely PC, is essential to the fusion process (Vidal & Hoekstra 1995). This exclusive effect of PE or DOPE to promote liposome–endosome interactions can be explained by the low hydration of its polar head group as compared with the significant repulsive forces associated with the hydration layer of PC or DSPC polar head groups (Schroit et al 1986). Hence, the presence of DOPE enhances the hydrophobicity of the liposomal membrane, thus supporting dynamically favorable interactions between lipid bilayers. Moreover, DOPE tends to assume a hexagonal inverted phase (H_{II}) leading to the formation of non-lamellar structures (Litzinger & Huang 1992). This may signify a key element to trigger endosomal destabilization, thus leading to cytoplasmic delivery of their contents. This idea partially explains observations on the striking similarity of the efficacy of intracellular delivery mediated by all DOPE-containing formulations. The treatment of endocytotic vesicles with trypsin strongly reduced their interaction with PE-containing liposomes (Vidal & Hoekstra 1995), thus providing proof that endosome-associated proteins play a major role in this process. Based on these investigations, it was proved that such proteins may not only be involved in the process of membrane fusion, but also promote liposome aggregation, which favours their destabilization. Similar results were obtained in lipid mixing studies involving pH-sensitive liposomes and human erythrocyte ghosts, where a decrease in the extent of lipid mixing was noticed by cleaving the sialic acid residues of the glycocalyx (Chu et al 1990). A better understanding of the mechanisms underlying liposome–cell interactions was obtained from the results of studies with agents that interfere with the endocytotic pathway. A drastic reduction of the efficacy of intracellular delivery was observed for all the formulations tested when their internalization was inhibited (using a mixture of antimycin A, sodium fluoride and sodium azide). This indicates that such liposomes utilize the endocytotic pathway to promote the intracellular release of their contents. In contrast, the strong inhibition observed for the calcein/

rhodamine fluorescence ratio when the cells were treated with lysosomotropic agents demonstrates clearly that acidification of the endocytotic vesicles is important to the intracellular delivery mediated by the liposomes (Slepushkin et al 1997; Simoes et al 2001). Despite the similarity of the efficacy of intracellular delivery assessed in terms of calcein release, observed among the different liposomes tested, it should be noted that such findings cannot be extrapolated directly to other types of encapsulated molecules, particularly to those with high molecular weights (Chu et al 1990). Additionally, it is also reported that the ability to mediate intracellular delivery of molecules with relatively high molecular weight (e.g. antisense oligonucleotides) is considerably higher for liposomes composed of DOPE/CHEMS compared with other DOPE-containing liposomes (Duzgunes et al 2001). Formulations containing DOPE/PG, DOPE/PS and DOPE/PC were shown not to be pH sensitive in buffer and proved to be less efficient in releasing large molecules into the cytoplasm.

DOPE in long-circulating pH-sensitive liposomes

Destabilization of DOPE/OA liposomes was extensively observed upon their incubation with 90% human plasma at physiological pH (Liu & Huang 1994). Extraction of OA by serum albumin from the liposomes was considered to be responsible for this destabilization process. However, this effect was shown to be dependent on the size of the liposomes (Drummond et al 1999), since liposomes with an average size < 200 nm were more stable in serum than larger liposomes, while the opposite was observed when serum was substituted with phosphate-buffered saline (PBS). The stabilizing effect evolves from the fact that the high membrane curvature of the liposomes of small size may favour protein incorporation, like apoprotein A1 of the high-density lipoproteins (HDL), to replace the extracted OA (Liu & Huang 1994). This increase in liposome stability leads to significant reduction in pH sensitivity, thus constituting a constraint for their appropriate in-vivo use. Different strategies considered for improving the biostability of pH-sensitive liposomes have been explained, including the inclusion of a third component to provide stability to the lipid bilayer. Addition of Chol in DOPE/OA liposome formulations resulted in a considerable increase in plasma stability, without decreasing their pH sensitivity (Liu & Huang 1989). The utilization of other amphiphilic stabilizers (such as Chol derivatives—CHEMS, or lipids with double acyl chains—DPSG), which were shown to be resistant to extraction by albumin, resulted in the formation of liposomes exhibiting higher stability in biological fluids, while maintaining their pH sensitivity (Collins et al 1990; Chu et al 1995). The in-vivo efficacy of pH-sensitive liposomes depends strongly on the interactions with serum components (opsonins) that influence their pharmacokinetics and biodistribution, apart from their limitations related to stability and changes in pH sensitivity. Whereas the number of studies describing the in-vivo use of pH-sensitive liposomes is limited, the acceptance is that upon their intravenous administration these liposomes are cleared quickly from blood circulation, accumulating in the liver and spleen (Connor & Huang 1986; Liu & Huang 1990; Torchilin et al 1993; Slepushkin et al 1997). On the contrary, the tendency of pH-sensitive liposomes to aggregate in the presence of biological fluids may justify their accumulation in the lungs (Connor & Huang 1986). Even though the fact that pH-sensitive liposomes exhibit a higher affinity to macrophages than non-pH-sensitive liposomes (Torchilin et al 1993), their pharmacokinetics and biodistribution pattern are basically the same (Allen 1992). Studies using pH-sensitive immunoliposomes (DOPE/OA) established in-vivo that the presence of an antibody at the liposomal surface neither affects their pharmacokinetics (namely the blood clearance rate) nor their biodistribution (Wang & Huang 1987). pH-Sensitivity and prolonged circulation time are highly desirable for the delivery of therapeutic macromolecules, such as nucleic acids, to cells. In this regard, pH-sensitive liposomes that can circulate in the blood for long periods and deliver encapsulated macromolecules to target cells may be useful. In-vivo studies with the ganglioside GM_1 were shown to give relatively prolonged residence in the circulation to pH-sensitive liposomes composed of DOPE and DPSG (Liu & Huang 1990). The addition of lipids with covalently attached PEG in liposomes of various non-pH-sensitive compositions has been shown to conquer the problem of their rapid removal by the RES (Blume & Cevc 1990; Klibanov et al 1990; Papahadjopoulos et al 1991; Senior et al 1991). One of the first compositions of liposomes with the combined properties of pH sensitivity and prolonged circulation in-vivo was developed and reported by Slepushkin et al (1997). The blood clearance curve of ^{111}In encapsulated in sterically stabilized pH-sensitive liposomes was similar to that of earlier developed liposomes with extended circulation time (Bakker-Woudenberg et al 1992; Woodle et al 1992). A sizeable percentage of liposomes (8.5%) remained after 24 h in the blood. On the contrary, the radioactive marker encapsulated in regular pH-sensitive liposomes was almost completely removed within 0.5 h from the bloodstream. The half-life of control DSPC/CHEMS/PE–PEG liposomes and sterically stabilized pH-sensitive DOPE/CHEMS/PE–PEG liposomes was similar (11.8 ± 0.7 and 11.1 ± 0.6 h, respectively). The area under the curve for control liposomes was greater than that for sterically stabilized pH-sensitive liposomes (1071 ± 151% and 629 ± 52% dose h mL^{-1}, respectively), most probably because a larger number of the latter was taken up quickly by the liver and spleen and therefore did not appear in blood samples. It is important to notice that the area under the curve for regular pH-sensitive liposomes (6.47 ± 1.24% dose h mL^{-1}) was about 100-fold lower than that for sterically stabilized pH-sensitive liposomes. The radioactive marker encapsulated in the former liposomes was removed from the circulation moreover because it leaks out as a result of the interaction of plasma proteins with liposomes or due to phagocytosis of the liposomes by the cells of the RES. ^{111}In associated with the lipid membrane of liposomes to differentiate between these possibilities. Blood clearance curves of lipid-labelled liposomes demonstrated rapid elimination from the circulation of the lipid component of the regular pH-sensitive liposomes but not that of sterically stabilized pH-sensitive liposomes. This observation revealed that the elimination of the liposome-encapsulated water-soluble marker was mostly due to removal of the liposomes by the RES from the blood. ^{111}In encapsulated in either long-circulating pH-sensitive or control liposomes primarily accumulated in the spleen and liver

