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Lipid-based systems for the intracellular delivery of genetic drugs

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Summary

Currently available delivery systems for genetic drugs have limited utility for systemic applications. Cationic liposome/plasmid DNA or oligonucleotide complexes are rapidly cleared from circulation, and the highest levels of activity are observed in 'first pass' organs, such as the lungs, spleen and liver. Engineered viruses can generate an immune response, which compromises transfection resulting from subsequent injections and lack target specificity. A carrier, which can accumulate at sites of diseases such as infections, inflammations and tumours, has to be a small, neutral and highly serum-stable particle, which is not readily recognized by the fixed and free macrophages of the reticuloendothelial system (RES). This review summarizes lipid-based technologies for the delivery of nucleic acid-based drugs and introduces a new class of carrier systems, which solve, at least in part, the conflicting demands of circulation longevity and intracellular delivery. Plasmid DNA and oligonucleotides are entrapped into lipid particles that contain small amounts of a positively charged lipid and are stabilized by the presence of a polyethylene glycol (PEG) coating. These carriers protect nucleic acid-based drugs from degradation by nucleases, are on average 70 nm in diameter, achieve long circulation lifetimes and are capable of transfecting cells.

Keywords: gene therapy, plasmid DNA, antisense oligonucleotides, cationic liposomes, DNA encapsulation.

Abbreviations: BamHI, restriction enzyme; CL, cardiolipin; chol, cholesterol; DMRIE, 1,2-dimyristyloxy-3-(N,N-dimethyl-N-hydroxyethylammonium) propane; DODAC, N,N-dioleyl-N,N-dimethylammonium chloride; DOPE, dioleoylphosphatidylethanolamine; DOPC, dioleoylphosphatidylcholine; DOTAP, 1,2-dioleoyl-3-trimethylammonium propane; EPC, egg phosphatidylcholine; GFP, green fluorescent protein; HII phase, inverted hexagonal phase; LUV, large unilamellar vesicle; pCMV-CAT and pCMV-βgal, plasmids containing the cytomegalovirus (CMV) promoter and coding for the marker enzymes chloramphenicol acetyltransferase (CAT) and β-galactosidase (βgal); pDNA, plasmid DNA; PC, phosphatidylcholine; PA, phosphatidic acid; PE, phosphatidylethanolamine; PEG, polyethylene glycol; PEG-CerC$_8$, C$_{14}$ and C$_{20}$, polyethyleneglycol-modified ceramides with variable fatty acid chain lengths; PG, phosphatidylglycerol; POPC, 1-palmitoyl-2-oleoyl-phosphatidylcholine; PS, phosphatidylserine; PS-oligos, phosphorothioate oligonucleotides; Rh-dextran, rhodamine-labelled dextran; SPLP, stabilized plasmid-lipid particles.

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Introduction

Genetic drugs, such as antisense oligonucleotides, and plasmids containing therapeutic genes, have considerable potential for treatment of human diseases such as cancer, infections and genetic disorders. Antisense oligonucleotides are used to decrease expression of disease-causing genes, whereas plasmids delivered to cells can cause expression of therapeutic proteins (Crooke and Bennett 1996, Akhtar and Agrawal 1997). However, rapid breakdown and clearance from the blood compromise the effectiveness of these molecules for systemic treatment of disease following intravenous injection. In addition, antisense oligonucleotides and plasmid DNA are large molecules that do not readily penetrate target cell membranes to reach their sites of action inside the cell. As a result, the development of appropriate delivery systems is critical to the clinical success of nucleic acids as pharmaceuticals. Currently, the favoured delivery systems for gene transfer are genetically engineered viruses including retroviruses, adenoviruses, adeno-associated virus (AAV) and Herpes virus (Miller and Vile 1995, Vile *et al.* 1996, Friedmann 1997, Robbins *et al.* 1998). Engineered viruses are efficient for inserting foreign genes into cells. Disadvantages of viral vectors include that they can generate an immune response, which compromises transfection resulting from subsequent injections, and that they may become pathogenic (Yei *et al.* 1994, Hope *et al.* 1998). Viral gene delivery systems are also rapidly cleared from the circulation, limiting potential transfection sites to first pass organs such as the lungs, liver and spleen. As a result of these and other limitations, there has been substantial effort focused on constructing non-viral vectors, particularly on the use of cationic lipids.

This review summarizes lipid-based technologies for the delivery of nucleic acid-based drugs. General concepts are explained first, followed by a description of the steps involved in cationic liposome-mediated gene transfer into cells. Subsequently, the role of dioleoylphosphatidylethanolamine (DOPE) and cationic lipids for the delivery of genetic drugs is described in the broader context of membrane fusion and/or destabilization (disruption). Finally, the approach taken for the systemic delivery of genetic drugs is outlined.

General concepts

Plasmid DNA and oligonucleotides

Plasmids for gene transfer consist of a bacterial plasmid backbone containing a gene encoding either a reporter protein, which allows easy quantitation of gene expression, or a therapeutic protein. The most commonly used reporters are the genes coding for luciferase, green fluorescent protein (GFP) and β-galactosidase (β-gal). Usually, the pDNA vector contains additional genetic sequences such as strong viral promoters/enhancers for efficient gene expression, select-

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able markers (antibiotic or drug resistance), transcript stabilizers, and targeting elements. One of the problems associated with DNA is the susceptibility of the phosphodiester linkage to degradation by nucleases present in serum or the intracellular environment. Unprotected DNA is degraded within minutes. Its plasma half-life after intravenous injection into mice is about 5–10 min (Kawabata *et al.* 1995).

Antisense oligonucleotide technology involves much shorter sequences of nucleic acids than the typical plasmid designed for gene therapy, and as a result has more in common with conventional drug treatment. Antisense oligonucleotides consist of short synthetic single-stranded sequences of DNA, which can bind to complementary sequences in DNA or mRNA, thereby preventing transcription and translation, respectively (Helene and Toulme 1990). In contrast to plasmid DNA oligonucleotides can be chemically modified to become more resistant against degradation by nucleases present in serum and inside cells. For example, if one of the non-esterified oxygen atoms of the natural phosphodiester backbone is replaced with sulphur, the sequence is protected from intracellular nucleases for 24–48 h, compared to an intracellular half-life for the phosphodiester of only minutes (Hoke *et al.* 1991, Fisher *et al.* 1993, Crooke 1998, Hope *et al.* 1998). The serum half-life increases by a factor of 30 to about 9 h (Campbell *et al.* 1990, Akhtar *et al.* 1991, Gilar *et al.* 1997). The resulting phosphorothioate oligodeoxynucleotides are the most frequently used class of chemically modified oligonucleotides. Table 1 summarizes physicochemical properties of a typical plasmid DNA and of oligonucleotides and compares these parameters with those of 100 nm LUVs. The dimensions of plasmid DNA and liposomes are shown in figure 1. The sizes (longest dimension) and structures of a 4.4 kbp plasmid were obtained from electron micrographs published by Lewis *et al.* (1985).

