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Eradication of human immunodeficiency virus type-1 (HIV-1)-infected cells

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On the formulation of pH-sensitive liposomes with long circulation times

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Abstract

Strategies used to enhance liposome-mediated drug delivery in vivo include the enhancement of stability and circulation time in the bloodstream, targeting to specific tissues or cells, and facilitation of intracytoplasmic delivery. pH-sensitive liposomes have been developed to mediate the introduction of highly hydrophilic molecules or macromolecules into the cytoplasm. These liposomes destabilize under acidic conditions found in the endocytotic pathway, and usually contain phosphatidylethanolamine (PE) and titratable stabilizing amphiphiles. Formulations without PE have also been developed. Encapsulated compounds are thought to be transported into the cytoplasm through destabilization of or fusion with the endosome membrane. Incorporation of a low mole percentage of poly(ethylene glycol) (PEG)-conjugated lipids into pH-sensitive liposomes confers prolonged circulation times to these liposomes, which are otherwise cleared rapidly. While the incorporation of PEG–lipids reduces the pH-dependent release of encapsulated fluorescent markers in vitro, it does not hinder the cytoplasmic delivery of the markers per cell-associated liposome. This suggests that intracellular delivery is not dictated simply by the destabilization of the liposomes. Antibodies or ligands to cell surface receptors can be coupled to pH-sensitive or sterically stabilized pH-sensitive liposomes for targeting. pH-sensitive liposomes have been used to deliver anticancer drugs, antibiotics, antisense oligonucleotides, ribozymes, plasmids, proteins and peptides to cells in culture or in vivo.

Keywords: Liposomes; Phosphatidylethanolamine, cholesteryl hemisuccinate; Sterically stabilized liposomes; Poly(ethylene glycol); Prolonged circulation; Endocytosis; Low pH compartment; Intracellular delivery; Neoplastic drugs; Gene delivery; Antisense oligonucleotides; Ribozymes

Contents

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1. Introduction

The development of strategies to increase the ability of liposomes to mediate intracellular delivery of biologically active molecules has been the subject of intensive research activity. The application of such strategies would result in liposomes that could constitute crucial tools to improve the therapeutic efficiency of many drugs that exert their effect at the intracellular level.

The inclusion of lipids with fusogenic properties results in the formation of the so-called "fusogenic" or polymorphic liposomes, since these undergo a phase transition under acidic conditions, either in the absence or presence of biological membranes. Such liposomes are able to interact and promote fusion or destabilization of target membranes (either at the level of the plasma or endosomal membrane) and have been described to release efficiently the encapsulated material into the cytoplasm.

pH-sensitive 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 concept of pH-sensitive liposomes emerged from the fact that certain enveloped viruses developed strategies to take advantage of the acidification of the endosomal lumen to infect cells, as well as from the observation that some pathological tissues (tumors, inflamed and infected areas) exhibit an acidic environment as compared to normal tissues [1].

Different classes of pH-sensitive liposomes have been proposed in the literature according to the mechanism triggering pH-sensitivity [1–3]. The most commonly recognized concept involves the combination of phosphatidylethanolamine (PE) or its derivatives with compounds containing an acidic group (e.g. carboxylic group) that act as a stabilizer at neutral pH [4–6]. More recently, the use of novel pH-sensitive lipids, synthetic fusogenic peptides/proteins either encapsulated [7,8] or incorporated in the lipid bilayer [9–13], and association of pH-sensitive polymers with liposomes [14–16] have been reported.

It should be emphasized, however, that when considering intravenous administration, in order to reach target cells (such as tumor cells) and mediate cytoplasmic delivery, liposomes need to be stable in biological fluids and exhibit long circulation times. The use of lipids with high transition temperatures (distearoylphosphatidylcholine, DSPC; hydrogenated soy PC, HSPC), and the incorporation of cholesterol (Chol) and lipid conjugates such as phosphatidylethanolamine–poly (ethylene glycol) (PE–PEG), have led to a significant decrease of leakage of the encapsulated drugs during circulation or in the extracellular milieu. Moreover, such lipids also reduce non-specific interactions between the liposomes and serum proteins (opsonins), thus preventing liposome clearance by the cells of the reticuloendothelial system (RES). In addition, the use of liposomes of adequate size (below 150 nm) can contribute to the increase of the circulation time [17–19].

2. Mechanisms of intracellular delivery mediated by pH-sensitive liposomes containing phosphatidylethanolamine

2.1. Biophysical properties underlying the pH-sensitivity of liposomes

In contrast to the majority of the phospholipids, PE presents a minimally hydrated and small headgroup which occupies a lower volume as compared to the respective hydrocarbon chains, exhibiting a cone

shape (as opposed to the cylinder shape of bilayer-stabilizing phospholipids), thus hampering the formation of a lamellar phase [20,21]. The cone shape of PE molecules favors the establishment of strong intermolecular interactions between the amine and phosphate groups of the polar headgroups, justifying the strong tendency of these molecules to acquire the inverted hexagonal phase above the phase transition temperature (T_H) (for DOPE the T_H is 10 °C). Intercalation of amphiphilic molecules containing a protonatable acidic group (negatively charged at physiological pH) among PE molecules favors electrostatic repulsion and allows the formation of bilayer structures, leading to liposome formation at physiological pH and temperature [22,23]. This latter approach constitutes the basis for the biophysical mechanisms underlying the pH-sensitivity exhibited by PE-containing liposomes. While at physiological pH stable liposomes are formed, acidification triggers protonation of the carboxylic groups of the amphiphiles, reducing their stabilizing effect and thus leading to liposomal destabilization, since under these conditions PE molecules revert into their inverted hexagonal phase [1,24].

The choice of the amphiphilic stabilizers as well as its molar percentage with respect to the PE content are imposed by the desired properties of the liposomes, including the extent of cellular internalization, the fusogenic ability, pH-sensitivity and stability in biological fluids. Such properties determine the liposome efficacy to mediate cytoplasmic delivery of the encapsulated molecules [1,2,25].

2.2. Binding and cell internalization

It has been suggested that pH-sensitive liposomes are internalized more efficiently than non-pH-sensitive formulations [26,27]. This has been attributed to the tendency of PE-containing liposomes to form aggregates, due to the poor hydration of its headgroup, which can explain their high affinity to adhere to cell membranes [25,28].