and had similar patterns of distribution in-vivo. A high level of ^{111}In in the liver resulted after injection of regular pH-sensitive liposomes. This observation and the rapid clearance of these liposomes from the circulation shows that liver cells take up most of the liposomes within an hour, and only a fraction of the encapsulated marker leaks out and is then excreted in urine. A higher fraction of the DOPE/CHEMS/PE–PEG liposomes was localized in the liver and spleen, even after 24 h of injection, compared with the non-pH-sensitive control DSPC/CHEMS/PE–PEG liposomes. Compared with the control liposomes, the total urinary excretion of ^{111}In was lowered by about 2 fold. This result, with the observation that the t½ of both sterically stabilized liposomes were similar, suggests that a higher fraction of the contents of pH-sensitive liposomes was accumulated and retained most likely in the liver and spleen because they were delivered into the cytoplasm and thus partially avoided the usual metabolic processing of liposome contents. In another attempt, DOPE-containing liposomes were stabilized in the bilayer form by the addition of a cleavable lipid derivative of PEG in which the polymer was attached to a lipid anchor via a disulfide linkage (PEG–S–S–DSPE) (Kirpotin et al 1996; Ishida et al 2001a). An encapsulated dye was retained by liposomes stabilized with either a non-cleavable PEG (PEG–DSPE) or PEG–S–S–DSPE at pH 5.5. Treatment at this pH of liposomes stabilized with PEG–S–S–DSPE, with either dithiothreitol or cell-free extracts, caused contents release due to cleavage of the PEG chains and related destabilization of the DOPE liposomes. PEG–S–S–DSPE was rapidly cleaved in circulation as suggested by the pharmacokinetic studies. Also, therapeutic studies performed in a murine model of B-cell lymphoma confirmed clearly that the developed pH-sensitive formulation targeted to the CD19 receptor was better than the stable, long-circulating, targeted non-pH-sensitive liposomes, even with the more rapid drug release and clearance of the pH-sensitive formulation (Ishida et al 2001a). A novel category of pH-sensitive liposomes based on the incorporation of a PEG–diortho ester–distearoyl glycerol conjugate (POD) has been represented (Guo & Szoka 2001). This conjugate, comprising of a head group, an acid-labile diortho ester linker and a hydrophobic tail, was shown to be stable at neutral pH for more than 3 h, but at pH 5 degraded completely within 1 h. Liposomes formulated from POD/DOPE (1:9) remained stable for up to 12 h in neutral buffer and in the presence of 75% fetal bovine serum (content release less than 25%), releasing their contents (84%) in the next 4 h. PEG head groups are cleaved off, leading to liposome aggregation at pH 5–6, releasing most of their contents in 10–100 min. A great potential for the rapid delivery of drugs/genes at therapeutic sites by these liposomes is due to the fast kinetics of acid catalysed POD hydrolysis, where the decrease of pH is perhaps only one pH unit or less. The liver and intestine are the main sites of accumulation and the blood clearance pattern of these liposomes was monophasic, with an elimination half-life of 200 min. Whether the incorporation of other lipids, such as Chol, is able to further improve the blood half-life of these liposomes remains to be seen. Liposomes composed of DOPE and Chol, together with the positively charged lipid didodecyldimethlyammonium chloride (DODAC), have been developed (Adlakha-Hutcheon et al 1999). The mixture was stabilized in a bilayer organization by including PE–PEG (30:45:15:10). Binding of the liposomes to the cells via electrostatic interactions was promoted by inclusion of cationic lipid. The use of different acyl chains with PE–PEG, leading to different exchange rates of this component from the lipid bilayer, gives the liposomes a time-dependent destabilization. For the delivery of mitoxantrone such an approach was evaluated. It was noted that the reduction in the length of the acyl chain of PE–PEG lipid (from DSPE to dimyristoylphosphatidylethanolamine (DMPE)) was associated with an increasing rate of liposome clearance from blood, as well as with an increasing drug leakage rate. Almost 50% of the injected dose accumulated in the liver at 1 h, with only a small increase at later times in the case of liposomes composed of PEG–DMPE, while for liposomes stabilized with PEG–DPPE liver accumulation occurred at later times (1–4 h). After 24 h less than 15% of the injected dose of PEG–DSPE-containing liposomes was observed in the liver (Adlakha-Hutcheon et al 1999). The therapeutic activity was tested either against a pseudometastatic leukaemia murine model (L1210) or a human colon subcutaneous xenograft model (LS180) for mitoxantrone-containing liposomes. Upon intravenous administration, liposomes containing PEG–DMPE or PEG–DSPE showed a higher therapeutic activity (most animals showing disease-free survival) than the free drug or mitoxantrone-containing DSPC/Chol liposomes. Despite the different pharmacokinetic properties referred to above, both fusogenic formulations exhibited similar therapeutic activity. This can be described by the fact that in the leukaemia model tumour cells are localized in readily accessible organs such as the liver and spleen (Adlakha-Hutcheon et al 1999). A different trend was observed in the treatment of distal (non-RES) tumour sites, such as in the case of the subcutaneous LS180 model. Among the several treatments tested, liposomes containing PEG–DSPE presented the highest activity, while the ability of liposomes containing PEG–DMPE to delay initiation of tumour growth was even lower than that observed for free drug. In this tumour model, the increased therapeutic activity was steady with the use of a moderately stable system that provides greater mitoxantrone bioavailability and delivery (Adlakha-Hutcheon et al 1999). In recent times, the same approach was used for both DNA and oligonucleotide delivery, except that another exchangeable PEG conjugate (PEG–ceramide) was incorporated into the lipid bilayer (Zhang et al 1999; Hu et al 2001).

The major drawback of liposomes with ligands attached to the distal end of PEGs is faster clearance from the circulation. This is due to the formation of high-molecular-weight immune complexes that leads to aggregation of liposomes, resulting in rapid clearance from the circulation by cells of the mononuclear phagocytic system. In solution, more than 90% of the PEGylated ligand molecules are intramolecularly blocked. If the vast majority of the ligand is so heavily masked by the PEG moiety that accessibility to the protein is significantly hindered, this may at least partially explain the lower immunogenicity and toxicity, higher proteolytic resistance and longer half-life often observed in-vivo with PEGylated analogues. In addition, at the high concentrations such masking would inhibit aggregation arising from protein–protein interactions.

Alternative strategies under acidic conditions to generate fusogenic liposomes

While the development of pH-sensitive liposomes has been correlated normally with the inclusion of DOPE in the liposomal formulations, other strategies have also been investigated.

Use of novel pH-sensitive lipids

Inference obtained with a new type of pH-sensitive liposome formulation, composed of egg yolk phosphatidylcholine (EPC) liposomes bearing succinylated poly(glycidol), a PEG derivative having carboxyl groups, proved that under weakly acidic and acidic conditions fusion ability of the liposomes increases (Kono et al 1994). This has been shown to result in intensive and diffuse cytoplasmic fluorescence as a consequence of intracellular delivery of calcein. These observations led to the conclusion that polymer-modified liposomes, upon endocytosis by CV-1 cells, transfer their content into the cytoplasm by fusing with the endosomal membrane (Kono et al 1997). Recently, this strategy has been utilized to target anti-BCG antibody-bearing pH-sensitive liposomes to tumour cells expressing BCG antigen (Mizoue et al 2002). More recently, three different techniques have been engaged to generate pH-sensitive liposomes in the absence of DOPE (Guo et al 2002; Shi et al 2002; Sudimack et al 2002). Formulations with cationic/anionic lipid combinations were shown to be highly efficient vehicles for intracellular drug and gene delivery. Liposomes composed of EPC, dimethyldioctadecylammonium bromide (DDAB), CHEMS and Tween-80 (25:25:49:1, mol/mol) were shown to stably entrap calcein at pH 7.4 and undergo destabilization and irreversible aggregation under acidic pH. These liposomes showed improved retention of pH sensitivity in the presence of serum, compared with pH-sensitive liposomes containing DOPE (Shi et al 2002). One more hopeful strategy consisted of preparing anionic pH-sensitive liposomes composed of diolein/CHEMS (6:4). The outcome proved that these liposomes were stable at physiological pH, although they underwent rapid aggregation and efficiently released encapsulated calcein at pH 5.0. Complexes formed upon association of the developed liposomes with DNA–protamine mixtures maintained their transfection activity in media containing up to 50% fetal bovine serum, contrary to what was observed for DOPE-containing liposomes (Guo et al 2002). Hopeful results in terms of pH sensitivity and resistance to serum were also gained using a novel liposome formulation composed of PC, CHEMS, oleyl alcohol (OAlc), Tween-80 (Sudimack et al 2002) and a monostearoyl derivative of morpholine (Asokan & Cho 2003). The above formulation showed much better retention of its pH-sensitive properties in the presence of 10% serum compared with DOPE-based pH-sensitive liposomes.

