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Cationic liposomes for gene delivery

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Cationic liposome–DNA complexes (lipoplexes) constitute a potentially viable alternative to viral vectors for the delivery of therapeutic genes. This review will focus on various parameters governing lipoplex biological activity, from their mode of formation to *in vivo* behaviour. Particular emphasis is given to the mechanism of interaction of lipoplexes with cells, in an attempt to dissect the different barriers that need to be surpassed for efficient gene expression to occur. Aspects related to new trends in the formulation of lipid-based gene delivery systems aiming at overcoming some of their limitations will be covered. Finally, examples illustrating the potential of cationic liposomes in clinical applications will be provided.

Keywords: biological barriers, clinical trials, gene therapy, lipoplexes, nonviral vectors

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1. Introduction

The efficient gene delivery into target cells is a critical issue for the success of gene therapy approaches. The drawbacks associated with the use of viral vectors, namely those related with safety problems, have prompted investigators to develop alternative methods for gene delivery, cationic lipid-based systems (lipoplexes) being the most representative. However, some disadvantages including limited efficiency of delivery and gene expression, toxicity at higher concentrations, potentially adverse interactions with negatively charged macromolecules in serum and on cell surfaces, and impaired ability to reach tissues beyond the vasculature unless directly injected into the tissue, represent restrictions to their wide successful application.

Despite extensive research in the last decade, which led to the development of elegant strategies to enhance lipoplex biological activity, these systems are still far from being viable alternatives to the use of viral vectors in gene therapy. Basic knowledge of the structure–activity relationships of lipoplexes and of the mechanisms involved in the process of intracellular gene delivery is still scarce. It is believed that such knowledge is crucial to further improve the biological performance of these systems; therefore, gaining insights into these mechanistic aspects should constitute one of the main goals in this field. **Table 1** summarises the main factors and related properties affecting the biological activity of the lipoplexes.

2. Factors governing the biological activity of lipoplexes: complex formation and related properties

It is well recognised that the mode of formation of the complexes strongly determines the final physicochemical features of the lipoplexes and, consequently, modulates their biological activity. In addition to the morphology of the lipoplexes, other properties, including size, charge density, colloidal stability and the ability to protect DNA, are strongly dependent on the mode of lipoplex formation. Therefore, understanding the parameters that modulate such properties is of

Table 1. Factors influencing the biological activity of lipoplexes.

Main stages and related properties	Affecting factors
Mode of lipoplex formation - morphology and structure	Nature of cationic and helper lipid, stoichiometry of cationic lipid and DNA, nature of the medium (ionic strength, pH and temperature), DNA structure
Control of physicochemical properties - size - net charge - colloidal stability	Mode of lipoplex preparation (type of liposomes, DNA structure, order of addition and rate of mixing, lipid and DNA concentration), role of adjuvants (polycations, surfactants, cryoprotectants)
In vitro and *in vivo* performance - stability in the presence of serum - resistance to DNA nuclease degradation	Route of administration, nonspecific interaction with serum components, interaction with blood cells, net charge of the lipoplexes, nature of the colipid (cholesterol versus dioleoylphosphatidylethanolamine)
-pharmacokinetics/biodistribution	Interaction with serum components, opsonisation, prolonged circulation time (inclusion of polyethyleneglycol)
- passive versus active targeting - surpass the endothelial barrier - transfection efficacy - cytotoxicity	Size, use of ligands and antibodies Unknown Level and duration of gene expression Type and concentration of lipid, type of cell
Lipoplex–cell interaction - mode of cellular internalisation	Size, liposome composition, net charge and topology of the complexes, presence of ligands
- escape from endosomes	Nature of helper lipid, use of endosome disrupting agents
- DNA dissociation from the complex	Net charge of the complex, nature and valency of the cationic lipid. Cellular factors unknown
- trafficking of DNA into the nucleus	Degree of DNA condensation/compaction, protection from nucleases, size of the plasmid, targeting to the nucleus (nuclear localisation signal peptides). Cellular factors unknown

crucial importance for the successful application of lipoplexes both *in vitro* and *in vivo*. Among such parameters, the nature of cationic and 'helper' lipids, the stoichiometry of cationic lipid and DNA, the DNA structure, the mode of lipoplex preparation and the nature of the medium in which they are prepared are considered the most relevant.