In an attempt to further improve their binding and cellular internalization, different strategies have been explored based on receptor-mediated targeting through specific ligands coupled to the liposome surface. 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, exhibited a higher efficacy in mediating cytoplasmic delivery of their aqueous contents as compared to the same formulations lacking the antibody [29–31]. These results demonstrate that: (i) the extent of liposome internalization is a crucial step in the process of intracellular delivery and (ii) receptor-mediated endocytosis is more efficient than non-specific endocytosis. Nevertheless, although non-pH-sensitive immunoliposomes (containing PC instead of DOPE) are internalized as extensively as pH-sensitive immunoliposomes, their capacity to mediate cytoplasmic delivery of the encapsulated molecules is significantly lower [32,33]. 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.

Fig. 1 illustrates the main steps involved in the internalization and intracellular delivery mediated by pH-sensitive liposomes. Following binding to cells, the liposomes are internalized through the endocytotic pathway, with or without the involvement of clathrin-coated vesicles. Independently of the internalization process, liposomes will be retained in early endosomes, which mature into late endosomes. The potential of pH-sensitive liposomes lies in their ability to undergo destabilization at this stage, thus preventing their degradation at the lysosomal level, and consequently increasing access to the cytosolic or nuclear targets [28,34].

2.3. Destabilization of liposomes at the endosomal level

Studies involving the incubation of cells with lysosomotropic agents (e.g. ammonium chloride or chloroquine, which prevent endosome acidification) demonstrate that the efficacy of pH-sensitive liposomes depends on the pH decrease upon endosome maturation. Furthermore, kinetic studies have shown that liposomes composed of DOPE/OA, DOPE/palmitoylhomocysteine (PHC) or DOPE/dipalmitoylsuccinylglycerol (DSPG) [35] or of DOPE/cholesteryl hemisuccinate (CHEMS) [36] 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 indicating that cytoplasmic delivery occurs from early and late endosomes. However, the molecular mechanisms by which liposomes overcome

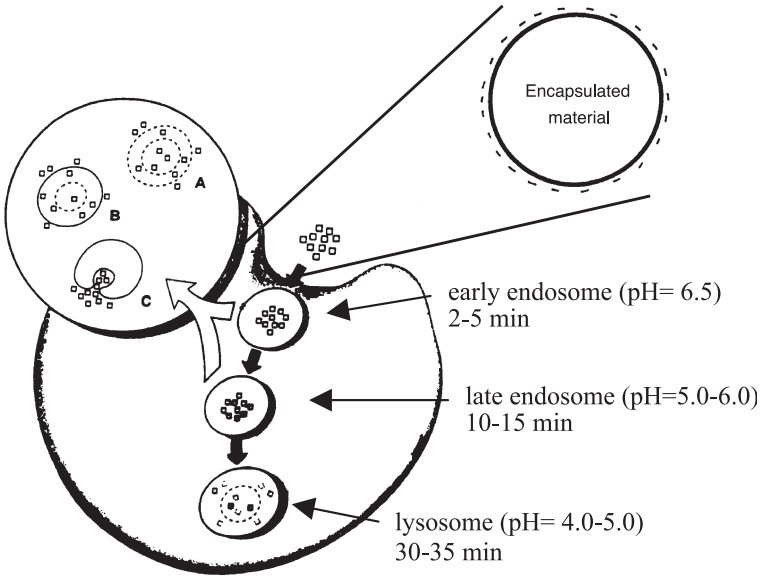

Fig. 1. Hypothetical mechanisms of internalization and intracellular delivery of pH-sensitive liposomes (adapted from Ref. [28]).

the barrier of cytoplasmic and endosomal membranes to release their contents into the intracellular space remain to be clarified. Three hypothetical mechanisms have been proposed (Fig. 1): (a) destabilization of pH-sensitive liposomes triggers the destabilization of the endosomal membrane, most likely through pore formation, leading to cytoplasmic delivery of their contents; (b) upon liposome destabilization, the encapsulated molecules diffuse to the cytoplasm through the endosomal membrane; and (c) fusion between the liposome and the endosomal membranes, leading to cytoplasmic delivery of their contents [28,37]. The fusogenic properties of PE associated with its tendency to form an inverted hexagonal phase under certain conditions suggest that hypotheses (a) and (c) are the most plausible.

It should be emphasized that the fusogenic properties of the liposomes do not always correlate with their efficacy in mediating intracellular delivery. Although aggregation, release of contents and lipid intermixing are observed at low pH (5.0) with DOPE/CHEMS liposomes, no intermixing of aqueous contents takes place [38]. Nevertheless, these liposomes are efficient in delivering their encapsulated contents into cultured cells [27].

Independently 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. Studies performed with high molecular weight proteins (e.g. DTA and BSA), demonstrate 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 (calcein) [27].

2.4. DOPE as a crucial component to promote cytosolic delivery

The incorporation of PEG (2000)–distearoyl-phosphatidylethanolamine into the membrane of DOPE/CHEMS (6:4 molar ratio) decreased significantly the pH-dependent release of a charged water-soluble fluorophore, calcein, from liposomes suspended in either buffer or cell culture medium (Fig. 2A) [39]. Surprisingly, the efficacy of such PEG-grafted pH-sensitive liposomes to promote intracellular delivery of their aqueous contents upon incubation with differentiated THP-1 cells remained unaltered (Fig. 2B), as demonstrated by a flow cytometry assay involving dual fluorescence labeling of the liposomes [39]. This lack of correlation between data obtained from biophysical assays generally used to assess pH-sensitivity, and the efficiency of the liposomes to interact with