Cationic liposomes

Cationic liposomes are the most widely and successfully used lipid-based vectors for gene transfer (Felgner *et al.* 1987, Gao and Huang 1995, Ledley 1995, Felgner 1997, Chonn and Cullis 1998). In addition, they have also been used for the delivery of RNA, antisense oligonucleotides and proteins (Malone *et al.* 1989, Debs *et al.* 1990, Bennett *et al.* 1992, Walker *et al.* 1992, Dwarki *et al.* 1993, Sells *et al.* 1995). The importance of cationic liposomes as gene carriers is reflected in the wide variety of commercially available cationic liposome formulations (see table II in Sorgi and Huang 1997). The vast majority of these formulations consists of a cationic lipid mixed with DOPE at a 1 : 1 molar ratio. The charge of the complexes is slightly positive to allow for interaction with negatively charged cell surfaces, thus increasing cellular uptake. The transfection efficiency of any given formulation is highly dependent on the cell line, type of cationic lipid (liposome formulation), and ratio of DNA to liposomes used (Felgner *et al.* 1987, Jarnagin *et al.* 1992, Stewart *et al.* 1992, Mok and Cullis 1997). The preparation procedure is simple. The cationic liposomes, usually vesicles with diameters ⩽100 nm, are mixed with DNA in a dilute

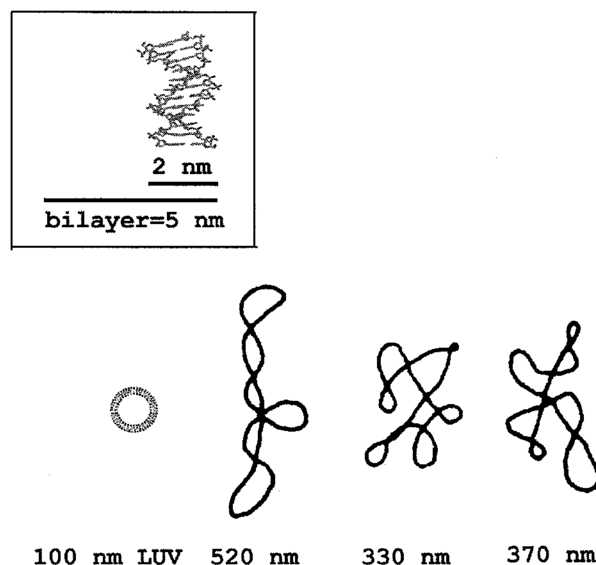

Figure 1. Comparison of the dimensions of a 100 nm LUV and a 4.4 kbp plasmid. Three different structures of the same 4.4 kpb plasmid are shown, together with their longest dimension. The bilayer thickness and DNA cross-section are not plotted according to their relative dimensions. They are shown on a correct scale in the inset. The plasmid structures were reproduced with permission from electron micrographs published by Lewis *et al.* (1985).

Table 1. Physico-chemical properties of plasmid DNA, oligonucleotides and liposomes. These parameters were obtained under the following conditions and assumptions: Plasmid DNA was modelled as a rodlike molecule with a circular cross-section of 2 nm. The contour (or extended) length of the plasmid was calculated using 0.34 nm/bp, and for the MW, an average MW of 650/bp was assumed. The MW of a 100 nm large unilamellar vesicle (LUV) was determined using 0.6 nm^2/lipid molecule for the lipid headgroup area and MWs between 630–760/lipid molecule, where 630 corresponds to the average molecular weight of a 1:1 DOPE/DODAC mixture and 670 is the MW of POPC. V_{in} is the internal aqueous volume of a 100 nm LUV, V_{mem} the volume of the lipid bilayer, O_{out} the outer surface area and O_{in} the inner surface area.

Physico-chemical property	Plasmid DNA pCMV-CAT	Typical oligo-nucleotide	100 nm LUV
Number of bases	8980	15–25	94700*
MW	2.9×10^6	$4.5–8 \times 10^3$	$60–70 \times 10^6$
Contour length (nm)	1500	< 10	100†
Volume (nm^3)	4.7×10^3	–	$V_{in}=3.8 \times 10^5$ $V_{mem}=1.4 \times 10^5$
Surface area (nm^2)	9.4×10^3	–	$O_{out}=3.1 \times 10^4$ $O_{in}=2.6 \times 10^4$

*Number of lipid molecules in a 100 nm LUV. †LUV diameter.

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solution. The complexes form spontaneously due to electrostatic charge interactions, which lead to liposome fusion and aggregation. The interaction between DNA and lipid is difficult to control, producing large complexes that have a very heterogeneous size distribution. Particle sizes range from 250 nm to> 1 μm. The major parameters determining the final product are the charge ratio, ionic strength of the media and the overall concentration of the reactants. Structural features revealed by electron microscopy include liposome-coated DNA strands (beads on a string), aggregates of liposomes intercalating DNA, DNA entrapped between the lamellae of aggregated multilamellar structures and tubular structures consisting of fused liposomes around DNA (Gershon *et al.* 1993, Sternberg *et al.* 1994, Gustafsson *et al.* 1995, Mok and Cullis 1997).

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Endocytosis

The primary route of internalization of liposomes by cells is the endocytic pathway via clathrin-coated pits (Straubinger *et al.* 1983, 1990, Daleke *et al.* 1990, Lee *et al.* 1992, Friend *et al.* 1996). Along this route, liposomes encounter compartments of progressively more acidic pH and are degraded together with their contents once they reach the lysosomes (Dijkstra *et al.* 1984, 1985, Yoshimura *et al.* 1995, Scherphof and Kamps 1998). Endocytosis is also the main mechanism by which DNA, as well as oligonucleotide-cationic liposome complexes, are taken up into the cell (Zhou and Huang 1994, Friend *et al.* 1996, Zelphati and Szoka 1996a,b). A main barrier in lipid-based drug delivery is the escape of hydrolytically sensitive material from degradation in lysosomes. The intracellular processing of liposomes and their contents is not well understood (Straubinger 1993). For example, how does the timing of release of liposomally entrapped material into endocytic compartments and its nature affect its intracellular distribution? Are there transport mechanisms other than these responsible for the rapid export of fluorescent dyes and drugs from cells (di Virgilio *et al.* 1989, Daleke *et al.* 1990, Cao *et al.* 1992, Steinberg 1994)? For example, Arabinoside C, entrapped in liposomes, is actively transported into the cytosol. Maximum cytotoxicity is attained if it is released from the liposomes into early endosomes (Brown and Silvius 1990). Furthermore, the differential effect of chloroquine with different cytofection formulations suggests that transfer to different endocytic compartments (early endosomes versus late endosomes or lysosomes) is necessary for transfection activity (Felgner *et al.* 1994).