2.1 Nature of the cationic liposome components

Cationic liposomes are frequently composed of a cationic lipid and a neutrally charged lipid (colipid). Cationic lipids include a group of amphiphiles that exhibit a positive charge, which triggers their interaction with negatively charged DNA leading to the formation of complexes containing condensed DNA. Since the first description by Felgner *et al.* [1] of the potential of such types of lipids (2,3-*bis*[oleyl]oxipropyl-trimethylammonium chloride [DOTMA]) for transfection, an increasing number of new cationic lipids of different structures have been synthesised and their respective transfection activities in a wide variety of cell types have been reported. Cationic lipids are frequently composed of a positively charged headgroup bridged by a linker group to a hydrophobic lipid anchor, with a specific role being attributed to each of these components. **Figure 1** shows the structure of DOTMA and illustrates some possible structures of each of the components of a typical cationic lipid [2].

The headgroup is composed of either single or multiple protonatable amines. Multivalent headgroups (e.g., 2,3 dioleyloxy-*N*-[2[sperminecarboxaminino]ethyl]-*N*,*N*-dimethyl-1-propanaminium trifluroacetate [DOSPA] and dioctadecyl amino glycyl spermine [DOGS]) [3] are more efficient in condensing DNA and more active than monovalent lipids (e.g., DOTMA, 1,2-dioleoyl-3-trimethylammonium propane [DOTAP], 3 β[*N*-[*N*′,*N*′-dimethyl amino ethane]-carbamoyl]cholesterol [DC-Chol] and 1,2-dimyristyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide [DMRIE]) [4], which may be related to the greater ability of the former to condense and protect DNA. Nevertheless, progressively increasing the number of positive charges may result in an interaction with DNA that is too strong and would hamper its subsequent dissociation. In addition, multivalent cationic lipids are more prone to form micelles, which may lead to the formation of less stable and more toxic complexes. The orientation of the headgroup in relation to the backbone was also shown to be an important aspect affecting structure–activity. Linear structures (formed on coupling of a primary amine to the lipid anchor) were shown to mediate lower levels of transfection as compared with T-shaped structures (in which a secondary amine is coupled to the lipid anchor) [5].

The hydrophobic moiety is composed of either a diacyl backbone or a cholesterol anchor. Although both groups have been extensively tested, cationic lipids containing cholesterol were shown to be more active than those containing diacyl chains, with this effect being more obvious for lipids with the T-shaped headgroups [5].