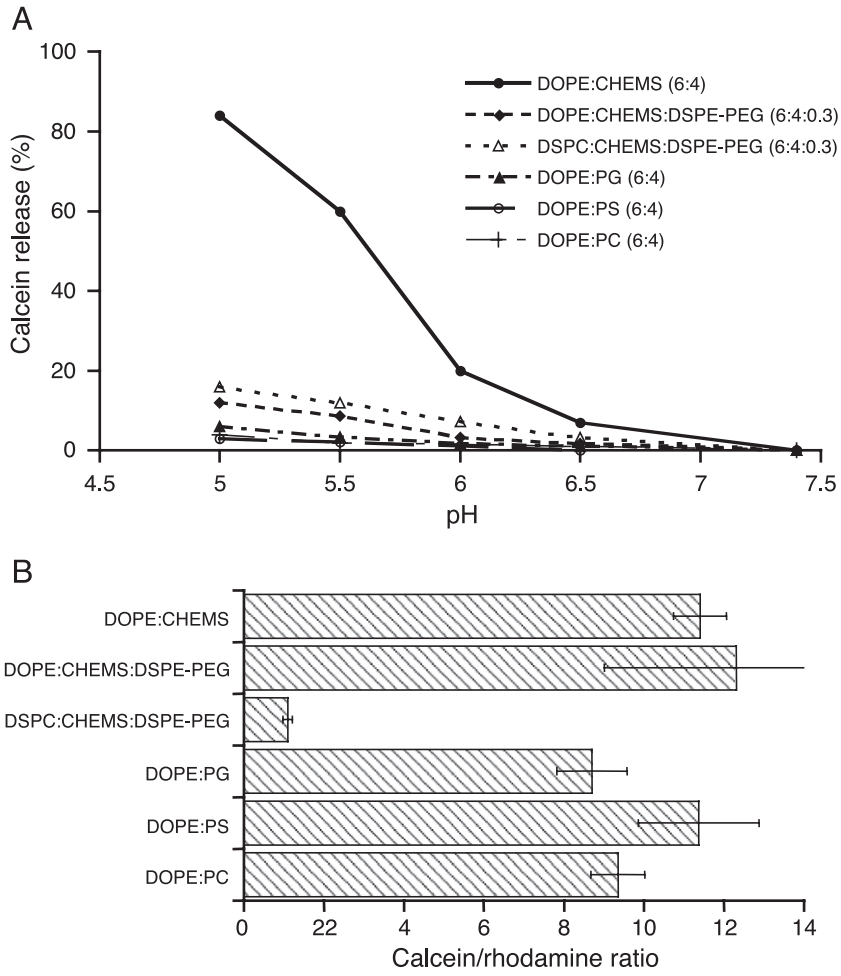

Fig. 2. (A) pH-sensitivity of DOPE-containing liposomes. Calcein-encapsulating liposomes were incubated for 10 min at 37 °C in MES-buffered saline at different pH values. The fluorescence intensity of calcein was measured before and after the addition of Triton X-100. (B) Efficacy of intracellular delivery mediated by different liposome formulations, using a flow cytometry-based assay. Liposomes encapsulating calcein at a self-quenched concentration (80 mM) and containing 1 mol% rhodamine–PE in the membrane were incubated with differentiated THP-1 cells and analyzed by flow cytometry. Mean rhodamine fluorescence values reflect the binding and uptake of liposomes, and the mean calcein fluorescence indicates intracellular dequenching of the dye fluorescence. The calculated ratio of calcein to rhodamine fluorescence is taken as a measure of the amount of aqueous marker released intracellularly per cell-associated liposome (reproduced with permission [39,41]).

cells suggests that the mechanisms by which pH-sensitive liposomes (both plain and sterically stabilized) mediate intracellular delivery of their contents are not simply dictated by the release of contents as a result of the decrease of endosomal pH.

These results were confirmed by Hong et al. [40], who showed that liposomes composed of DOPE, DPSG and DSPE–PEG (up to 5%) are pH-sensitive, plasma stable and have a long circulation time in the blood. Furthermore, they are able to release an entrapped marker more rapidly in tumor tissue homogenates (where the pH is lower than normal healthy tissues) in comparison with non-pH-sensitive DPPC/Chol/DSPE–PEG liposomes.

We have investigated the mechanisms by which pH-sensitive liposomes interact with cells and mediate the intracellular delivery of their aqueous contents. In contrast to what was observed for DOPE/CHEMS liposomes, liposomes composed of DOPE/phosphatidylglycerol (PG), DOPE/phosphatidylserine (PS), or

DOPE/PC did not exhibit any pH-sensitivity, even when incubated under very acidic conditions (Fig. 2A). Surprisingly, however, DOPE-containing liposomes, shown to be non-pH-sensitive by biophysical assays, mediated cytoplasmic delivery of their contents as efficiently as well known pH-sensitive formulations (e.g. DOPE/CHEMS) (Fig. 2B). However, among the different formulations studied, DOPE/CHEMS liposomes were those with the highest extent of cell association [41].

Overall, these findings suggest that the processes underlying the intracellular efficacy of the different DOPE-containing liposomes involve more complex mechanisms than the mere decrease of the endosomal pH. On the other hand, the combination of these results with those reported previously [39] indicates that the presence of DOPE is the crucial factor determining the ability of such liposomes to undergo destabilization upon acidification of the endosomes. In fact, 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 inability to mediate intracellular delivery was observed.

In this context, it is interesting to note that studies performed by Vidal and Hoekstra [42] 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 crucial to the fusion process. This specific effect of PE or DOPE to promote liposome–endosome interactions can be explained by the low hydration of its polar headgroup as compared to the significant repulsive forces associated with the hydration layer of PC or DSPC polar headgroups [26]. Therefore, the presence of DOPE enhances the hydrophobicity of the liposomal membrane, thus facilitating energetically favorable interactions between lipid bilayers. Furthermore, DOPE tends to assume an hexagonal inverted phase (H_{II}) leading to the formation of non-lamellar structures [43]. This may constitute a key element to trigger endosomal destabilization, thus leading to cytoplasmic delivery of their contents. This proposal partially explains our observations on the striking similarity of the efficacy of intracellular delivery mediated by all DOPE-containing formulations.

Vidal and Hoekstra [42], have also demonstrated that treatment of endocytotic vesicles with trypsin strongly reduced their interaction with PE-containing liposomes, thus providing evidence that endosome-associated proteins play a major role in this process. Based on these findings, these authors suggest that such proteins may not only be involved in the process of membrane fusion, but also promote liposome aggregation, which favors their destabilization. Assuming that this hypothesis can be extended to liposome–endosome interactions at the lumenal surface of endocytotic vesicles, it could justify the disparity of results between the biophysical studies (in buffer) and those involving cultured cells, in terms of acidification-induced destabilization. Similar conclusions were obtained in lipid mixing studies involving pH-sensitive liposomes and human erythrocyte ghosts, where a decrease in the extent of lipid mixing was observed by cleaving the sialic acid residues of the glycocalyx [27].

Results obtained from studies with agents that interfere with the endocytotic pathway also contributed to a better understanding of the mechanisms underlying liposome–cell interactions. The drastic reduction of the efficacy of intracellular delivery observed for all the formulations tested when their internalization was inhibited (using a mixture of antimycin A, sodium fluoride and sodium azide) indicates that such liposomes utilize the endocytotic pathway (either mediated by clathrin or non-clathrin coated vesicles) to promote the intracellular release of their contents. On the other hand, the strong inhibition observed for the calcein/rhodamine fluorescence ratio when the cells were treated with lysosomotropic agents demonstrate clearly that acidification of the endocytotic vesicles is crucial to the intracellular delivery mediated by the liposomes [39,41].