Liposomal co-encapsulation of synthetic fusogenic peptides and therapeutic molecules

To promote cytosolic delivery of hydrophilic molecules with limited access to sub-cellular compartments, the liposomal co-encapsulation of therapeutic molecules with peptides has turned out to be a promising strategy. Gelonin, a type I plant toxin, represents an example of such a compound known to inactivate ribosomes and arrest protein synthesis. The failure of this compound to permeate the plasma membrane and to escape efficiently from endosomes into the cytosol leads to its rapid degradation within endosomes and lysosomes, thus compromising its anti-tumour activity. To overcome this problem, gelonin was co-encapsulated inside pH-sensitive liposomes with listeriolysin O, the pore-forming protein that mediates escape of the intracellular pathogen *Listeria monocytogenes* from the endosome into the cytosol (Provoda et al 2003). This technique resulted in a significant improvement in the cytotoxicity of encapsulated gelonin against the murine B16 melanoma cell line, compared with free gelonin or gelonin encapsulated alone in pH-sensitive liposomes. The inclusion of PEG strongly decreased the cytotoxicity of the pH-sensitive formulation co-encapsulating gelonin and listeriolysin O. It is to be noticed whether the inclusion of PEG compromises the cell uptake or the pH-sensitivity properties of the liposomes (Provoda et al 2003). A similar strategy has been used (Mastrobattista et al 2002) to demonstrate that co-encapsulation of a pH-dependent fusogenic peptide (diINF-7) and diphtheria toxin A chain (DTA) in non pH-sensitive immunoliposomes promotes cytosolic delivery of the encapsulated macromolecule. This peptide (resembling the NH_2-terminal domain of influenza virus hemagglutinin HA-2 subunit) was used, as functional characterization studies showed its capability to induce fusion between liposome membranes and leakage of liposome-entrapped compounds when exposed to low pH. In addition, co-encapsulation of diINF-7 into DTA-containing immunoliposomes resulted in significantly increased cytotoxicity toward ovarian carcinoma cells, indicating that this peptide can be used to obtain cytosolic delivery of liposome entrapped drugs with poor membrane permeation capacities (Mastrobattista et al 2002).

Synthetic fusogenic peptides/proteins with cationic liposomes

Immunogenicity is the major problem with using pH sensitive proteins/peptides in the formulation. This drawback can be overcome by using cationic liposomes (lipoplexes) and polymers (polyplexes). As cationic liposome/DNA complexes are believed to enter cells primarily through endocytosis, it has been theorized that the use of peptides that can destabilize endosomes or facilitate the fusion of the liposome/DNA complexes with the endosomal membrane would enhance gene delivery. Incubation of COS-7 cells with a ß-gal-expressing plasmid, and anionic or cationic derivatives of the N-terminal peptide of the HA-2 subunit of the influenza virus fusion protein, hemagglutinin, in the presence of Lipofectin, resulted in the improvement of transfection activity by a factor of 2–7 over that of Lipofectin alone (Kamata et al 1994). The degree of Transfectam (lipopolyamine)-mediated transfection of H225 human melanoma cells could be increased by up to 1000-fold (over that obtained with a sub-optimal charge-equivalent (1.5) of Transfectam/DNA) by adding the hemagglutinin-derived peptide INF6 to the preformed lipoplexes (Kichler et al 1997). The correlation of the pH-sensitive peptide GALA (Subbarao et al 1987; Parente et al 1988) with DOTAP/DOPE (1:1) liposomes before complexation with plasmid DNA resulted in a significant enhancement in luciferase expression in COS-7 cells, which depended on the cationic liposome/DNA (+/–) charge ratio (Simoes et al 1998). The capacity of certain peptides to cause endosomal

destabilization due to their fusogenic properties may also be extended to proteins. Actually studies on the methods of gene delivery mediated by transferrin-associated lipoplexes indicated that besides triggering internalization of the complexes, transferrin may also play a role in the cytoplasmic delivery of DNA by facilitating endosome destabilization (Aronsohn & Hughes 1998). Indeed, transfection experiments carried out with cells pre-treated with drugs that prevent acidification of the endosomal lumen (bafilomycin A1 and chloroquine) demonstrated that a significant inhibition was observed not only in the levels of transfection activity, but also in the extent of release of complexed DNA into the cytoplasm, as assessed by fluorescence microscopy. Contradictory to the observation of diffused cytoplasmic fluorescence in the absence of the lysosomotropic drugs, treatment of cells resulted in punctate fluorescence restricted to intracellular organelles, suggesting that complexed DNA was unable to escape from endosomes. Also, recent studies on the kinetics of the initial steps involved in lipoplex–cell interactions have suggest that association of transferrin to lipoplexes increases the extent of fusion with endosomes. Generally, these results support the hypothesis that transferrin acquires fusogenic properties under acidic conditions by exposure of hydrophobic domains, thus facilitating endosomal disruption and intracellular release of DNA (Schenkman et al 1981; Aronsohn & Hughes 1998). Alarmed by these results and by previous reports describing the ability of albumin to promote membrane fusion under acidic conditions (Zanta et al 1999; Chan & Jans 1999), further studies showed that albumin could also function as a fusogenic protein that destabilizes endosomes under acidic conditions, thus enhancing intracellular gene delivery and transfection activity (Plank et al 1994).

pH-Sensitive polymers with liposomes

In recent years, a number of studies have confirmed the potential of alkylated N-isopropylacrylamide (NIPAM) copolymers to confer pH sensitivity on liposomes. Complexation of hydrophobically modified copolymers of NIPAM (either randomly or terminally alkylated) with EPC/Chol liposomes resulted in an improvement of in-vitro release of both highly-water-soluble markers and amphipathic drugs upon acidification (Zignani et al 2000; Leroux et al 2001; Roux et al 2002b, 2004). Randomly alkylated NIPAM-anchored liposomes containing ara-C were shown to mediate a higher cytotoxicity towards J774 macrophage-like cells than non-pH-sensitive liposomes (Roux et al 2002b). Recently, different NIPAM-based copolymers were synthesized and estimated in terms of their ability to give pH sensitivity, serum stability (Roux et al 2002a, b, 2003) and steric stabilization to liposomes (Roux et al 2002b, 2003). Terminally alkylated NIPAM copolymer coating promotes steric stabilization of liposomes, resulting in prolonged blood circulation (Roux et al 2004). However, as this effect was still considered insufficient for in-vivo purposes, co-incorporation of PEG–lipid derivatives into the liposomal surface was evaluated. As anticipated, a considerable increase in blood circulation time was observed, blood clearance profiles being essentially analogous to those observed for stealth liposomes without NIPAM. Similar to earlier observations, the stabilizing effect of PEG noticeably reduced the liposome pH sensitivity contributed by the copolymers (Slepushkin et al 1997; Roux et al 2003).

Application of long circulating pH-sensitive liposomes for the delivery of anti-cancer drugs and other therapeutic molecules

The usefulness of pH-sensitive liposomes has been well exhibited in a wide variety of applications. These include: the transport of fluorescent probes to estimate the efficacy of different liposome compositions and also to explain the mechanisms involved in intracellular trafficking; the effective delivery of neoplastic drugs or recombinant proteins; the intracellular transport of antigens, targeting intracellular pathways involved in processing and presentation of antigens and enhancing the immune response to tumour cells; and the intracellular transport of genetic material for application in gene and antisense therapies. Applications of pH-sensitive liposomes for the transport and intracellular delivery of drugs for cancer therapy are shown in Table 1. A large extent of the work has been dedicated to improving the therapeutic efficacy of drugs entrapped in pH-sensitive liposomes, to confer tissue and cell specificity by directing the liposomes to cell surface receptors. Different ligands have been coupled covalently to the liposome surface or to the distal end of PEG–lipid conjugates for this purpose. These ligands comprise monoclonal antibodies against the H-2K^k receptor (expressed in several types of tumour cell) (Wang & Huang 1987), E-selectin (on activated vascular endothelial cells) (Spragg et al 1997), CD-19 (on B-lymphoma cells) (Ishida et al 2001b), CD3 (on T-leukaemia cells) (Turner et al 2002), P-glycoproteins (on endothelial cells) (Ng et al 2000) and BCG antigen (Mizoue et al 2002). Folate-receptor-targeted pH-sensitive liposomes, via coupling of folic acid to the distal end of PEG molecules, have been recently used to deliver antineoplastic drugs (Shi et al 2002; Sudimack et al 2002) and plasmid DNA (Lee & Huang 1996; Reddy & Low 2000; Shi et al 2002). pH-Sensitive liposomes have been revealed to be less effective than cationic liposomes in mediating intracellular gene delivery into mammalian cells under the same experimental conditions (Legendre & Szoka 1992). Different factors, not commonly unique, can explain this dissimilarity in transfection activity, including the lower amount of DNA encapsulated into pH-sensitive liposomes, their lower extent of cell internalization and the fact that once in the cytoplasm cationic liposomes could be more useful in protecting DNA against nucleases and in mediating its nuclear entry. Different methods have been developed recently, to avoid the limitations of pH-sensitive liposomes for nucleic acid delivery. These approaches share an identical strategy that basically consists of complexing preformed pH-sensitive liposomes with plasmid DNA pre-condensed with a cationic polymer (Reddy et al 1999; Reddy & Low 2000; Guo et al 2002; Shi et al 2002; Turner et al 2002). These approaches allow the efficient condensation and protection of plasmid DNA, and targeting to a specific cell (through coupling of a PEG–lipid conjugate to a ligand), in comparison with conventional liposome formulations, resulting in improved transfection efficiency. These strategies were further optimized by promoting nuclear entry of DNA, which was achieved by incorporation of a nuclear targeting sequence into the plasmid DNA (Reddy et al 1999). While this generated satisfactory results in-vitro, their pharmacokinetics, biodistribution and in-vivo gene delivery