All eukaryotic cells exhibit one or more forms of endocytosis (Mellman 1996, Robinson *et al.* 1996, Mukherjee *et al.* 1997). The best-characterized mechanism is receptor-mediated endocytosis via clathrin-coated vesicles. Clathrin-coated vesicles are also involved in liposome endocytosis (Chin *et al.* 1989). The clathrin-coated pit pathway is described below. The overall organization of the endocytic pathway is shown in figure 2. Extracellular macromolecules (ligands) bind to complementary cell-surface receptors and enter the cell together with solutes in clathrin coated vesicles (CCV) that pinch off from the cell surface. Their contents are delivered to early endosomes (EE) spread throughout the peripheral cytoplasm, where ligands and receptors are sorted to a variety of destinations. Their internal pH is only slightly acidic, ranging from 6.3–6.8. From here, endocytosed material is either directly sorted back to the plasma membrane, or may also pass through a separate, highly tubulated recycling compartment (RE), which in many cell types is located in the perinuclear region. Transit through EEs is very rapid (2–3 min) but takes longer through perinuclear REs. The transfer of material from EEs to late endosomes (LEs) involves carrier vesicles (CV) originating from EEs, which migrate on microtubules (MT) to the perinuclear region where they fuse with LEs. Ligands accumulate in LEs with a halftime of 10–25 min and encounter a pH of 5–5.5 (Schmid *et al.* 1988). The relationship between LEs and lysosomes is dynamic and not easily defined. Lysosomes contain a wide variety of hydrolytic enzymes and are the principal sites of intracellular digestion. It takes about 35 min for ligands to reach the lysosomes (pH $\leqslant$5) (Schmid *et al.* 1988). Endosomes communicate also with the biosynthetic pathway by vesicular transport. Newly synthesized lysosomal enzymes are delivered from the ER via the

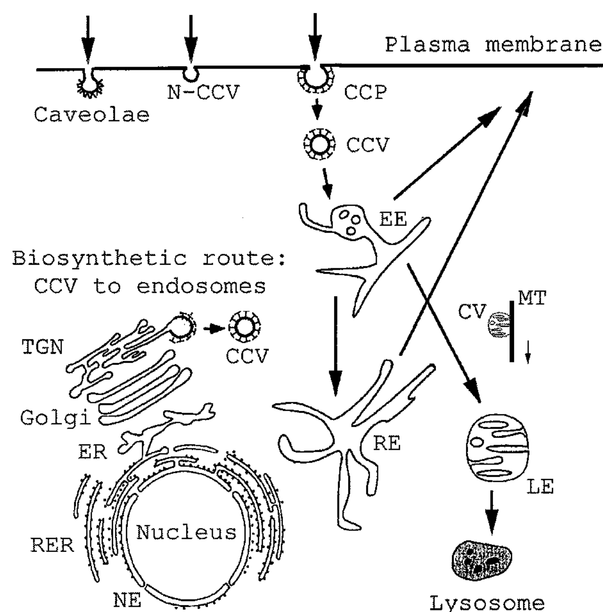

Figure 2. Organization of the endocytic pathway. Cells constantly take up material such as essential nutrients, chemical signals and also pathogens, from the extracellular medium by different forms of endocytosis. The best-characterized mechanism is the entry through clathrin-coated vesicles (CCV). However, other, non-clathrin-mediated mechanisms occur in parallel. CCV deliver their cargo to early endosomes. From there, part of the material is either directly recycled back to the plasma membrane or with a delay via recycling endosomes (RE) located in the peri-nuclear region. Material which is destined for degradation is further transported by carrier vesicles (CV) along microtubule tracks (MT) to late endosomes (LE) and finally to lysosomes, the principal sites of intracellular digestion. The endocytic pathway merges with the biosynthetic (secretory) pathway. CCV deliver digestive enzymes synthesized at the endoplasmic reticulum (ER) to endosomes. Further abbreviations found in this figure are: TGN, trans-Golgi-network; NE, nuclear envelope; N-CCV, non-clathrin coated vesicles and CCP, clathrin coated pits. This figure was adapted from Robinson *et al.* (1996).

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Golgi apparatus and the trans-Golgi-network (TGN) to endosomes and then routed towards lysosomes (Kornfeld and Mellman 1989). Cells such as macrophages and fibroblasts internalize their entire plasma membrane surface area twice every hour. The extracellular fluid thereby taken up corresponds to 25% of their internal volume. Each LDL receptor makes one round trip every 12 min, or 150 round trips in its 30 h lifespan (Brown *et al.* 1983).

Cationic liposome-mediated delivery of genetic drugs into cells

Plasmid DNA

Cationic liposomes are the most intensively investigated non-viral gene transfer vectors (Felgner *et al.* 1987, Gao and Huang 1995, Felgner 1997). Many steps of the cationic liposome-mediated gene transfer process have been identified. Factors which influence gene expression are: (1) uptake of the DNA-liposome complexes into the cell, (2) release from endo/lysosomal compartments, (3) dissociation of the DNA from its interaction with the cationic lipid, (4) DNA transport across the nuclear envelope, and (5) gene expression (transcription and translation).