It should be noted that, despite the similarity of the efficacy of intracellular delivery evaluated in terms of calcein release, observed among the different liposomes tested, such findings cannot be extrapolated directly to other types of encapsulated molecules, particularly to those with high molecular weights [27]. In addition, we have reported that the ability to mediate intracellular delivery of molecules with relatively high molecular weight (e.g. antisense oligonucleotides) is significantly higher for liposomes composed of DOPE/CHEMS compared to other

DOPE-containing liposomes [44]. Formulations that were shown not to be pH-sensitive in buffer (DOPE/PG, DOPE/PS and DOPE/PC) were those that proved to be less efficient in releasing large molecules into the cytoplasm.

3. Long-circulating DOPE-containing pH-sensitive liposomes

A critical issue in the in vitro and in vivo use of pH-sensitive liposomes relates to the possibility that properties like stability, pH-sensitivity and cell affinity may be changed as a consequence of their interaction with components of biological fluids or with cells [1]. Extensive destabilization of DOPE/OA liposomes was observed upon their incubation with 90% human plasma at physiological pH [45]. Extraction of OA from the liposomes by serum albumin was considered to be responsible for this destabilization process. Nevertheless, this effect was shown to be dependent on the size of the liposomes, since liposomes with an average size smaller than 200 nm were more stable in serum than larger liposomes, although the opposite was observed when phosphate buffered saline (PBS) was substituted for serum. According to Liu and Huang [45], this stabilizing effect arises from the fact that the high membrane curvature of the liposomes of small size may favor protein incorporation, like apoprotein A1 of the high density lipoproteins (HDL), to replace the extracted OA. This increase in liposome stability is accompanied, however, by a significant reduction in pH-sensitivity, thus constituting a limitation for their appropriate use in vivo.

Several different strategies aimed at improving the biostability of pH-sensitive liposomes have been described, including the incorporation of a third component to confer stability to the lipid bilayer. Inclusion of Chol in DOPE/OA liposome formulations resulted in a significant increase in plasma stability, without decreasing their pH-sensitivity [5]. The use of other amphiphilic stabilizers that were shown to be resistant to the extraction by albumin (such as Chol derivatives—CHEMS, or lipids with double acyl chains—DPSG) resulted in the formation of liposomes exhibiting higher stability in biological fluids, while maintaining their pH-sensitivity [25,46].

In addition to the limitations related to stability and changes in pH-sensitivity, the in vivo efficacy of pH-sensitive liposomes depends strongly on the interactions with serum components (opsonins) that influence their pharmacokinetics and biodistribution. Although the number of studies describing the in vivo use of pH-sensitive liposomes is scarce, the consensus is that upon their intravenous administration these liposomes are cleared rapidly from blood circulation, accumulating in the liver and spleen [1,30,39,47]. On the other hand, the tendency of pH-sensitive liposomes to aggregate in the presence of biological fluids may explain their accumulation in the lungs [30]. Despite the fact that pH-sensitive liposomes exhibit a higher affinity to macrophages than non-pH-sensitive liposomes [1], their pharmacokinetics and biodistribution pattern are essentially the same [48]. In vivo studies using pH-sensitive immunoliposomes (DOPE/OA) demonstrated that the presence of an antibody at the liposomal surface affects neither their pharmacokinetics (namely the blood clearance rate) nor their biodistribution [49].

Both properties, i.e. pH-sensitivity and prolonged circulation time, are highly desirable for the delivery of therapeutic macromolecules, such as nucleic acids, to cells. pH-sensitive liposomes that can circulate in the blood for long periods and deliver encapsulated macromolecules to target cells may be useful in this regard. In vivo studies using the ganglioside GM_1 were shown to confer relatively prolonged residence in circulation to pH-sensitive liposomes composed of DOPE and DPSG [47].

The inclusion of lipids with covalently attached PEG in liposomes of various (non-pH-sensitive) compositions has been shown to overcome the problem of their rapid removal by the RES [50–53]. One of the first compositions of liposomes with the combined properties of pH-sensitivity and prolonged circulation in vivo was developed in our laboratory [39]. The blood clearance curve of ^{111}In encapsulated in sterically stabilized pH-sensitive liposomes (Fig. 3A) was similar to that of previously developed liposomes with prolonged circulation time [54,55]. A substantial percentage of liposomes (8.5%) remained in the blood after 24 h. In contrast, the radioactive marker encapsulated in regular pH-sensitive liposomes was almost completely eliminated from the bloodstream within 0.5 h. The $t_{1/2}$ of control