Table 1 Applications of pH-sensitive liposomes in the delivery of anti-cancer drugs

Liposome formulation (composition and method of preparation)	Encapsulated material (drug)	Target disease/therapeutic applications	Reference
DOPE/DSPG/DSPE–PEG (7:3:5) (reverse-phase evaporation)	Methotrexate	Cancer therapy	Hong et al 2002
PC/DDAB/CHEMS/Tween-80/ folate–PE–PEG (25:25:49:1:0.1) PC/CHEMS/Tween-80/OAlc/ folate–PEG–Chol (50:50:2:80:0.5) (freeze–thawing)	Cytosine-β-D-arabinofuranoside	Cancer therapy (KB human oral cancer cells)	Shi et al 2002; Sudimack et al 2002
DOPE/CHEMS/PEG-S-S-DSPE/ MAL–PEG–DSPE[anti-CD19] (6:4:0.12:0.06) (hydration method)	Doxorubicin	Cancer therapy (haematological malignancies, B-lymphoma)	Ishida et al 2001b
DOPE/CHEMS (3:2) (freeze–thawing)	FICT-labeled antisense oligonucleotide	Cancer therapy (NG 108-15 neuroblastoma and glioma cells)	Skalko-Basnet et al 2000, 2002
DOPE/N-citraconyl-DOPE/ Chol/folate–PEG–DOPE (45.8:10:40:0.1) (freeze–thawing)	pDTS β-gal condensed with poly-L-lysine	Cancer therapy (transfection of KB human oral cancer cells)	Reddy et al 1999
DDAB/CHEMS/folate–PE–PEG (30:70:0.1) (freeze–thawing)	pCMV-Luc condensed with poly-L-lysine		Guo et al 2002
Diolein/CHEMS (6:4) (ethanol injection)	pCMV-Luc condensed with poly-L-lysine		Shi et al 2002
DOPE/CHEMS (2:1) (reverse-phase evaporation)	pSV2 luc, pRSV luc, pC luc and pCMV-β-Gal	Transfection of plasmid DNA into various mammalian cell lines	Legendre & Szoka 1992
DOPE/OA/Chol/DSPE–PEG–MAL [anti-CD3] (40:20:39.9:0.1) (hydration method)	pEGlacZ β-Gal condensed with poly-L-lysine	Transfection of Jurkat T-leukaemia cells	Turner et al 2002
POPE/CHOH/MPL (7:3:0.01) (freeze–thawing)	Cytotoxic T lymphocytes (CTL) epitope peptides from Hantaan nucleocapsid protein (M6) or human papilloma virus E7; FITC-conjugated H-2K CTL	Tumour therapy mediated by CTL that recognize tumour-associated antigen	Chang et al 2001
Epitope peptide POPE/CHOH/MPL (7:3:0.01) (freeze–thawing)		Induction of antigen specific CTL response in-vivo	Lee et al 2002
DOPE/HSPC/CHEMS/CHOL/mPEG$_{2000}$-DSPE (4:2:2:2:0.3) DOPE/HSPC/ CHEMS/CHOL (4:2:2:2) (hydration method)	Doxorubicin	Cancer therapy (B lymphoma cells)	Ishida et al 2006

potential remain to be established. For nucleic acid delivery, the use of liposomes exhibiting both pH sensitivity and steric stabilization has not been reported significantly. The approach of intracellular delivery of antisense oligonucleotides and ribozymes to inhibit virus production in HIV-infected macrophages derived from human peripheral blood monocytes has been assessed (Duzgunes et al 2001). 15-*mer* Anti-Rev-responsive element (RRE) phosphorothioate oligonucleotide hindered viral p24 production by 91% when delivered by pH-sensitive liposomes, whereas the free (unencapsulated) oligonucleotide was not active against HIV infection in macrophages. The oligonucleotide was also efficient when delivered through sterically stabilized pH-sensitive DOPE/CHEMS/PE–PEG liposomes, but not when encapsulated in non-pH-sensitive liposomes. A non-specific oligonucleotide encapsulated in pH-sensitive liposomes had no effect at 1 μM, but at 3 μM it inhibited HIV infection by 53%. Identical trials were executed with a 38-*mer* chimeric ribozyme complementary to HIV 5′-LTR. Cationic liposome-mediated delivery of this ribozyme to HIV-1-infected cells could not effectively reduce virus production under conditions where the delivery method was not toxic to the cells (Konopka et al 1998). When this ribozyme was delivered by pH-sensitive liposomes to HIV-infected macrophages, virus production was inhibited by 88%, while the free ribozyme caused a decrease of only 10% (Duzgunes et al 2001). The inclusion of a low mole fraction of PE–PEG in the membrane of pH-sensitive liposomes composed of DOPE/CHEMS results in prolonged circulation, without compromising their ability to deliver encapsulated molecules into macrophage-like cells (Slepushkin et al 1997). As these liposomes circulate for prolonged periods and can localize in lymph nodes after intravenous or subcutaneous injection (Allen et al 1993), they may be useful for the delivery of antisense molecules to lymph nodes where active HIV replication takes place (Pantaleo et al 1993; Pantaleo & Fauci 1995). As reported earlier, other fusogenic liposome formulations exhibiting long circulation times have also been utilized for nucleic acid delivery. Programmable fusogenic vesicles (PFVs), formerly developed for the delivery of mitoxantrone, are liposomes composed of DOPE, Chol, DODAC and an

exchangeable PEG–ceramide conjugate. When incorporating PEG–ceramide C_8, these systems were able to mediate high in-vitro transfection levels of COS-7 and HepG2 cells. High levels of luciferase expression were also observed upon intraperitoneal administration into B16 intraperitoneal tumour-bearing mice. It is necessary to point out that gene expression in tumour tissue was considerably higher than that observed for cationic liposome–DNA complexes (Zhang et al 1999). PFVs have also been assessed as carriers for the intracellular delivery of antisense oligonucleotides. PFVs containing PEG–ceramide C_{14} were shown to boost intracellular delivery of oligonucleotides relative to PFVs displaying faster (with PEG–ceramide C_8) or lower (with PEG–ceramide C_{20}) rates of destabilization. A substantial drop (about 20% and 25%) in the levels of bcl-2 mRNA was found upon treatment of cells with PEG–ceramide C_{14} liposomes containing the antisense oligonucleotide against the target proto-oncogene (Hu et al 2001).

Conclusion

Liposomes are a viable delivery system in targeting and accumulating anti-cancer drugs in malignant tumours. In the process of rectifying the various drawbacks, modified forms were developed, including pH-sensitive liposomes. The biopharmaceutical properties of PE, especially DOPE, have been favorable in eliciting pH sensitivity, which eventually resulted in effective cytosolic delivery of anti-cancer drugs. DOPE has been tried in combination with several other lipids and evaluation has revealed that the combination with CHEMS provides a better fusogenic property in comparison with other combinations. The same combination has performed well with the PEGylated lipids to give long-circulating and serum-stable formulations. Liposomal formulations using succinylated poly(glycidol), synthetic fusogenic peptides/proteins with cationic liposomes like Lipofectin, and alkylated N-isopropylacrylamide (NIPAM) copolymers have been effective alternatives in this area. The applicability of this mechanism has not only been effective with respect to drugs but also DNA, which has been evident by various transfection studies with DNA-loaded pH-sensitive liposomes.

To summarize, fusogenic liposomes using pH-sensitive materials has been a potentially useful approach that continues to generate much research interest in the treatment of cancer, due to their ability to be serum stable, long acting and target into the cytosol. The mechanism has the potential to be used as an effective alternative to conventional liposomal delivery when research is focused and issues related to clinical therapeutical aspects are addressed.