The main route of entry of cationic liposome/DNA complexes into cells is by endocytosis (Zhou and Huang 1994, Friend *et al.* 1996). Uptake is facilitated by a net positive charge on the complexes. In a typical transfection experiment using DMRIE/DOPE, COS-1 cells take up approximately 3×10^5 plasmids/cell, while only 50% of the cells expressed the transgene (Zabner *et al.* 1995). Once the complexes are taken up into the cell they are transported to the lysosomes for degradation. In order for gene expression to occur, DNA has to escape the endocytic pathway and redistribute into the nucleus in intact form. Endosomal membranes appear to be extensively destabilized upon interaction with cationic liposome/DNA complexes. Transmission electron microscopy pictures, for example, show disrupted endosomal membranes (Zhou and Huang 1994, El Ouahabi *et al.* 1997). The authors' own experiments demonstrate release of rhodamine-labelled dextran (Rh-dextran, MW 10000) into the cytosol upon co-incubation with DOPE/DODAC liposome/DNA complexes (figure 3, bottom). At the same time, extensive mixing of the lipids in the complex with cellular membranes takes place (Wrobel and Collins 1995, Stegmann and Legendre 1997). It was further proposed that the destabilization of the endosomal membrane by the internalized complexes induces flip-flop of anionic lipids from the cytoplasmic-facing monolayer. Formation of a charge-neutral ion pair would then result in displacement of the DNA from the cationic lipid and release of the DNA into the cytoplasm (Xu and Szoka 1996). Most of the DNA, however, remains localized in endocytic compartments and is degraded. Only a small fraction escapes into the cytoplasm in intact form.

A further barrier in cationic liposome-mediated gene transfer is the translocation of the plasmid across the nuclear envelope (Zabner *et al.* 1995, Wrobel and Collins 1995, Zabner 1997, Pollard *et al.* 1998). It was found that microinjection of plasmid DNA into the cytoplasm of cells does not support substantial levels of gene expression. Direct introduction of DNA into the nucleus is required for high levels of expression to occur (Capecchi 1980, Mirzayans *et al.* 1992). Gene expression following cytoplasmic microinjection of naked DNA reaches a maximum 24 h post-injection (Pollard

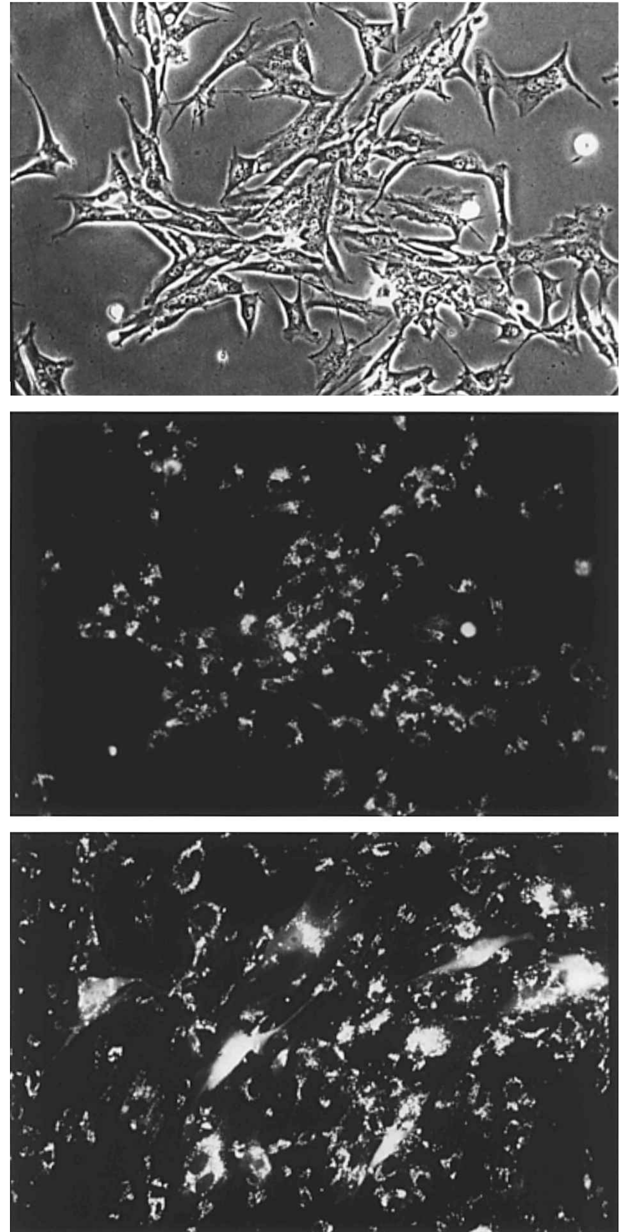

Figure 3. Membrane-destabilizing effect of DOPE, demonstrated by the cationic liposome mediated release of fluorescently labelled dextran into the cytosol. BHK cells were co-incubated with DOPE/DODAC or DOPC/DODAC cationic liposome/DNA complexes (±charge ratio 1.5 and 0.5 μg pCMV-βgal plasmid) and 1 mg/ml rhodamine-labelled dextran (Rh-dextran, MW 10000). After 24 h, release of Rh-dextran into the cytosol can only be observed for PE-containing complexes (bottom panel) but not for PC-containing complexes (middle panel). The top panel shows the corresponding phase contrast picture of the cells incubated with the PC complexes. Incubation with Rh-dextran alone resulted in punctate fluorescence (not shown).

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et al. 1998). Its efficacy is lower by a factor of 1000 compared to direct nuclear injection (10 000 copies of naked plasmid injected into the cytoplasm of COS-7 cells gave the same level of transfection (13%) as 10 copies injected into the nucleus) (Zabner *et al.* 1995, Pollard *et al.* 1998). Also, transgene expression is greater with plasmids (naked as well as complexed with cationic liposomes) that do not require nuclear transcription (e.g. T7 promoter-driven gene expression system together with T7 RNA polymerase) (Rose *et al.* 1991, Gao and Huang 1993, Zabner *et al.* 1995). Mitotic cells show increased transfectability (Zabner *et al.* 1995, Wilke *et al.* 1996, Mortimer *et al.* 1998, Zelphati *et al.* 1998). This limits the number of cells amenable to transfection to the fraction of cells undergoing cell division. These results demonstrate that trafficking of DNA from the cytoplasm to the nucleus is very inefficient and is facilitated by the disassembly of the nuclear envelope during cell division. The inefficient transfer of plasmid DNA to the nucleus increases its exposure time to cellular nucleases. Naked plasmid DNA has a half-life of about 2 h in the cell cytoplasm (75% degraded after 4 h) (Mirzayans *et al.* 1992, Lechardeur *et al.* 1999). Increased transgene expression following cytoplasmic microinjection of DNA has been observed when DNA was complexed with polyethyleneimine, but not for DNA complexed with cationic liposomes (Page *et al.* 1995, Zabner *et al.* 1995, Pollard *et al.* 1998). Further, it was shown that the microinjection of DNA/liposome complexes into the nucleus of oocytes resulted in no detectable level of expression when compared to free DNA, indicating that the lipid coating of the DNA inhibits transcription and has to be removed before the DNA enters the nucleus (Zabner *et al.* 1995).