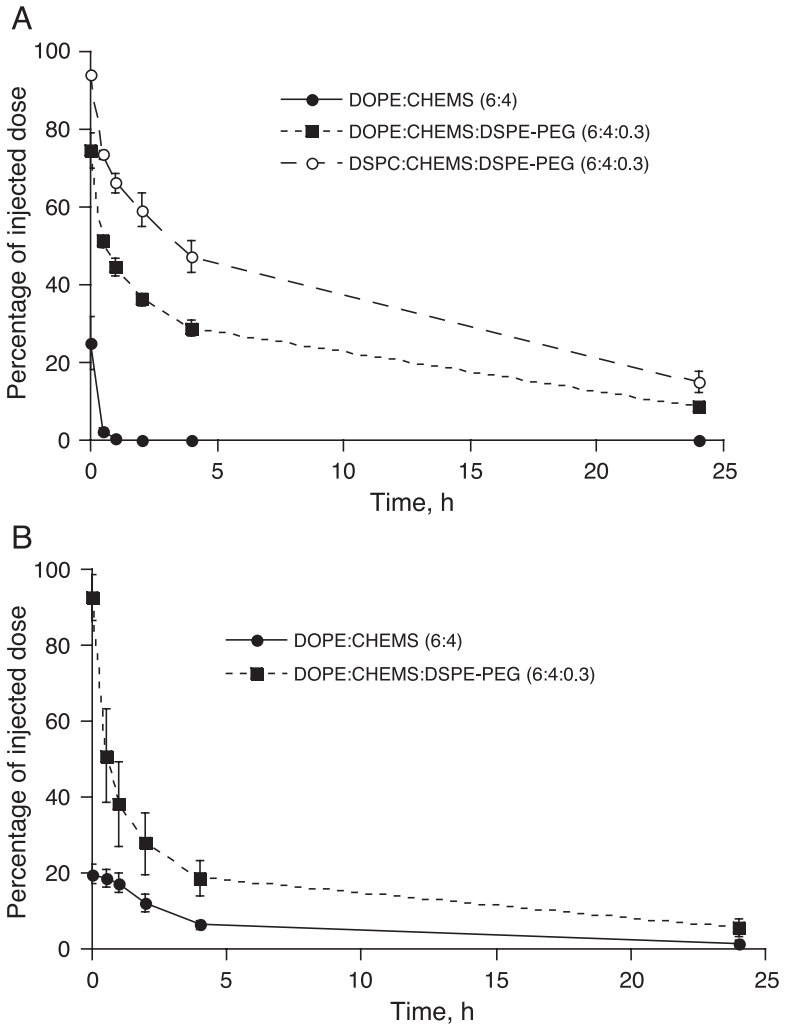

Fig. 3. Blood clearance curves of regular pH-sensitive, sterically stabilized pH-sensitive, and control sterically stabilized liposomes. ^{111}In-loaded liposomes (A) or liposomes containing lipid-associated ^{111}In (B) were injected intravenously into rats. Blood samples were taken after various times, and radioactivity was determined in a gamma counter (the first blood sample was drawn immediately after injection at time 0). Percent of injected dose was calculated and plotted against time. ▲, DOPE/CHEMS (6:4) liposomes; ●, DOPE/CHEMS/PE–PEG (6:4:0.3) liposomes; ■, DSPC/CHEMS/PE–PEG (6:4:0.3) liposomes (reproduced with permission [39]).

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 ($1071 \pm 151\%$ and $629 \pm 52\%$ dose h/ml, respectively), presumably because a larger number of the latter was taken up rapidly by the liver and spleen (Table 1 and Fig. 3A, time = 0 point), and therefore did not appear in blood samples. It is important to note that the area under the curve for regular pH-sensitive liposomes ($6.47 \pm 1.24\%$ dose h/ml) was about 100-fold lower than that for sterically stabilized pH-sensitive liposomes. The radioactive marker encapsulated in the former liposomes was eliminated from the circulation either 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. To discriminate between these possibilities we associated

Table 1
Tissue distribution of liposome-encapsulated [^{111}In] 24 h after intravenous injection in rats (adapted from [39])

Tissue	Percent of injected ^{111}In dose per g in tissues		
	DOPE/CHEMS	DOPE/CHEMS/PE–PEG	DSPC/CHEMS/PE–PEG
Liver	5.46 ± 1.83	2.01 ± 0.23	0.76 ± 0.28
Spleen	4.15 ± 0.6	22.06 ± 2.68	6.27 ± 0.33
Heart	0.03 ± 0.02	0.16 ± 0.01	0.27 ± 0.08
Kidneys	0.12 ± 0.01	0.24 ± 0.03	0.33 ± 0.04
Lung	0.03 ± 0.01	0.17 ± 0.02	0.25 ± 0.01
Skin	0.01 ± 0	0.03 ± 0.01	0.05 ± 0.01
Bone	0.05 ± 0.02	0.53 ± 0.17	0.25 ± 0.03
Muscle	0.02 ± 0.01	0.02 ± 0	0.02 ± 0
Urine	2.22 ± 0.94	1.36 ± 0.28	1.89 ± 0.83

^{111}In with the lipid membrane of liposomes. Blood clearance curves of lipid-labeled liposomes (Fig. 3B) showed 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 indicates that the elimination of the liposome-encapsulated water-soluble marker, described above, was mostly due to removal of the liposomes from the blood by the RES.

^{111}In encapsulated in either long circulating pH-sensitive or control liposomes had similar patterns of distribution in vivo in that it primarily accumulated in the spleen and liver (Table 1). Injection of regular pH-sensitive liposomes resulted in a high level of ^{111}In in the liver. This observation and the rapid clearance of these liposomes from the circulation (Fig. 3) indicate that most of the liposomes are taken up within an hour by liver cells and that only a fraction of the encapsulated marker leaks out and is then excreted in urine (Table 1). Although 24 h after injection a higher fraction of the DOPE/CHEMS/PE–PEG liposomes was localized in the liver and especially in the spleen, compared with the non-pH-sensitive control DSPC/CHEMS/PE–PEG liposomes, the total urinary excretion of ^{111}In was also about 2-fold lower compared with the control liposomes (Table 1). This result, together with the observation that the $t_{1/2}$ (and hence the clearance rate) of both sterically stabilized liposomes were similar, suggests that a higher fraction of the contents of pH-sensitive liposomes was accumulated and retained in the liver and spleen, most likely because they were delivered into the cytoplasm and thus partially avoided the usual metabolic processing of liposome contents.

In another approach, DOPE-containing liposomes were stabilized in the bilayer form through 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) [56,57]. Liposomes stabilized with either a non-cleavable PEG (PEG−DSPE) or PEG−S−S−DSPE retained an encapsulated dye at pH 5.5. However, 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 concomitant destabilization of the DOPE liposomes. Pharmacokinetic studies suggested that PEG−S−S−DSPE was rapidly cleaved in circulation. In addition, therapeutic studies performed in a murine model of B-cell lymphoma demonstrated clearly that the developed pH-sensitive formulation targeted to the CD19 receptor was superior to stable, long-circulating, targeted non-pH-sensitive liposomes, despite the more rapid drug release and clearance of the pH-sensitive formulation [57].

Recently, a novel category of pH-sensitive liposomes based on the incorporation of a PEG–diortho ester–distearoyl glycerol conjugate (POD) has been described [58]. This conjugate, composed of a headgroup, an acid-labile diortho ester linker and a hydrophobic tail was shown to be stable at neutral pH for greater than 3 h, but degraded completed within 1 h at pH 5. Liposomes composed of 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 following 4 h. At pH 5–6, PEG headgroups are cleaved off, leading to liposome aggregation and release of most of their contents in 10–100 min. According to the authors, the fast kinetics of acid-catalyzed POD hydrolysis, gives these liposomes a great potential for the rapid delivery of drugs/genes at therapeutic sites where the decrease of pH is perhaps only one unit pH or less. The blood clearance pattern of these liposomes was monophasic, with an elimination half-life of 200 min, the liver and intestine being the main sites of accumulation of these liposomes. It remains to be seen whether the incorporation of other lipids such as Chol is able to further improve the blood half-life of these liposomes.