References

Abra, R. M., Bankert, R. B., Chen, F., Egilmez, N. K., Huang, K., Saville, R., Slater, J. L., Sugano, M., Yokota, S. J. (2002) The next generation of liposome delivery systems: recent experience with tumor-targeted, sterically stabilized immunoliposomes and active-loading gradients. *J. Liposome Res*. **12**: 1–3

Adlakha-Hutcheon, G., Bally, M. B., Shew, C. R., Madden, T. D. (1999) Controlled destabilization of a liposomal drug delivery system enhances mitoxantrone antitumor activity. *Nat. Biotechnol*. **17**: 775–779

Allen, T. M. (1992) Stealth liposomes: five years on. *J. Liposome Res*. **2**: 289–294

Allen, T. M., Hansen, C. (1991) Pharmacokinetics of stealth versus conventional liposomes: effect of dose. *Biochim. Biophys. Acta* **1068**: 133–141

Allen, T. M., Hansen, C. B., Guo, L. S. (1993) Subcutaneous administration of liposomes: a comparison with the intravenous and intraperitoneal routes of injection. *Biochim. Biophys. Acta* **1150**: 9–16

Aronsohn, A. I., Hughes, J. A. (1998) Nuclear localization signal peptides enhance cationic liposome-mediated gene therapy. *J. Drug Target*. **5**: 163–169

Asokan, A., Cho, M. J. (2003) Cytosolic delivery of macromolecules. II. Mechanistic studies with pH-sensitive morpholine lipids. *Biochim. Biophys. Acta* **1611**: 151–160

Bailey, A. L., Monck, M. A., Cullis, P. R. (1997) pH-induced destabilization of lipid bilayers by a lipopeptide derived from influenza hemagglutinin. *Biochim. Biophys. Acta* **1324**: 232–244

Bakker-Woudenberg, I. A., Lokerse, A. F., ten Kate, M. T., Storm, G. (1992) Enhanced localization of liposomes with prolonged blood circulation time in infected lung tissue. *Biochim. Biophys. Acta* **1138**: 318–326

Blume, G., Cevc, G. (1990) Liposomes for the sustained drug release in vivo. *Biochim. Biophys. Acta* **1029**: 91–97

Blume, G., Cevc, G. (1993) Molecular mechanism of the lipid vesicle longevity *in vivo*. *Biochim. Biophys. Acta* **1146**: 157–168

Blume, G., Cevc, G., Crommelin, M. D., Bakker-Woudenberg, I. A., Kluft, C., Storm, G. (1993) Specific targeting with poly(ethylene glycol)-modified liposomes: coupling of homing devices to the ends of polymeric chains combines effective target binding with long circulation times. *Biochim. Biophys. Acta* **1149**: 180–184

Chan, C. K., Jans, D. A. (1999) Enhancement of polylysine-mediated transferrinfection by nuclear localization sequences: polylysine does not function as a nuclear localization sequence. *Hum. Gene Ther*. **10**: 1695–1702

Chang, J. S., Choi, M. J., Cheong, H. S., Kim, K. (2001) Development of Th1-mediated CD8+ effector T cells by vaccination with epitope peptides encapsulated in pH-sensitive liposomes. *Vaccine* **19**: 3608–3614

Chu, C. J., Szoka, F. C. (1994) pH-sensitive liposomes. *J. Liposome Res*. **4**: 361–395

Chu, C. J., Dijkstra, J., Lai, M. Z., Hong, K., Szoka, F. C. (1990) Efficiency of cytoplasmic delivery by pH-sensitive liposomes to cells in culture. *Pharm. Res*. **7**: 824–834

Collins, D. (1995) pH-sensitive liposomes as tools for cytoplasmic delivery. In: Philippot, J. R., Schuber, F. (eds) *Liposomes as tools in basic research and industry*. CRS Press, Boca Raton, pp 201–214

Collins, D., Huang, L. (1987) Cytotoxicity of diphtheria toxin A fragment to toxin-resistant murine cells delivered by pH-sensitive immunoliposomes. *Cancer Res*. **47**: 735–739

Collins, D., Maxfield, F., Huang, L. (1989) Immunoliposomes with different acid sensitivities as probes for the cellular endocytic pathway. *Biochim. Biophys. Acta* **987**: 47–55

Collins, D., Litzinger, D. C., Huang, L. (1990) Structural and functional comparisons of pH-sensitive liposomes composed of phosphatidylethanolamine and three different diacylsuccinylglycerols. *Biochim. Biophys. Acta* **1025**: 234–242

Collins, D. S., Findlay, K., Harding, C. V. (1992) Processing of exogenous liposome-encapsulated antigens in vivo generates class I MHC-restricted T cell responses. *J. Immunol*. **148**: 3336–3341

Connor, J., Huang, L. (1986) pH-sensitive immunoliposomes as an efficient and target-specific carrier for antitumor drugs. *Cancer Res*. **46**: 3431–3435

Cullis, P. R., de Kruijff, B. (1979) Lipid polymorphism and the functional roles of lipids in biological membranes. *Biochim. Biophys. Acta* **559**: 399–420

Derycke, A. S., De Witte, P. A. (2002) Transferrin-mediated targeting of hypericin embedded in sterically stabilized PEG-liposomes. *Int. J. Oncol*. **20**: 181–187

Drummond, D. C., Meyer, O., Hong, K., Kirpotin, D. B., Papahadjopoulos, D. (1999) Optimizing liposomes for delivery of chemotherapeutic agents to solid tumors. *Pharmacol. Rev*. **51**: 691–743

Drummond, D. C., Zignani, M., Leroux, J. C. (2000) Current status of pH-sensitive liposomes in drug delivery. *Prog. Lipid Res*. **39**: 409–460

Duzgunes, N., Straubinger, R. M., Baldwin, P. A., Friend, D. S., Papahadjopoulos, D. (1985) Proton-induced fusion of oleic acid–phosphatidylethanolamine liposomes. *Biochemistry* **24**: 3091–3098

Duzgunes, N., Simoes, S., Slepushkin, V., Pretzer, E., Rossi, J. J., De Clercq, E., Antao, V. P., Collins, M. L., de Lima, M. C. (2001) Enhanced inhibition of HIV-1 replication in macrophages by antisense oligonucleotides, ribozymes and acyclic nucleoside phosphonate analogs delivered in pH-sensitive liposomes, *Nucleosides Nucleotides Nucleic Acids* **20**: 515–523

Eavarone, D. A., Yu, X., Bellamkonda, R. V. (2000) Targeted drug delivery to C6 glioma by transferrin-coupled liposomes. *J. Biomed. Mater. Res*. **51**: 10–14

Ellens, H., Bentz, J., Szoka, F. C. (1985) H^+- and Ca^{2+}-induced fusion and destabilization of liposomes. *Biochemistry* **24**: 3099–3106

Fattal, E., Couvreur, P., Dubernet, C. (2004) Smart delivery of antisense oligonucleotides by anionic pH-sensitive liposomes. *Adv. Drug Deliv. Rev*. **56**: 931–946

Gabizon, A. A. (2001) Pegylated liposomal doxorubicin: metamorphosis of an old drug into a new form of chemotherapy. *Cancer Invest*. **19**: 424–436

Gabizon, A., Papahadjopoulos, D. (1988) Liposome formulations with prolonged circulation time in blood and enhanced uptake by tumors, *Proc. Natl Acad. Sci. USA* **85**: 6949–6953

Gabizon, A., Shmeeda, H., Horowitz, A. T., Zalipsky, S. (2004) Tumor cell targeting of liposome-entrapped drugs with phospholipid-anchored folic acid-PEG conjugates. *Adv. Drug Deliv. Rev*. **56**: 1177–1192

Gijsens, A., Derycke, A., Missiaen, L., De Vos, D., Huwyler, J., Eberle, A., de Witte, P. (2002) Targeting of the photocytotoxic compound AlPcS4 to Hela cells by transferring conjugated PEG-liposomes. *Int. J. Cancer* **101**: 78–85

Goncalves, A, Braud, A. C., Viret, F., Genre, D., Gravis, G., Tarpin, C., Giovannini, M., Maraninchi, D., Viens, P. (2003) Phase I study of pegylated liposomal doxorubicin (Caelyx) in combination with carboplatin in patients with advanced solid tumors. *Anticancer Res*. **23**: 3543–3548

Grant, G. J., Barenholz, Y. B., Elijah M., Bansinath, M., Turndorf, H. P., Boris B. S., Davidson, E. M. (2004) A novel liposomal bupivacaine formulation to produce ultra long-acting analgesia. *Anesthesiology* **101**: 133–137

Guo, X., Szoka, F. C. (2001) Steric stabilization of fusogenic liposomes by a low-pH sensitive PEG–diortho ester–lipid conjugate. *Bioconjug. Chem*. **12**: 291–300

Guo, W., Gosselin, M. A., Lee, R. J. (2002) Characterization of a novel diolein-based LPDII vector for gene delivery. *J. Control. Release* **83**: 121–132

Harrington, K. J., Lewanski, C., Northcote, A. D., Whittaker, J., Peters, A. M., Vile, R. G., and Stewart, J. S. (2001) Phase II study of PEGylated liposomal doxorubicin (Caelyx) as induction chemotherapy for patients with squamous cell cancer of the head and neck. *Eur. J. Cancer* **37**: 2015–2022

Hatakeyama, H., Akita, H., Maruyama, K., Suhara, T., Harashima, H. (2004) Factors governing the *in vivo* tissue uptake of transferrin-coupled polyethylene glycol liposomes *in vivo. Int. J. Pharm*. **281**: 25–33

Hong, M. S., Lim, S. J., Oh, Y. K., Kim, C. K. (2002) pH-sensitive, serum-stable and long-circulating liposomes as a new drug delivery system. *J. Pharm. Pharmacol*. **54**: 51–58

Hu, Q., Shew, C. R., Bally, M. B., Madden, T. D. (2001) Programmable fusogenic vesicles for intracellular delivery of antisense oligodeoxynucleotides: enhanced cellular uptake and biological effects. *Biochim. Biophys. Acta* **1514**: 1–13