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Oligonucleotides

Phosphodiester oligonucleotides are rapidly degraded in biological fluids. Therefore, most antisense studies and clinical trials have involved chemically modified, more resistant oligonucleotides, in particular phosphorothioate oligonucleotides (Akhtar and Agrawal 1997, Crooke 1997, Stein 1998). A potential problem associated with free oligonucleotides is their poor uptake by most cell lines *in vitro* and export from cells (Crooke 1991, Marti *et al.* 1992, Stein and Cheng 1993, Tonkinson and Stein 1994). In addition, sequestration of oligonucleotides into endosomal compartments decreases their intracellular availability. Cationic lipids have been shown to enhance uptake of oligonucleotides into cells (Bennett *et al.* 1992).

When antisense oligonucleotides are given to cells in the form of 'complexes' with cationic liposomes, endocytosis and rapid movement to the nucleus occur (Zelphati and Szoka 1996a,b). This is shown in figure 4. The oligonucleotide/cationic lipid complex dissociates, with the oligodeoxynucleotide entering the nucleus and the cationic lipid as well as DOPE remaining in the endosomal compartments (Marcusson *et al.* 1998). Rapid nuclear accumulation of fluorescently labelled oligonucleotides can also be observed upon microinjection into the cell cytoplasm (Chin *et al.* 1990, Leonetti *et al.* 1991, Fisher *et al.* 1993, Sixou *et al.* 1994). Nuclear transport occurs mainly by diffusion and does not appear to be severely affected by depletion of the intracellular ATP pool or excess unlabelled oligomer (Chin *et al.* 1990, Leonetti *et al.* 1991). Binding to nuclear structures is responsible for retention (accumulation) in the nucleus (diffuse and bind model). PS-oligonucleotides were found to be unevenly distributed across the nucleus, concentrated in small foci (Lorenz *et al.* 1998). In contrast to plasmid DNA, the smaller size of the oligonucleotides allows them to translocate across the nuclear envelope through the nuclear pores. These pores act like a size exclusion sieve, with

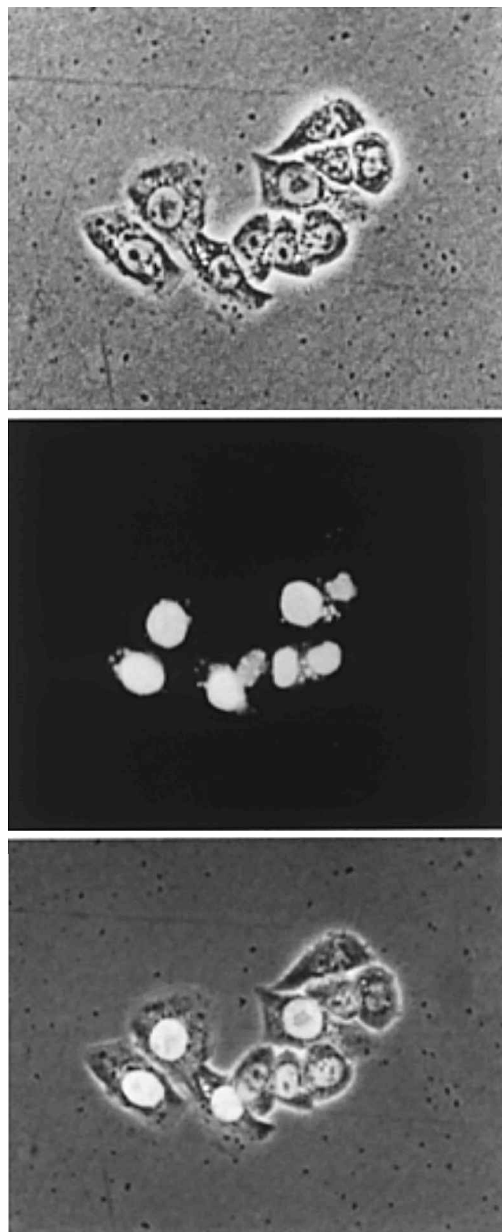

Figure 4. Rapid nuclear accumulation of fluorescently labelled phosphorothioate oligonucleotides (PS-oligos) mediated by DOPE/DODAC cationic liposomes. SK-BR-3 cells were incubated for 4 h with 3 μg of FITC-labelled PS-oligos complexed with DODAC/DOPE (1 : 1) at a positive-to-negative charge ratio of 1.5. Cells were washed and observed by phase contrast (upper panel) or fluorescence microscopy (middle panel). The merged photographs are presented in the bottom panel.

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macromolecules as large as 60 kDa being able to pass through (Peters 1986, Dingwall 1991).

Membrane fusion versus membrane destabilization

DOPE and its ability to induce lipid mixing are automatically associated with membrane fusion. Membrane fusion in biological systems is usually thought to be a non-leaky process. However, there is evidence that this is not always the case. For example, influenza virus-mediated fusion appears to be a highly leaky process with extensive disruption of the lipid membrane at the fusion site (Shangguan *et al.* 1996). The classical picture of a non-leaky fusion event certainly does not apply to the cationic liposome-mediated gene transfer process, where the endosomal membrane is significantly disrupted upon interaction with the cationic liposome/DNA complexes (see figure 3). In both cases, extensive lipid mixing was observed. Lipid mixing, often equated with fusion, is in these cases only an expression of extensive membrane destabilization. Under these circumstances the definition of fusion has to be broadened to allow for massive membrane destabilization (disruption).

Membrane fusion

Membrane fusion is a fundamental process in many cellular functions, including endocytosis, exocytosis/secretion, and cell division and the mechanism by which viruses enter cells (White 1992). Membrane fusion in biological systems is an extremely fast and local event that involves only a very small surface area of the interacting membrane (Burger 1997). It is essentially non-leaky and involves proteins that bring the fusing membranes into close proximity and promote fusion through membrane destabilization. Proteins determine when and where membrane fusion occurs. The central event in membrane fusion, however, is the merger of two membranes. This requires a transient reorganization of membrane lipids (disruption of the lipid bilayer structure). During this reorganization intermediate structures are formed (Cullis and Hope 1978, Siegel 1986, Siegel and Epand 1997).