Adlakha-Hutcheon et al. [60] developed fusogenic liposomes composed of DOPE and Chol, together with the positively charged lipid, didodecyldimethylammonium chloride (DODAC). The mixture was stabilized in a bilayer organization by including PE–PEG (30:45:15:10). The cationic lipid was included to promote binding of the liposomes to the cells via electrostatic interactions. The use of PE–PEG with different acyl chains, leading to different exchange rates of this component from the lipid bilayer, provides the liposomes a time-dependent destabilization. Such a strategy was evaluated for the delivery of mitoxantrone. It was observed that the decrease in the length of the acyl chain of PE–PEG lipid (from DSPE to dimyristoylphosphatidylethanolamine (DMPE)) correlates with an increasing rate of liposome clearance from blood, as well as with an increasing drug leakage rate. In the case of liposomes composed of PEG–DMPE, almost 50% of the injected dose accumulated in the liver at 1 h, with only a small increase at later times, while for liposomes stabilized with PEG–DPPE liver accumulation occurred at later times (between 1 and 4 h). Less than 15% of the injected dose of PEG–DSPE-containing liposomes was observed in the liver after 24 h [59].

The therapeutic activity of mitoxantrone-containing liposomes was tested either against a pseudometastatic leukemia murine model (L1210) or a human colon subcutaneous xenograft model (LS180). In the former study, 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. Interestingly, despite the different pharmacokinetic properties referred to above, both fusogenic formulations exhibited similar therapeutic activity. This can be explained by the fact that in the leukemia model tumor cells are localized in readily accessible organs such as the liver and spleen [59].

In the treatment of distal (non-RES) tumor sites, such as in the case of the subcutaneous LS180 model, a different trend was observed. Liposomes containing PEG–DSPE presented the highest activity among the several treatments tested, while the ability of liposomes containing PEG–DMPE to delay initiation of tumor growth was even lower than that observed for free drug. In this tumor model, the increased therapeutic activity is consistent with the use of a fairly stable system that provides greater mitoxantrone bioavailability and delivery [59].

More recently, 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 [60,61].

4. Alternative strategies to generate liposomes that are fusogenic under acidic conditions

Although the development of pH-sensitive liposomes has been associated frequently with the incorporation of DOPE in the liposome formulation, other strategies have also been explored.

4.1. The use of novel pH-sensitive lipids

Results 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, showed that fusion ability of the liposomes increases under weakly acidic and acidic conditions [62]. This has been shown to result in intensive and diffuse cytoplasmic fluorescence as a consequence of intracellular delivery of calcein. According to their observations, the authors concluded that polymer-modified liposomes, upon endocytosis by CV-1 cells, transfer their content into the cytoplasm by fusing with the endosomal membrane [63]. This strategy has been utilized recently to target anti-BCG antibody-bearing pH-sensitive liposomes to tumor cells expressing BCG antigen [16].

More recently, three different approaches have been taken to generate pH-sensitive liposomes in the absence of DOPE [64–66]. Formulations consisting of 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. Compared to pH-sensitive liposomes incorporating DOPE, these liposomes showed improved

retention of pH-sensitivity in the presence of serum [65].

Another promising strategy consisted of preparing anionic pH-sensitive liposomes composed of diolein/CHEMS (6:4). The results show that these liposomes are stable at physiological pH, but undergo rapid aggregation and efficiently release 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, in contrast to what was observed for DOPE-containing liposomes [64].

Promising results in terms of pH-sensitivity and resistance to serum were also obtained using a novel liposome formulation composed of PC, CHEMS, oleyl alcohol (OAlc), and Tween-80 [66]. Compared to DOPE-based pH-sensitive liposomes, the above formulation showed much better retention of its pH-sensitive properties in the presence of 10% serum.

4.2. Co-encapsulation of synthetic fusogenic peptides and therapeutic molecules into liposomes

The liposomal co-encapsulation of therapeutic molecules with peptides has turned out to be a promising strategy to promote cytosolic delivery of hydrophilic molecules with limited access to subcellular compartments. Gelonin, a type I plant toxin, constitutes an example of such a compound known to inactivate ribosomes and arrest protein synthesis. The inability 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 antitumor activity. In order 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 [8]. Such a strategy resulted in a dramatic improvement on the cytotoxicity of encapsulated gelonin against the murine B16 melanoma cell line, relative to free gelonin or gelonin encapsulated alone in pH-sensitive liposomes. Interestingly, the inclusion of PEG strongly decreased the cytotoxicity of the pH-sensitive formulation co-encapsulating gelonin and listeriolysin O. It remains to be seen whether the inclusion of PEG compromises the cell uptake and/or the pH-sensitivity properties of the liposomes [8].

A similar strategy has been explored by Mastrobattista et al. [7], who have demonstrated 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 because functional characterization studies showed its ability to induce fusion between liposome membranes and leakage of liposome-entrapped compounds when exposed to low pH. Moreover, co-encapsulation of diINF-7 into DTA-containing immunoliposomes resulted in drastically 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 [7].

4.3. Association of synthetic fusogenic peptides/proteins with cationic liposomes

Since cationic liposome/DNA complexes are thought to enter cells primarily via endocytosis, it has been hypothesized 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 enhancement of transfection activity over that of Lipofectin alone, by a factor of 2–7 [67]. The levels 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 [68]. The association of the pH-sensitive peptide GALA [69,70] 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 [71].

The ability of certain peptides to induce endosomal destabilization due to their fusogenic properties may also be extended to proteins. In fact, studies on the mechanisms of gene delivery mediated by transferrin-associated lipoplexes performed in our laboratory have indicated that besides triggering internalization of the complexes, transferrin may also play a role in the cytoplasmic delivery of DNA by facilitating endosome destabilization [72]. In fact, transfection experiments carried out with cells pre-treated with drugs that prevent acidification of the endosomal lumen (bafilomycin A1 and chloroquine) showed 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 [P. Pires, S. Simões, N. Düzgüneş, M.C. Pedroso de Lima, unpublished data]. As opposed to the observation of diffuse 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. Furthermore, recent studies on the kinetics of the initial steps involved in lipoplex–cell interactions have indicated that association of transferrin to lipoplexes increases the extent of fusion with endosomes. Overall, these results reinforce the hypothesis that transferrin acquires fusogenic properties under acidic conditions via exposure of hydrophobic domains, thus facilitating endosomal disruption and intracellular release of DNA [72,73].

Alerted by these results and by earlier reports describing the ability of albumin to promote membrane fusion under acidic conditions [74,75], we demonstrated that albumin could also function as a fusogenic protein that destabilizes endosomes under acidic conditions, thus enhancing intracellular gene delivery and transfection activity [76].