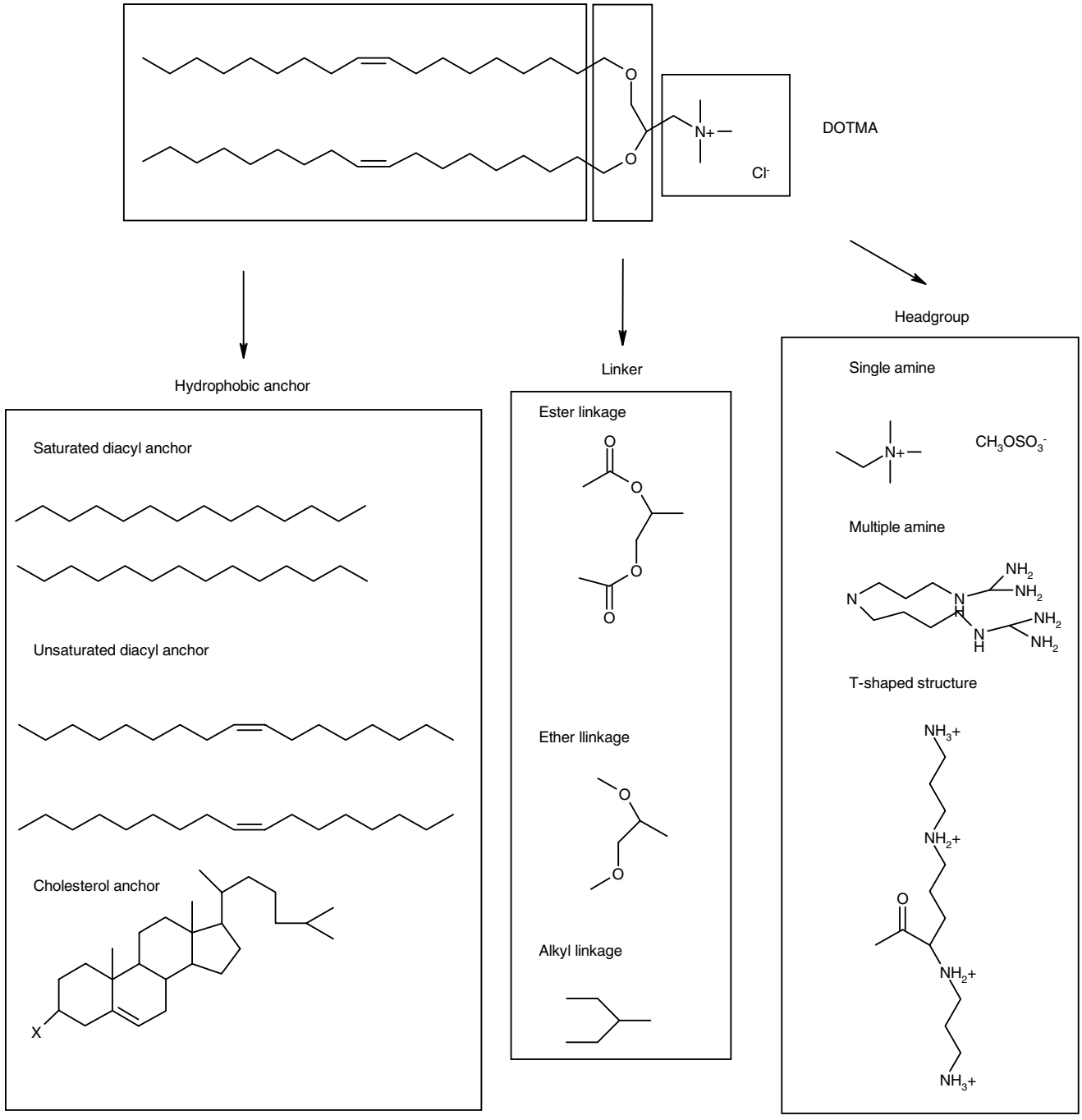

Figure 1. Representative structure of the cationic lipid DOTMA. Some possible structures of each of the components of a typical cationic lipid are also included. Reproduced with permission from PEDROSO DE LIMA MC, NEVES S, FILIPE A, DUZGUNES N, SIMOES S: Cationic liposomes for gene delivery: from biophysics to biological applications. *Curr. Med. Chem.* (2003) **10**(14):1221-1231.
DOTMA: 2,3-*bis*[oleyl]oxipropyl-trimethylammonium chloride.

In general, the transfection activity of cationic lipids decreases with increasing alkyl chain length and saturation. Shorter alkyl chain length favours higher rates of intermembrane transfer of lipid monomers and lipid membrane mixing [6,7].

The nature of the linker group that bridges the hydrophobic anchor with the cationic headgroup has been correlated with the transfection activity and cytotoxicity of the cationic lipid [5]. This latter effect has been attributed to differences in the degree of stability and biodegradability of the lipid. Lipids with stable ether linkages (e.g., DOTMA, DMRIE) are more toxic than those containing labile ester linkages (e.g., DOTAP) [6,8,9]. Nevertheless, incorporation of linkers that are chemically unstable can limit their application, as the stability of lipoplexes on storage or in biological fluids can be drastically affected.