DOPE and cationic lipids as membrane-destabilizers

Unsaturated phosphatidylethanolamines (PEs) are common membrane phospholipids which, in isolation, spontaneously adopt the inverted hexagonal phase (H_{II} phase). They have been proposed to play a key role in biomembrane fusion facilitating the formation of highly curved semifusion intermediates and stimulating membrane fusion (Hope and Cullis 1981, de Kruijff *et al.* 1985, Ellens *et al.* 1986, Bailey and Cullis 1997a,b). Unsaturated PEs can adopt a bilayer structure in the presence of stabilizing lipids such as PCs, detergents and PEG-lipid constructs (Madden and Cullis 1982, Seddon 1990, Litzinger and Huang 1992, Holland *et al.* 1996a). Loss of the stabilizing function results in H_{II} phase formation and is, for liposomes, accompanied by leakage of contents. Formation of non-bilayer structures also results in extensive lipid mixing.

Most of the lipofectin formulations presently studied require PE for optimal activity. The dependence of efficient transfection on the presence of unsaturated PE as a helper lipid has been observed for a variety of cationic lipids in many different cell lines *in vitro* (Felgner *et al.* 1994, Gao and Huang 1995). The strong DOPE dependence is related to the membrane-destabilizing activity of DOPE (Farhood *et al.* 1995). This is demonstrated in figure 3. Cytoplasmic release of Rh-dextran requires DOPE (figure 3, bottom) and does not occur to a detectable level in the presence of dioleoylphosphatidylcholine (DOPC) (figure 3, middle). Successive methylation of the PE headgroup and increasing acyl chain saturation reduces transfection efficiency (Felgner *et al.* 1994). Cationic liposome formulations containing DOPE were also more active in inducing lipid mixing than DOPC formulations in model membrane studies as well as in *in vitro* experiments (Stamatatos *et al.* 1988, Düzgüneş *et al.* 1989, Leventis and Silvius 1990, Wrobel and Collins 1995, Bailey and Cullis 1997a,b). Cationic liposomes are capable of inducing haemolysis of erythrocytes and of destabilizing isolated lysosomes (Yoshihara and Nakae 1986, Wattiaux *et al.* 1997, Mui, unpublished observations). Lipid mixing between cationic liposomes and anionic liposomes, as well as the haemolytic activity of cationic liposomes, is strongly reduced by DNA and in the presence of serum (Leventis and Silvius 1990, van der Woude *et al.* 1995, Bailey and Cullis 1997, Mui, unpublished observations). Free DOPE-containing cationic liposomes (helper liposomes) can significantly enhance the transfection activity of the complexes (Farhood *et al.* 1995, Li *et al.* 1998).

The cationic lipid component mediates association of the liposomes with the nucleic acid polyanions through electrostatic interactions, thus allowing the complexes to be formed. The interaction with the cationic liposomes results in a partial protection of these molecules against hydrolytic enzymes. A net positive charge of the complexes facilitates uptake into cells by allowing efficient interaction with the negatively charged cell surface. The cationic lipid-mediated association with cell membranes may initiate membrane fusion and disruption. It was shown that anionic liposomes can dissociate DNA from its interaction with cationic liposomes (Xu and Szoka 1996). Destabilization of the endosomal membrane by the complexes with concomitant displacement of the cationic lipid by ion pair formation with anionic cellular lipids may be responsible for the release of nucleic acid-based drugs from endocytic compartments into the cytosol (Zelphati and Szoka 1996). In general, the transfection activity of cationic lipids decreases with increasing alkyl chain length and saturation. It was suggested that the higher intermembrane lipid transfer rates and faster rates of intermembrane lipid mixing of cationic lipids with shorter alkyl chain lengths could contribute to the destabilization of the endosome (Silvius and Leventis 1993, Felgner *et al.* 1994).

Towards systemic delivery of genetic drugs

Currently available delivery systems for genetic drugs have limited utility for systemic applications. The large size and positively charged character of cationic liposome/plasmid DNA or oligonucleotide complexes result in rapid clearance from circulation, and the highest levels of activity are

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observed in 'first pass' organs, particularly the liver and lungs (Mahato *et al.* 1995, Huang and Li 1997, Chonn and Cullis 1998). Oligonucleotides show efficacy in free form *in vivo*, however, they also lack site-selective accumulation (Agrawal 1996, Akhtar and Agrawal 1997). The need for a delivery system for treatment of systemic disease is obvious. For example, for cancer gene and oligonucleotide therapy there is a vital need to access metastatic disease sites as well as primary tumours. Similar considerations apply to other systemic disorders, such as inflammatory diseases. The design features for lipid-based delivery systems that preferentially access such disease sites are increasingly clear from studies on liposomal systems containing conventional drugs, where it has been shown that small (diameter < 100 nm), long-circulating vesicles preferentially accumulate at sites of infection, inflammation and tumours (Gabizon and Papahadjopoulos 1988, Chonn and Cullis 1995). Thus, the carrier containing genetic drugs should be a small, neutral and highly serum-stable particle, which is not readily recognized by the fixed and free macrophages of the reticuloendothelial system (RES). However, in order to maximize potency after arrival at a disease site, the particle should interact readily with cells and have the ability to destabilize cell membranes to promote intracellular delivery of the active agent. The approach taken to solve these problems is described below.

Encapsulation of plasmid DNA in stabilized plasmid-lipid particles

Plasmid DNA has been encapsulated by a variety of methods (see table 2). None of these procedures yields small, serum-stable particles at high plasmid concentrations and plasmid-to-lipid ratios in combination with high plasmid encapsulation efficiencies. In particular, passive encapsulation of plasmid DNA in liposomes is very inefficient due to the large size of these molecules. Efficient entrapment requires the interaction of the lipid components with the DNA with a concomitant reduction in DNA size. Cationic lipids fulfil these requirements (Düzgüneş and Felgner 1993, Bloomfield 1996, Lasic 1997). Neutralization of the negative phosphate charges through association with cationic lipids decreases repulsion between DNA segments and allows bending of DNA and a reduction in size. However, the strong electrostatic interaction between DNA and cationic lipid is difficult to control. Membrane fusion events and aggregation usually result in the production of large and heterogeneous aggregates. Therefore, regulatory components, which allow the control of these processes, are required. For example, macromolecules can be sterically excluded from the liposomal surface by incorporation of PEG-lipid conjugates into the liposomal membrane. This forms the basis of sterically stabilized liposomes, where reduction of the level of plasma

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Table 2. Procedures for encapsulating pDNA in lipid-based systems. The following table was adapted from Wheeler et al. (1999). ND stands for not determined; DLS for dynamic light scattering and EM for electron microscopy. *Some values were calculated based on presented data.