Huwyler, J., Wu, D., Pardridge, W. M. (1996) Brain drug delivery of small molecules using immunoliposomes. *Proc. Natl Acad. Sci. USA* **93**: 14164–14169

Iinuma, H., Maruyama, K., Okinaga, K., Sasaki, K., Sekine, T., Ishida, O., Ogiwara, N., Johkura, K., Yonemura, Y. (2002) Intracellular targeting therapy of Cisplatin encapsulated transferrin-polyethylene glycol liposome on peritoneal dissemination of gastric cancer. *Int. J. Cancer* **99**: 130–137

Ishida, O., Maruyama, K., Tanahashi, H., Iwatsuru, M., Sasaki, K., Eriguchi, M., Yanagie, H. (2001a) Liposomes bearing polyethylene glycol coupled transferrin with intracellular targeting property to the solid tumors *in vivo. Pharm. Res*. **18**: 1042–1048

Ishida, T., Kirchmeier, M. J., Moase, E. H., Zalipsky, S., Allen, T. M. (2001b) Targeted delivery and triggered release of liposomal doxorubicin enhances cytotoxicity against human B lymphoma cells. *Biochim. Biophys. Acta* **1515**: 144–158

Ishida, T., Okada, Y., Kobayashi, T., Kiwada, H. (2006) Development of pH-sensitive liposomes that efficiently retain encapsulated doxorubicin (DXR) in blood. *Int. J. Pharm*. **309**: 94–100

Ishiguro, R., Matsumoto, M., Takahashi, S. (1996) Interaction of fusogenic synthetic peptide with phospholipid bilayers: orientation of the peptide alpha-helix and binding isotherm. *Biochemistry* **35**: 4976–4983

Johnston, S. R., Gore, M. E. (2001) Caelyx: phase II studies in ovarian cancer. *Eur. J. Cancer* **37** (Suppl. 9): S8–S14

Joshee, N., Bastola, D. R., Cheng, P. W. (2002) Transferrin facilitated lipofection gene delivery strategy: characterization of the transfection complexes and intracellular trafficking. *Hum. Gene Ther*. **13**: 1991–2004

Kakudo, T., Chaki, S., Futaki, S., Nakase, I., Akaji, K., Kawakami, T., Maruyama, K., Kamiya, H., Harashima, H. (2004) Transferrin-modified liposomes equipped with a pH-sensitive fusogenic peptide: an artificial viral-like delivery system. *Biochemistry* **43**: 5618–5628

Kamata, H., Yagisawa, H., Takahashi, S., Hirata, H. (1994) Amphiphilic peptides enhance the efficiency of liposome-mediated DNA transfection. *Nucleic Acids Res*. **22**: 536–537

Kamps, J. A., Koning, G. A., Velinova, M. J., Morselt, H. W., Wilkens, M., Gorter, A., Donga, J., Scherphof, G. L. (2000) Uptake of long-circulating immunoliposomes, directed against colon adenocarcinoma cells, by liver metastases of colon cancer. *J. Drug Target*. **8**: 235–245

Kichler, A., Mechtler, K., Behr, J. P., Wagner, E. (1997) Influence of membrane-active peptides on lipospermine/DNA complex mediated gene transfer. *Bioconjug. Chem*. **8**: 213–221

Kirpotin, D., Hong, K., Mullah, N., Papahadjopoulos, D., Zalipsky, S. (1996) Liposomes with detachable polymer coating: destabilization and fusion of dioleoylphosphatidylethanolamine vesicles triggered by cleavage of surface-grafted poly(ethylene glycol). *FEBS Lett*. **388**: 115–118

Klibanov, A. L., Maruyama, K., Torchilin, V. P., Huang, L. (1990) Amphipatic polyethyleneglycols effectively prolong the circulation time of liposomes. *FEBS Lett*. **268**: 235–238

Kono, K., Zenitani, K., Takagishi, T. (1994) Novel pH-sensitive liposomes: liposomes bearing a poly(ethylene glycol) derivative with carboxyl groups. *Biochim. Biophys. Acta* **1193**: 1–9

Kono, K., Igawa, T., Takagishi, T. (1997) Cytoplasmic delivery of calcein mediated by liposomes modified with a pH-sensitive poly(ethylene glycol) derivative. *Biochim. Biophys. Acta* **1325**: 143–154

Konopka, K., Rossi, J. J., Swiderski, P., Slepushkin, V. A., Duzgunes, N. (1998) Delivery of an anti-HIV-1 ribozyme into HIV infected cells via cationic liposomes. *Biochim. Biophys. Acta* **1372**: 55–68

Lai, M. Z., Duzgunes, N., Szoka, F. C. (1985) Effects of replacement of the hydroxyl group of cholesterol and tocopherol on the thermotropic behavior of phospholipid membranes. *Biochemistry* **24**: 1646–1653

Lasic, D. D. (1998) Novel applications of liposomes. *Trends Biotechnol*. **16**: 307–321

Leamon, C. P., Low, P. S. (1991) Delivery of macromolecules into living cells: a method that exploits folate receptor endocytosis. *Proc. Natl Acad. Sci. USA* **88**: 5572–5576

Leamon, C. P., Cooper, S. R., Hardee, G. E. (2003) Folate liposome-mediated antisense oligodeoxynucleotide targeting to cancer cells: evaluation *in vitro* and *in vivo*. *Bioconjug. Chem*. **14**: 738–747

Lee, R. J., Low, P. S. (1994) Delivery of liposomes into cultured KB cells via folate receptor-mediated endocytosis. *J. Biol. Chem*. **269**: 3198–3204

Lee, R. J., Huang, L. (1996) Folate-targeted, anionic liposome entrapped polylysine-condensed DNA for tumor cell-specific gene transfer. *J. Biol. Chem*. **271**: 8481–8487

Lee, K. Y., Chun, E., Seong, B. L. (2002) Investigation of antigen delivery route in vivo and immune-boosting effects mediated by pH-sensitive liposomes encapsulated with K(b)-restricted CTL epitope. *Biochem. Biophys. Res. Commun*. **292**: 682–688

Legendre, J. Y., Szoka, F. C. (1992) Delivery of plasmid DNA into mammalian cell lines using pH-sensitive liposomes: comparison with cationic liposomes. *Pharm. Res*. **9**: 1235–1242

Leroux, J. C. (2000) Current status of pH-sensitive liposomes in drug delivery. *Prog. Lipid Res*. **39**: 409–460

Leroux, J., Roux, E., Le Garrec, D., Hong, K., Drummond, D. C. (2001) *N*-Isopropylacrylamide copolymers for the preparation of pH-sensitive liposomes and polymeric micelles. *J. Control. Release* **72**: 71–84

Levchenko, T. S., Rammohan, R., Lukyanov, A. N., Whiteman, K. R., Torchilin, V. P. (2002) Liposome clearance in mice: the effect of a separate and combined presence of surface charge and polymer coating. *Int. J. Pharm*. **240**: 95–102

Litzinger, D. C., Huang, L. (1992) Phosphatidylethanolamine liposomes: drug delivery, gene transfer and immunodiagnostic applications. *Biochim. Biophys. Acta* **1113**: 201–227

Liu, D. X., Huang, L. (1989) Small, but not large, unilamellar liposomes composed of dioleoylphosphatidylethanolamine and oleic acid can be stabilized by human plasma. *Biochemistry* **28**: 7700–7707

Liu, D., Huang, L. (1990) pH-sensitive, plasma-stable liposomes with relatively prolonged residence in circulation. *Biochim. Biophys. Acta* **1022**: 348–354

Liu, D., Huang, L. (1994) Interaction of pH-sensitive liposomes with blood comp. *J. Liposome Res*. **987**: 47–55

Lu, Y., Low, P. S. (2002a) Folate targeting of haptens to cancer cell surfaces mediates immunotherapy of syngeneic murine tumors. *Cancer Immunol. Immunother*. **51**: 153–162

Lu, Y., Low, P. S. (2002b) Folate-mediated delivery of macromolecular anticancer therapeutic agents. *Adv. Drug Deliv. Rev*. **54**: 675–693

Lukyanov, A. N., Elbayoumi, T. A., Chakilam, A. R., Torchilin, V. P. (2004) Tumor-targeted liposomes: doxorubicinloaded long-circulating liposomes modified with anti-cancer antibody. *J. Control. Release* **100**: 135–144

Maeda, H., Sawa, T., Konno, T. (2001) Mechanism of tumor targeted delivery of macromolecular drugs, including the EPR effect in solid tumor and clinical overview of the prototype polymeric drug SMANCS. *J. Control. Release* **74**: 47–61

Mastrobattista, E., Koning, G. A., van Bloois, L., Filipe, A. C., Jiskoot, W., Storm, G. (2002) Functional characterization of an endosome disruptive peptide and its application in cytosolic delivery of immunoliposome-entrapped proteins. *J. Biol. Chem*. **277**: 27135–27143