Linkers should thus be selected so that a balance between stability and biodegradability in the cell can be achieved.

The importance of associating a colipid to improve the ability of cationic liposomes to transfect cells has been demonstrated. *In vitro* studies show clearly that liposomes composed of an equimolar mixture of dioleoylphosphatidylethanolamine (DOPE) and cationic lipids (e.g., DOTMA, DOTAP) can mediate higher levels of transfection than those containing only the cationic lipid or a different helper lipid such as DOPC [10-12]. This fact has been attributed to the ability of DOPE to undergo a transition from a bilayer to an hexagonal configuration under acidic pH, which may facilitate fusion with or destabilisation of target membranes, in particular endosomal membranes [6,13,14]. In addition, the motional properties of DOPE, in contrast to DOPC, were correlated with the transfection potential of DOPE-containing complexes [11]. More recently, it was suggested that DOPE can also play a role in facilitating the disassembling of the lipid-based DNA formulations after their internalisation and escape of DNA from endocytic vesicles [15,16]. This was based on the assumption that the amine group of polyethylene (PE) can interact with DNA phosphate groups, thus leading to weakening of the binding of cationic lipids to DNA [15]. Although the benefits of using DOPE have been demonstrated empirically, recent work has shown that the choice of the helper lipid can dictate the structure and activity of cationic liposome–DNA complexes. Cholesterol has also been employed as a colipid to prepare cationic liposomes, resulting in the formation of more stable complexes than those containing DOPE. In contrast to what has been observed in *in vitro* studies, the inclusion of cholesterol in the bilayer of cationic liposomes resulted in very active complexes on *in vivo* administration [17-22]. Moreover, inclusion of cholesterol in the liposome composition enables the use of increased concentrations of lipid and DNA without affecting lipoplex stability. This, in turn, allows increased doses of DNA to be delivered and expressed. On the other hand, the choice of DOPE as the helper lipid for cationic liposomes was described to result in a decrease of the levels of transfection *in vivo* [22]. These findings suggest that the function of the helper lipid in liposomes is different *in vivo* from that *in vitro*, also supporting the hypothesis that the *in vivo* behaviour of lipoplexes cannot necessarily be established from *in vitro* data. Incorporation of polyethylene glycol phospholipid conjugates (PEG–PE) into the liposomal membrane has been explored aiming at improving the colloidal stability of lipoplexes both *in vitro* and *in vivo* [19,20] and will be discussed in more detail in Section 2.3.

2.2 Stoichiometry of cationic lipid and DNA

Several studies have shown that highly positively charged complexes, in which DNA is completely sequestered and condensed, exhibit a homogeneous size distribution (mean diameter of 100 – 450 nm). A similar size distribution is also observed when complexes are prepared with an excess of DNA over cationic lipids (i.e., negatively charged complexes), although in this case the presence of free DNA is generally observed [23-26]. On the other hand, complexes prepared from a lipid–DNA charge ratio of ~ 1:1 exhibit a neutral zeta potential, suggesting that all the cationic lipid molecules are neutralised by DNA [12,26-28]. Such neutral complexes are characterised by a heterogenous size distribution (mean diameter of 350 – 1200 nm) and usually present a much lower colloidal stability than those exhibiting an excess of net positive or negative charge. This can be attributed to a lack of electrostatic repulsive forces among the complexes that would prevent their aggregation [6,12,26,29,30]. It should be noted that some controversy has been reported regarding the (+/-) charge ratio at which neutral complexes are formed. In fact, the surface charge of the lipoplexes (usually assessed by zeta potential measurements) does not always correspond to the theoretically calculated charge ratio. Among other factors, differences in the mode of lipoplex formation resulting from variations in the experimental conditions used by different research groups, or differences in the cationic lipid concentration among batches, can be responsible for such discrepancy. The influence of lipid–DNA stoichiometry on the physicochemical properties of the complexes becomes even more difficult to evaluate considering that, for a fixed lipid–DNA charge ratio, the increase in concentration of lipid and DNA results in a significant change of their size and colloidal stability, which can be attributed to enhanced precipitation at higher concentrations due to smaller interparticle separation [23]. It seems, therefore, that charge ratio alone is not sufficient to predict the mode of formation and physicochemical features of the complexes and, consequently, their biological activity.