Procedure	Lipid composition	Trapping efficiency*, size of DNA	DNA-to-lipid ratio*	Diameter	References
Reverse phase evaporation	PS or PS:Chol (50:50)	30–50%, SV40 DNA	< 4.2 μg/μmol	400 nm	Fraley et al. 1980
Reverse phase evaporation	PC:PS:Chol (40:10:50)	13–16%, 11.9 kbp	0.23 μg/μmol	100 nm to 1 μm	Soriano et al. 1983
Reverse phase evaporation	PC:PS:Chol (50:10:40)	10%, 8.3 and 14.2 kbp	0.97 μg/μmol	ND	Nakanishi et al. 1985
Reverse phase evaporation	EPC:PS:Chol (40:10:50)	12%,3.9 kbp	0.38 μg/μmol	400 nm	Cudd and Nicolau 1985
Ether injection	EPC:EPG (91:9)	2–6%, 3.9 kbp	< 1 μg/μmol	0.1–1.5 μm	Fraley et al. 1979
Ether injection	PC:PS or PG:Chol (40:10:50)	15%, 3.9 kbp	15 μg/μmol	ND	Nicolau and Rottem 1982
Detergent dialysis	EPC:Chol:stearylamine (43.5:43.5:13)	11% , sonicated genomic DNA	0.26 μg/μmol	50 nm	Stavridis et al. 1986
Detergent dialysis, extrusion	DOPC or DOPE:Chol: oleic acid (40:40:20)	14–17%, 4.6 kbp	2.25 μg/μmol	180 nm (DOPC) 290 nm (DOPE)	Wang and Huang 1987
Lipid hydration	EPC:Chol (65:35) or EPC	ND, 3.9 and 13 kbp	ND	0.5–7.5 μm	Lurquin 1979
Dehydration-rehydration, extrusion (400 or 200 nm filters)	Chol:EPC:PS (50:40:10)	ND	0.83–1.97 μg/μmol	54.6 nm and 142.5 nm	Alino et al. 1993
Dehydration-rehydration	EPC	35–40%, 2.96, 7.25 kbp	2.65–3.0 μg/μmol	1–2 μm	Baru et al. 1995
Sonication (in the presence of lysozyme)	Asolectin (soybean phospholipids)	50%, 1 kbp linear DNA	0.08 μg/μmol	100–200 nm	Jay and Gilbert 1987
Sonication	EPC:Chol:lysine-DPPE (55:30:15)	60–95% , 6.3 kbp ssDNA	13 μg/μmol ssDNA	100–150 nm	Puyal et al. 1995
Spermidine-condensed DNA, sonication, extrusion	EPC:Chol:PS or EPA or CL (40:50:10)	46–52%, 4.3 and 7.2 kbp	2.53–2.87 μg/μmol	400–500 nm	Ibanez et al. 1997
Ca^{2+}-EDTA entrapment of DNA-protein complexes	PS:Chol (50:50)	52–59%, 42.1 kbp bacteriophage	22 μg/μmol	ND	Szelei and Duda 1989
Freeze-thaw, extrusion	POPC:DDAB (99:1)	17–50%, 3.4 kbp linear	ND	80–120 nm	Monnard *et al.* 1997
Stabilized plasmid-lipid particles (SPLP)	DOPE:PEG-Cer: DODAC (84:10:6)	60–70%, 4.4–10 kbp plasmid	62.5 μg/mmol	65–75 nm (DLS, EM)	Wheeler *et al.* 1999

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protein binding results in much longer blood circulation lifetimes. Further, PEG-lipid constructs can inhibit fusion and aggregation of charged liposomes by preventing close membrane approach (Holland *et al.* 1996b). In the following, it is demonstrated that it is possible to use PEG-lipid constructs to regulate fusion and aggregation events following the interaction of plasmid DNA with cationic lipids.

Figure 5 demonstrates that plasmid DNA can be efficiently entrapped in DOPE/DODAC/PEG-ceramide (84 : 6 : 10 mol%) systems termed 'stabilized plasmid-lipid particles' (SPLP) employing a detergent dialysis procedure (Wheeler *et al.* 1999). The trapping efficiencies are a function of the relative amounts of cationic lipid and PEG-ceramide and the ionic strength of the medium. In the absence of PEG-ceramide precipitation occurs. In these system the plasmid DNA is protected from degradation by DNase I and serum nucleases. Long circulation lifetimes can be achieved when PEG-ceramide C_{20} is used, which does not readily exchange out of the membrane. These systems thus rely on the stabilizing effects of PEG coatings and become progressively destabilized as the PEG coating dissociates from the liposomes. The rate of exchange of PEG-lipid conjugates out of the membrane depends on the acyl chain lengths and their degree of saturation (Silvius and Zuckermann 1993, Holland *et al.* 1996b). Typical exchange half-times of PEG-ceramides with acyl chain lengths from 8 to 20 carbons range from several minutes to several days (Wheeler *et al.* 1999). This allows one to adjust the rate at which the stabilizing coating dissociates from the liposomes.

The cryo-EM picture in figure 6 shows that SPLP have the morphological features of large unilamellar liposomes (LUV).

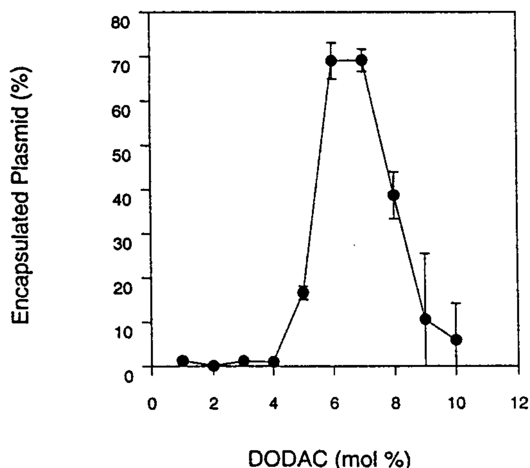

Figure 5. Effect of cationic lipid concentration on the encapsulation efficiency of plasmid DNA (pCMV-CAT) in DOPE/DODAC/PEG-ceramideC_{20} SPLP. Ten milligrams of lipid were dissolved in 200 mm octylglucoside and mixed with 50 μg plasmid DNA in a total volume of 1 ml to form an optically clear solution. This was then placed in a dialysis tube and dialysed against HBS for 36 h at 20°C. Encapsulation efficiency was determined following removal of unencapsulated plasmid by anion exchange chromatography. DNA was quantitated using either ^{3}H-labelled plasmid or the DNA intercalating fluorescent dye, PicoGreen (Molecular Probes). Lipid concentrations were determined by chromatography or with radiolabelled lipids. This figure was taken from Wheeler *et al.* (1998).