4.4. Association of pH-sensitive polymers with liposomes

Over the last few years, a number of studies from Leroux and collaborators have demonstrated the potential of alkylated *N*-isopropylacrylamide (NIPAM) copolymers to confer pH-sensitivity to liposomes. Complexation of hydrophobically-modified copolymers of NIPAN (either randomly or terminally alkylated) with EPC/Chol liposomes resulted in an enhancement of in vitro release of both highly-water soluble markers and amphipathic drugs upon acidification [14,77,78]. Liposomes anchoring randomly-alkylated NIPAM and containing ara-C were shown to mediate a higher cytotoxicity towards J774 macrophage-like cells as compared to non-pH-sensitive liposomes [78]. More recently, different NIPAM based copolymers were synthesized and evaluated in terms of their ability to confer pH-sensitivity and serum stability [78–80] as well as steric stabilization to liposomes [79,80].

Coating of liposomes with terminally alkylated NIPAM copolymer promotes steric stabilization of liposomes, thus leading to prolonged blood circulation. Nevertheless, since this effect was still considered insufficient for in vivo purposes, co-incorporation of PEG–lipid derivatives into the liposomal surface was assessed. As expected, a significant increase in blood circulation time was observed, blood clearance profiles being essentially similar to that observed for stealth liposomes lacking NIPAM. Consistent with previous observations, the stabilizing effect of PEG significantly reduced the liposome pH-sensitivity conferred by the copolymers [39,79].

5. Applications of long circulating pH-sensitive liposomes for the delivery of therapeutic molecules

The versatility of pH-sensitive liposomes has been well illustrated in a wide range of applications. These include: (a) the transport of fluorescent probes that allowed not only to evaluate the efficacy of different liposome compositions, but also to clarify the mechanisms involved in intracellular trafficking; (b) the efficient delivery of neoplastic drugs or recombinant proteins; (c) the intracellular transport of antigens, aiming at clarifying the intracellular pathways involved in processing and presentation of antigens as well as at enhancing the immune response to tumor cells; and (d) the intracellular transport of genetic material for application in gene and antisense therapies. Examples of applications of pH-sensitive liposomes for the transport and intracellular delivery of therapeutic agents are shown in Table 2.

To improve the therapeutic efficacy of drugs entrapped in pH-sensitive liposomes, a large amount

Table 2
Therapeutic applications of pH-sensitive liposomes

Liposome formulation (composition and method of preparation)	Encapsulated material	Target disease/therapeutic applications	Reference
	Drugs		
DOPE/DSPG/DSPE–PEG (7:3:5) (reverse-phase evaporation)	Methotrexate	Cancer therapy	[40]
PC/DDAB/CHEMS/Tween-80/ folate–PE–PEG (25:25:49:1:0.1)	Cytosine-β-D-arabinofuranoside	Cancer therapy (KB human oral cancer cells)	[65,66]
PC/CHEMS/Tween-80/OAlc/folate–PEG–Chol (50:50:2:80:0.5) (freeze–thawing)			
DOPE/CHEMS/PEG–S–S–DSPE/ MAL–PEG–DSPE[anti-CD19] (6:4:0.12:0.06) (hydration method)	Doxorubicin	Cancer therapy (hematological malignancies, B-lymphoma)	[57]
DOPE/*N*-succinyl-DOPE (70:30)	Gentamicin	Intracellular infections	[93]
DOPE/*N*-glutaryl-DOPE (70:30)			
DOPE/*N*-succinyl DOPE/Chol/PEG–ceramide (35:30:30:5) (freeze–thawing)			[94]
DOPE/CHEMS	Staurosporin	Inflammatory processes (endotoxin shock). Suppression of tumor necrosis factor (TNF) production	[95]
	Antisense oligonucleotides		
DOPE/CHEMS (6:4)	Against the HIV-1 RRE (15-*mer*)	Inhibition of HIV-1 replication in macrophages	[44]
DOPE/CHEMS/PE–PEG(6:4:03) (reverse-phase evaporation)			
DOPE/OA/Chol (1:1.3:0.4) (freeze–thawing)	Against the start codon AUC of Env gene mRNA of Friend retrovirus (15-*mer*)	Inhibition of the translation of Env protein of Friend retrovirus in cultured cells	[96–99]
DOPE/CHEMS (3:2) (freeze–thawing)	Against sodium/myo-inositol cotransporter mRNA (20-*mer*)	Bipolar affective disorders (inhibition of inositol uptake in astrocytes)	[100]
DOPE/CHEMS (3:2) (freeze–thawing)	FICT-labeled antisense oligonucleotide	Cancer therapy (NG 108-15 neuroblastoma and glioma cells)	[101,102]
PE/CHEMS/Chol (7:4:2) (reverse-phase evaporation)	^{32}P-TJU-2755 against TNF-α mRNA	Endotoxemia: immune reactions: inflammatory diseases	[103]
	Reporter genes		
DOPE/*N*-citraconyl-DOPE/Chol/folate–PEG–DOPE (45.8:10:40:0.1)	pDTS β-gal condensed with poly-L-lysine	Cancer therapy (transfection of KB human oral cancer cells)	[87]
DDAB/CHEMS/folate–PE–PEG (30:70:0.1) (freeze–thawing)	pCMV-Luc condensed with poly-L-lysine		[64]
Diolein/CHEMS (6:4) (ethanol injection)	pCMV-Luc condensed with poly-L-lysine		[65]
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	[86]
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-leukemia cells	[82]
	Proteins, peptides		
DOPE/DOSG/surfactant protein A	Superoxide dismutase	Respiratory insufficiency	[104]

(continued on next page)

Table 2 (*continued*)

Liposome formulation (composition and method of preparation)	Encapsulated material	Target disease/therapeutic applications	Reference
DOPE/DOSG (1:1) (hydration method)		Prevention of nitric oxide-dependent motor neuron death induced by trophic factor withdrawal	[105]
POPE/CHOH/MPL (7:3:0.01) (freeze–thawing)	V3 peptides	Immunomodulating adjuvant system for the development of HIV and other viral vaccines	[106]
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 epitope peptide	Tumor therapy mediated by CTL that recognize tumor-associated antigen	[107]
POPE/CHOH/MPL (7:3:0.01) (freeze–thawing)		Induction of antigen specific CTL response in vivo	[108]

of work has been devoted to confer tissue and cell specificity by targeting the liposomes to cell surface receptors. For this purpose, different ligands have been coupled covalently to the liposome surface or to the distal end of PEG–lipid conjugates. These include monoclonal antibodies against the H-2K^k receptor (expressed in several types of tumor cells) [49], E-selectin (on activated vascular endothelial cells) [81], CD-19 (on B-lymphoma cells) [57], CD3 (on T-leukemia cells) [82], P-glycoproteins (on endothelial cells) [83] and BCG antigen [16]. pH-sensitive liposomes targeted to the folate receptor through coupling of folic acid to the distal end of PEG molecules have been used recently to deliver neoplastic drugs [65,66] and plasmid DNA [65,84,85].