Metselaar, J. M., Bruin, P., de Boer, L. W., de Vringer, T., Snel, C., Oussoren, C., Wauben, M. H., Crommelin, D. J., Storm, G., Hennink, W. E. (2003) A novel family of L-amino acid based biodegradable polymer-lipid conjugates for the development of long-circulating liposomes with effective drug-targeting capacity. *Bioconjug. Chem*. **14**: 1156–1164

Mizoue, T., Horibe, T., Maruyama, K., Takizawa, T., Iwatsuru, M., Kono, K., Yanagie, H., Moriyasu, F. (2002) Targetability and intracellular delivery of anti-BCG antibody-modified, pH-sensitive fusogenic immunoliposomes to tumor cells. *Int. J. Pharm*. **237**: 129–137

Ng, K., Zhao, L., Liu, Y., Mahapatro, M. (2000) The effects of polyethyleneglycol (PEG)-derived lipid on the activity of target-sensitive immunoliposome. *Int. J. Pharm*. **193**: 157–166

Ni, S., Stephenson, S. M., Lee, R. J. (2002) Folate receptor targeted delivery of liposomal daunorubicin into tumor cells. *Anticancer Res*. **22**: 2131–2135

Nir, S., Nico, F., Szoka, F. C. (1999) Surface aggregation and membrane penetration by peptides: relation to pore formation and fusion. *Mol. Membr. Biol*. **16**: 95–101

O'Shaughnessy, J. A. (2003) Pegylated liposomal doxorubicin in the treatment of breast cancer. *Clin. Breast Cancer* **4**: 318–328

Omori, N., Maruyama, K., Jin, G., Li, F., Wang, S.J., Hamakawa, Y., Sato, K., Nagano, I., Shoji, M., Abe, K. (2003) Targeting of post-ischemic cerebral endothelium in rat by liposomes bearing polyethylene glycol coupled transferrin. *Neurol. Res*. **25**: 275–279

Pan, X. Q., Zheng, X., Shi, G., Wang, H., Ratnam, M., Lee, R. J. (2002) Strategy for the treatment of acute myelogenous leukemia based on folate receptor beta-targeted liposomal doxorubicin combined with receptor induction using all-trans retinoic acid. *Blood* **100**: 594–602

Pan, X. Q., Wang, H., Lee, R. J. (2003) Antitumor activity of folate receptor-targeted liposomal doxorubicin in a KB oral carcinoma murine xenograft model. *Pharm. Res*. **20**: 417–422

Pantaleo, G., Fauci, A. S. (1995) New concepts in the immunopathogenesis of HIV infection. *Annu. Rev. Immunol*. **13**: 487–512

Pantaleo, G., Graziosi, C., Demarest, J. F., Butini, L., Montroni, M., Fox, C. H., Orenstein, J. M., Kotler, D. P., Fauci, A. S. (1993) HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of disease. *Nature* **362**: 355–358

Papahadjopoulos, D., Allen, T. M., Gabizon, A., Mayhew, E., Matthay, K., Huang, S. K., Lee, K. D., Woodle, M. C., Lasic, D. D., Redemann, C., Martin, F. J. (1991) Sterically stabilized liposomes: improvements in pharmacokinetics and antitumor therapeutic efficacy. *Proc. Natl Acad. Sci. USA* **88**: 11460–11464

Parente, R. A., Nir, S., Szoka, F. C. (1988) pH-dependent fusion of phosphatidylcholine small vesicles. Induction by a synthetic amphipathic peptide. *J. Biol. Chem*. **263**: 4724–4730

Park, J. W., Kirpotin, D. B., Hong, K., Shalaby, R., Shao, Y., Nielsen, U. B., Marks, J. D., Papahadjopoulos, D., Benz, C. C. (2001) Tumor targeting using anti-HER2 immunoliposomes. *J. Control. Release* **74**: 95–113

Perez, A. T., Domenech, G. H., Frankel, C., Vogel, C. L. (2002) Pegylated liposomal doxorubicin (Doxil) for metastatic breast cancer: the Cancer Research Network, Inc., experience. *Cancer Invest*. **20** (Suppl. 2): 22–29

Plank, C., Oberhauser, B., Mechtler, K., Koch, C., Wagner, E. (1994) The influence of endosome-disruptive peptides on gene transfer using synthetic virus-like gene transfer systems. *J. Biol. Chem*. **269**: 12918–12924

Provoda, C. J., Stier, E. M., Lee, K. D. (2003) Tumor cell killing enabled by listeriolysin O-liposome-mediated delivery of the protein toxin gelonin. *J. Biol. Chem*. **278**: 35102–35108

Raffaghello, L., Pagnan, G., Pastorino, F., Cosimo, E., Brignole, C., Marimpietri, D., Bogenmann, E., Ponzoni, M., Montaldo, P. G.

(2003) Immunoliposomal fenretinide: a novel antitumoral drug for human neuroblastoma. *Cancer Lett*. **197**: 151–155

Reddy, J. A., Low, P. S. (2000) Enhanced folate receptor mediated gene therapy using a novel pH-sensitive lipid formulation. *J. Control. Release* **64**: 27–37

Reddy, J. A., Dean, D., Kennedy, M. D., Low, P. S. (1999) Optimization of folate-conjugated liposomal vectors for folate receptor mediated gene therapy. *J. Pharm. Sci*. **88**: 1112–1118

Reddy, J. A., Abburi, C., Hofland, H., Howard, S. J., Vlahov I., Wils, P., Leamon, C. P. (2002) Folate-targeted, cationic liposome mediated gene transfer into disseminated peritoneal tumors. *Gene Ther*. **9**: 1542–1550

Ropert, C., Malvy, C., Couvreur, P. (1995) pH sensitive liposomes as efficient carriers for intracellular delivery of oligonucleotides. In: Gregoriadis, G. (ed.) *Strategies for oligonucleotide and gene delivery in therapy*. Vol. 290, Plenum Press, New York, pp 151–162

Roux, E., Francis, M., Winnik, F. M., Leroux, J. C. (2002a) Polymer based pH-sensitive carriers as a means to improve the cytoplasmic delivery of drugs. *Int. J. Pharm*. **242**: 25–36

Roux, E., Stomp, R., Giasson, S., Pezolet, M., Moreau, P., Leroux, J. C. (2002b) Steric stabilization of liposomes by pH-responsive N-isopropylacrylamide copolymer. *J. Pharm. Sci*. **91**: 1795–1802

Roux, E., Lafleur, M., Lataste, E., Moreau, P., Leroux, J. C. (2003) On the characterization of pH-sensitive liposome/polymer complexes. *Biomacromolecules* **4**: 240–248

Roux, E., Passirani, C., Scheffold, S., Benoit, J. P., Leroux, J. C. (2004) Serum-stable and long-circulating, PEGylated, pH-sensitive liposomes. *J. Control. Release* **94**: 447–451

Sapra, P., Allen, T. M. (2002) Internalizing antibodies are necessary for improved therapeutic efficacy of antibody-targeted liposomal drugs. *Cancer Res*. **62**: 7190–7194

Schenkman, S., Arau'jo, P. S., Dijkman, R., Quina, F. H, Chaimovich, H. (1981) Effects of temperature and lipid composition on the serum albumin-induced aggregation and fusion of small unilamellar vesicles. *Biochim. Biophys. Acta* **649**: 633–647

Schroit, A. J., Madsen, J., Nayar, R. (1986) Liposome–cell interactions: in vitro discrimination of uptake mechanism and in vivo targeting strategies to mononuclear phagocytes. *Chem. Phys. Lipids* **40**: 373–393

Schwonzen, M., Kurbacher, C. M., Mallmann, P. (2000) Liposomal doxorubicin and weekly paclitaxel in the treatment of metastatic breast cancer. *Anticancer Drugs* **11**: 681–685

Seddon, J. M., Cevc, G., Marsh, D. (1983) Calorimetric studies of the gel-fluid (L beta–L alpha) and lamellar-inverted hexagonal (L alpha-HII) phase transitions in dialkyl- and dia- cylophosphatidylethanolamines. *Biochemistry* **22**: 1280–1289

Senior, J., Delgado, C., Fisher, D., Tilcock, C., Gregoriadis, G. (1991) Influence of surface hydrophilicity of liposomes on their interaction with plasma protein and clearance from the circulation: studies with poly(ethylene glycol)-coated vesicles. *Biochim. Biophys. Acta* **1062**: 77–82

Shi, G., Guo, W., Stephenson, S. M., Lee, R. J. (2002) Efficient intracellular drug and gene delivery using folate receptor-targeted pH-sensitive liposomes composed of cationic/anionic lipid combinations. *J. Control. Release* **80**: 309–319

Simoes, S., Slepushkin, V., Gaspar, R., de Lima, M. C., Duzgunes, N. (1998) Gene delivery by negatively charged ternary complexes of DNA, cationic liposomes and transferrin or fusogenic peptides. *Gene Ther*. **5**: 955–964