The average lipid bilayer thickness is 5.1 nm, as determined by small-angle X-ray scattering. The average diameter from dynamic light scattering measurements and freeze-fracture electron microscopy studies is approximately 70 nm. Analysis of the plasmid-to-lipid ratio reveals that each SPLP contains one plasmid (Wheeler *et al.* 1999).

Procedures have also been developed for the efficient entrapment of antisense oligonucleotides. ICAM-1 entrapped in liposomes containing a protonable cationic lipid and PEG-ceramide has been shown to elicit a strong anti-inflammatory effect in an *in vivo* ear inflammation model upon intravenous injection, where the efficacy of the liposomally entrapped ICAM-1 is much higher than for the free oligonucleotide (unpublished observations).

Circulation life-times and tumour accumulation of SPLP

Stabilized plasmid/lipid particles show long circulation lifetimes and facilitate the preferential accumulation of intact plasmid DNA in disease sites such as tumours following intraveneous injection. Figure 7a shows that 80% of the lipid with roughly the same amount of intact plasmid DNA remained in circulation after 1 h and slowly decreased to 10–20% in the course of 24 h (unpublished observations). Using a mouse tumour model and PEG-CerC_{20} 6% of the injected dose (30 μg plasmid, 2 mg lipid) was found in the tumour (figure 7b) (upublished observations).

The low levels of transfection observed with these systems appeared to be related to the low level of cellular uptake (unpublished observations). Increasing cellular uptake will be the focus of future work.

Conclusion

Delivery systems for systemic applications must have the potential to selectively deliver genetic drugs to specific target

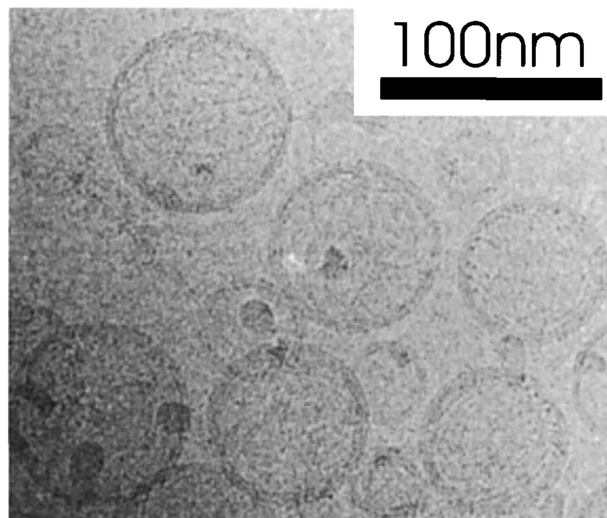

Figure 6. Cryo-electron microscopic picture of stabilized plasmid-lipid particles (SPLP). The plasmid-lipid system has the morphological features of conventional LUV. The picture was taken by Holger Stark, Imperial College, London.

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sites while avoiding recognition by cells of the reticuloendothelial system. The stabilized plasmid-lipid particles introduced above solve, at least in part, the conflicting demands of circulation longevity and intracellular delivery. Future work will focus on the improvement of cellular uptake employing specific targeting ligands attached to the surface of these carriers.

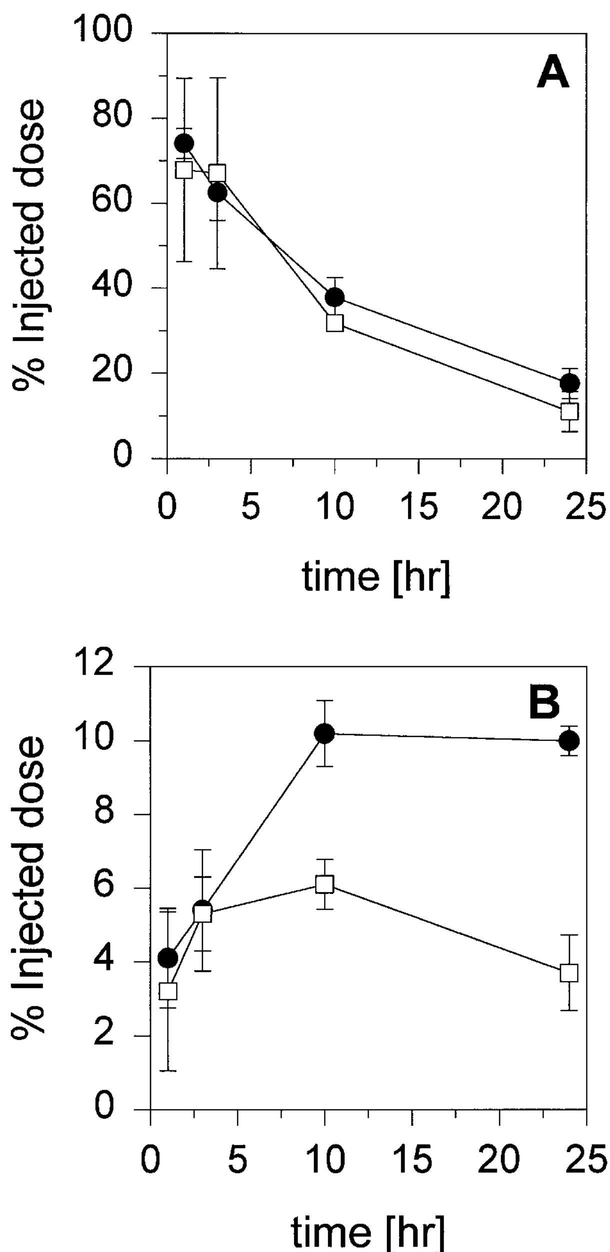

Figure 7. Tumour accumulation and plasma clearance of SPLP containing PEG-$CerC_{20}$ in BDF-1 mice bearing a Lewis lung tumour. Mice were seeded with tumour cells and, after 14 days, injected with the stabilized plasmid/lipid system at a dose of 30 μg plasmid DNA (pCMV-CAT) and 2 mg lipid. Animals were sacrificed 1, 3, 10 and 24 h post-injection. Plasma samples (a), and tumour tissue (b) were analysed for ^{3}H-labelled lipids by scintillation counting and for intact plasmid DNA by Southern blot analysis. The amount of lipid (full circles) and intact plasmid (open squares) recovered from tumour tissue and blood are given as per cent injected dose and plotted as a function of time following injection.

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