Under the same experimental conditions, pH-sensitive liposomes have been described to be less efficient than cationic liposomes to mediate intracellular gene delivery into mammalian cells [86]. Different factors, not mutually exclusive, can explain this difference 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 efficient in protecting DNA against nucleases and in mediating its nuclear entry.

Recently, different approaches have been developed to circumvent the limitations of pH-sensitive liposomes for nucleic acid delivery. These approaches share a similar strategy that essentially consists of complexing preformed pH-sensitive liposomes with plasmid DNA precondensed with a cationic polymer [64,65,82,84,87]. In comparison with "conventional" liposome formulations, the reported 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), resulting in improved transfection efficiency. Reddy et al. [87] were able to further optimize this strategy by promoting nuclear entry of DNA, which was achieved by incorporation of a nuclear targeting sequence into the plasmid DNA. Although these approaches generated satisfactory results in vitro, their pharmacokinetics, biodistribution and in vivo gene delivery potential remain to be demonstrated.

The use of liposomes exhibiting both pH-sensitivity and steric stabilization for nucleic acid delivery has not been reported extensively. This approach was evaluated in our laboratory aiming at mediating intracellular delivery of antisense oligonucleotides and ribozymes to inhibit virus production in HIV-infected macrophages derived from human peripheral blood monocytes [44]. As can be observed in Fig. 4, a 15-*mer* anti-Rev-responsive element (RRE) phosphorothioate oligonucleotide inhibited viral p24 production by 91% when delivered by pH-sensitive liposomes, while the free (unencapsulated) oligonucleotide was not active against HIV infection in macrophages. The oligonucleotide was also effective when delivered by sterically stabilized pH-sensitive DOPE/CHEMS/PE–PEG liposomes, but not when encapsulated in non-pH-sensitive liposomes. A non-specific oligonucleotide encap-

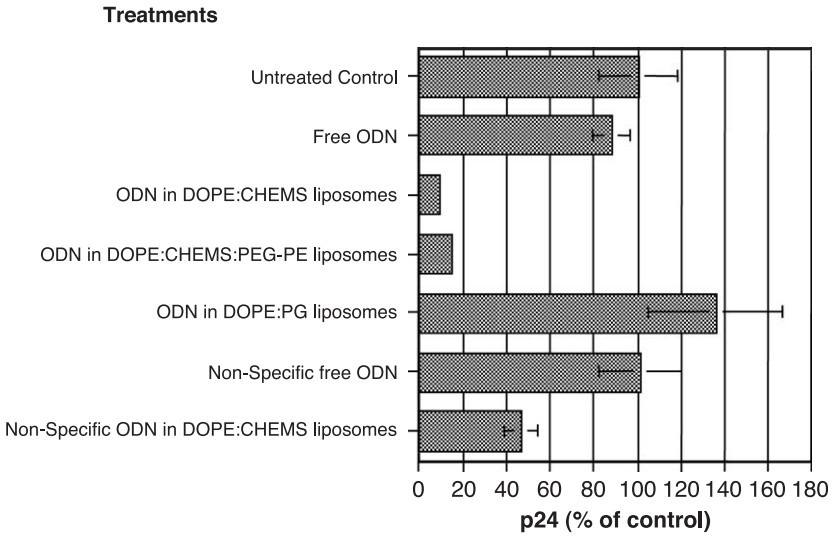

Fig. 4. Inhibition of HIV-1 production in human macrophages by free and liposome-encapsulated anti-RRE 15-*mer* oligonucleotide (ODN) at a concentration of 3 μM. The viral p24 values are given as the percent of untreated controls (reproduced with permission [44]).

sulated in pH-sensitive liposomes had no effect at 1 μM, but at 3 μM it inhibited HIV infection by 53%.

Similar experiments were performed with a 38-*mer* chimeric ribozyme complementary to HIV 5′-LTR. Cationic liposome-mediated delivery of this ribozyme to HIV-1-infected cells was not effective in reducing virus production under conditions where the delivery method was not toxic to the cells [88]. When this ribozyme was delivered to HIV-infected macrophages by pH-sensitive liposomes, however, virus production was inhibited by 88%, while the free ribozyme caused a decrease of only 10% [44]. As referred to above, 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 [39]. Since these liposomes circulate for prolonged periods and can localize in lymph nodes after intravenous or subcutaneous injection [89], they may be useful for the delivery of antisense molecules to lymph nodes where active HIV replication takes place [90,91].

As reported previously, other fusogenic liposome formulations exhibiting long circulation times have also been applied for nucleic acid delivery. Programmable fusogenic vesicles (PFV), previously mentioned 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 tumor-bearing mice. It is important to point out that gene expression in tumor tissue was significantly higher than that observed for cationic liposome/DNA complexes [60].

PFV have also been evaluated as carriers for the intracellular delivery of antisense oligonucleotides. PFV containing PEG–ceramide C_{14} were shown to enhance intracellular delivery of oligonucleotides relative to PFV displaying faster (with PEG–ceramide C_8) or slower (with PEG–ceramide C_{20}) rates of destabilization. A significant reduction (about 20% and 25%) in the levels of bcl-2 mRNA was demonstrated upon treatment of cells with PEG–ceramide C_{14} liposomes containing the antisense oligonucleotide against the target proto-oncogene [61].

6. Concluding remarks

Soon after liposomes were introduced by Bangham et al. as models of biological membranes in biophys-

ical studies [92], their potential as carriers for biologically active molecules was recognized due to their biocompatibility and chemical and structural versatility. Different applications were envisaged, including vaccination (as immunological adjuvants), cosmetics, imaging (carrying contrasting agents) and for the transport and specific delivery of potent drugs (for cancer, ophthalmic, pulmonary and infectious diseases), as well as of nucleic acids, aiming at gene therapy applications. The liposomal delivery of highly charged or macromolecular drugs into cells presents the challenge of penetration through the endosomal membrane. This problem has been resolved partially by the development of pH-sensitive liposomes, and sterically stabilized pH-sensitive liposomes. The use of the latter liposomes, as well as their targeting to specific tissues, is very likely to facilitate the application of ribozymes, antisense oligonucleotides, triple helix forming oligonucleotides, short interfering RNAs and other macromolecular drugs to the therapy of infectious diseases and cancer.

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