Simoes, S., Slepushkin, V., Duzgunes, N., Pedroso de Lima, M. C. (2001) On the mechanisms of internalization and intracellular delivery mediated by pH-sensitive liposomes. *Biochim. Biophys. Acta* **1515**: 23–37

Simoes, S., Moreira, J. N., Fonseca, C., Duzgunes, N., Pedroso de Lima, M. C. (2004) On the formulation of pH-sensitive liposomes with long circulation times. *Adv. Drug Del. Rev*. **56**: 947–965

Skalko-Basnet, N., Tohda, M., Watanabe, H. (2000) Delivery of antisense oligonucleotides to neuroblastoma cells. *Neuroreport* **11**: 3117–3121

Skalko-Basnet, N., Tohda, M., Watanabe, H. (2002) Uptake of liposomally entrapped fluorescent antisense oligonucleotides in NG108-15 cells: conventional versus pH-sensitive. *Biol. Pharm. Bull*. **25**: 1583–1587

Slepushkin, V. A., Simoes, S., Dazin, P., Newman, M. S., Guo, L. S., Pedroso de Lima, M. C., Duzgunes, N. (1997) Sterically stabilized pH-sensitive liposomes. Intracellular delivery of aqueous contents and prolonged circulation in vivo. *J. Biol. Chem*. **272**: 2382–2388

Spragg, D. D., Alford, D. R., Greferath, R., Larsen, C. E., Lee, K. D., Gurtner, G. C., Cybulsky, M. I., Tosi, P. F., Nicolau, C., Gimbrone, M. A. (1997) Immuno targeting of liposomes to activated vascular endothelial cells: a strategy for site-selective delivery in the cardiovascular system. *Proc. Natl Acad. Sci. USA* **94**: 8795–8800

Stephenson, S. M., Yang, W., Stevens, P. J., Tjarks, W., Barth, R. F., Lee, R. J. (2003) Folate receptor-targeted liposomes as possible delivery vehicles for boron neutron capture therapy. *Anticancer Res*. **23**: 3341–3345

Subbarao, N. K., Parente, R. A., Szoka, F. C., Nadasdi, L., Pongracz, K. (1987) pH-dependent bilayer destabilization by an amphipathic peptide. *Biochemistry* **26**: 2964–2972

Sudimack, J. J., Guo, W., Tjarks, W., Lee, R. J. (2002) A novel pH sensitive liposome formulation containing oleyl alcohol. *Biochim. Biophys. Acta* **1564**: 31–37

Sundar, S., Jha, T. K., Thakur, C. P., Mishra, M., Singh, V. P., Buffels, R. (2003) Single-dose liposomal amphotericin B in the treatment of visceral leishmaniasis in India: a multicenter study. *Clin. Infect. Dis*. **37**: 800–804

Symon, Z., Peyser A, Tzemach, D., Lyass, O., Sucher, E., Shezen, E., Gabizon, A. (1999) Selective delivery of doxorubicin to patients with breast carcinoma metastases by stealth liposomes. *Cancer* **86**: 72–78

Takeuchi, H., Kojima, H., Yamamoto, H., Kawashima, Y. (2001) Evaluation of circulation profiles of liposomes coated with hydrophilic polymers having different molecular weights in rats. *J. Control. Release* **75**: 83–91

Tan, P. H., Manunta, M., Ardjomand, N., Xue, S. A., Larkin, D. F. P., Haskard, D. O., Taylor, K. M., George, A. J. T. (2003) Antibody targeted gene transfer to endothelium. *J. Gene Med*. **5**: 311–323

Torchilin, V. P. (1985) Liposomes as targetable drug carriers. *CRC Crit. Rev. Ther. Drug Carrier Syst*. **1**: 65–115

Torchilin, V. P. (2005) Recent advances with liposomes as pharmaceutical carriers. *Nature* **4**: 145–160

Torchilin, V. P., Trubetskoy, V. S. (1995) Which polymers can make nanoparticulate drug carriers long-circulating? *Adv. Drug. Deliv. Rev*. **16**: 141–155

Torchilin, V. P., Klibanov, A. L., Huang, L. (1992) Targeted accumulation of polyethylene glycol-coated immunoliposomes in infarcted rabbit myocardium. *FASEB J*. **6**: 2716–2719

Torchilin, V. P., Zhou, F., Huang, L. (1993) pH-sensitive liposomes. *J. Liposome Res*. **3**: 201–255

Torchilin, V. P., Omelyanenko, V. G., Papisov, M. I., Bogdanov, A. A., Trubetskoy, V. S., Herron, J. N., Gentry, C. A. (1994) Poly(ethylene glycol) on the liposome surface: on the mechanism of polymer-coated liposome longevity. *Biochim. Biophys. Acta* **1195**: 11–20

Torchilin, V. P., Levchenko, T. S., Lukyanov, A. N., Khaw, B. A., Klibanov, A. L., Rammohan, R., Samokhin, G. P., Whiteman, K. R. (2001a) p-Nitrophenylcarbonyl-PEG-PE liposomes: fast and simple attachment of specific ligands, including monoclonal antibodies, to distal ends of PEG chains via p-nitrophenylcarbonyl groups. *Biochim. Biophys. Acta* **1511**: 397–411

Torchilin, V. P., Levchenko, T. S., Whiteman, K. R., Yaroslavov, A. A., Tsatsakis, A. M., Rizos, A. K., Michailova, E. V., Shtilman, M. I. (2001b) Amphiphilic poly-*N*-vinylpyrrolidones: synthesis, properties and liposome surface modification. *Biomaterials*. **22**: 3035–3044

Turk, M. J., Reddy, J. A., Chmielewski, J. A., Low, P. S. (2002) Characterization of a novel pH-sensitive peptide that enhances drug release from folate-targeted liposomes at endosomal pHs. *Biochim. Biophys. Acta* **1559**: 56–68

Turner, C., Weir, N., Catterall, C., Baker, T. S., Jones, M. N. (2002) The transfection of Jurkat T-leukemic cells by use of pH-sensitive immunoliposomes. *J. Liposome Res*. **12**: 45–50

Vidal, M., Hoekstra, D. (1995) In vitro fusion of reticulocyte endocytic vesicles with liposomes. *J. Biol. Chem*. **270**: 17823–17829

Wang, C. Y., Huang, L. (1987) pH-sensitive immunoliposomes mediate target-cell-specific delivery and controlled expression of a foreign gene in mouse. *Proc. Natl Acad. Sci. USA* **84**: 7851–7855

Wang, C. Y., Huang, L. (1989) Highly efficient DNA delivery mediated by pH-sensitive immunoliposomes. *Biochemistry* **28**: 9508–9514

Whiteman, K. R., Subr, V., Ulbrich, K., Torchilin, V. P. (2001) Poly(HPMA)-coated liposomes demonstrate prolonged circulation in mice. *J. Liposome Res*. **11**: 153–164

Woodle, M. C. (1995) Sterically stabilized liposome therapeutics. *Adv. Drug Deliv. Rev*. **16**: 249–266

Woodle, M. C., Matthay, K. K., Newman, M. S., Hidayat, J. E., Collins, L. R., Redemann, C., Martin, F. J., Papahadjopoulos, D. (1992) Versatility in lipid compositions showing prolonged circulation with sterically stabilized liposomes. *Biochim. Biophys. Acta* **1105**: 193–200

Xu, L., Huang, C. C., Huang, W., Tang, W. H., Rait, A., Yin, Y. Z., Cruz, I., Xiang, L. M., Pirollo, K. F., Chang, E. H. (2002) Systemic tumor-targeted gene delivery by antitransferrin receptor scFv-immunoliposomes. *Mol. Cancer Ther*. **1**: 337–346

Yoshimura, T., Shono, M., Imai, K., Hong, K. (1995) Kinetic analysis of endocytosis and intracellular fate of liposomes in single macrophages. *J. Biochem*. **117**: 34–41

Zalipsky, S. (1995) Chemistry of polyethylene glycol conjugates with biologically active molecules, *Adv. Drug Deliv. Rev*. **16**: 157–182

Zanta, M. A., Belguise-Valladier, P., Behr, J. P. (1999) Gene delivery: a single nuclear localization signal peptide is sufficient to carry DNA to the cell nucleus. *Proc. Natl Acad. Sci. USA* **96**: 91–96

Zhang, Y. P., Sekirov, L., Saravolac, E. G., Wheeler, J. J., Tardi, P., Clow, K., Leng, E., Sun, R., Cullis, P. R., Scherrer, P. (1999) Stabilized plasmid-lipid particles for regional gene therapy: formulation and transfection properties. *Gene Ther*. **6**: 1438–1447

Zignani, M., Drummond, D. C., Meyer, O., Hong, K., Leroux, J. C. (2000) In vitro characterization of a novel polymeric-based pH-sensitive liposome system. *Biochim. Biophys. Acta* **1463**: 383–394