The ability to protect the carried DNA against nuclease degradation is considered to be a crucial feature affecting the biological activity of the complexes [31]. Besides size and charge, the stoichiometry of cationic liposome–DNA complexes determines their resistance to the inhibitory effect of the nucleases. Negatively charged complexes (prepared with an excess of DNA over cationic lipid) cannot protect DNA efficiently, which can be explained by the susceptibility of noncomplexed DNA to nuclease degradation. In contrast, positively charged complexes (containing an excess of positive over negative charges) are able to fully condense and coat DNA, thus acquiring a high degree of resistance to DNases. Curiously, complexes prepared so that a balance between positive and negative charges is achieved (neutrally charged complexes) have been shown to be capable of protecting DNA. The degree of DNA protection obtained for different formulations can be assessed by evaluating the accessibility of ethidium bromide to the DNA associated with the complexes [32]. A more biologically relevant assay to evaluate the protection conferred by

cationic liposomes to DNA degradation is based on the assessment of DNase I resistance mediated by the complexes [32].

2.3 *In vivo* lipoplex behaviour

Positively charged complexes have been described as being able to completely condense DNA and to mediate the highest levels of transfection *in vivo* [12,21,26]. Favourable interactions with and binding to the cell surface, as well as an efficient protection of the foreign DNA against nucleases, can partially explain these observations [1,4,23]. Surprisingly, it has been recognised that within this type of complex, those exhibiting large sizes (> 200 nm) are more effective in mediating transfection than small complexes (50 – 100 nm) [23,33,34]. Whether these findings are due to more efficient lipoplex–cell interactions (presumably favoured by a more extensive deposition of the large complexes at the cell surface), to the ability of certain size classes of the complexes to trigger cellular internalisation events (such as phagocytosis), or to the fact that more copies of the plasmids may be carried in the larger complexes, are still open questions. It was demonstrated recently that, for highly positively charged complexes, free liposomes coexist with the cationic liposome–DNA complexes and play an important role in mediating transfection *in vivo*, namely on intravenous administration in mice [21]. This enhancing effect was attributed to an increase of the retention time and efficient protection of DNA, and presumably to the ability of the free liposomes in promoting intracellular gene delivery by the lipoplexes [21,35]. In addition, it was shown that lipoplexes prepared at high cationic lipid/DNA (+/-) charge ratios were also able to overcome the inhibitory effect of serum on lipofection [33,36]. This resistance to the inhibitory effect of serum on transfection can also be achieved by prolonging the time of complex formation [37]. Curiously, this time-dependent maturation was only observed for monovalent cationic lipids. Moreover, the process of maturation, which was accelerated by high charge ratios, high concentration and high temperature, resulted in the formation of homogeneous particles with a mean diameter of 170 – 400 nm.

Different approaches have been explored aiming at enhancing the biological activity of lipoplexes and overcoming some of the biological barriers faced by the lipoplexes on their *in vivo* administration. Whether coating of lipoplexes with fusogenic peptides or with negatively charged proteins, in the presence or absence of polycations (including protamine sulphate) would constitute a promising strategy to modulate their colloidal stability and transfection efficacy in the presence of serum has been evaluated. These studies show a significant enhancement of transfection and resistance to the presence of serum when human serum albumin was associated to lipoplexes, independently of the composition of the cationic liposomes used in their preparation [38,39]. Transferrin–lipoplexes were also found to mediate extensive transfection in the presence of serum [12].

The use of cationic liposomes modified with the phospholipid derivative of the polymer PEG (PEG–PE) may constitute a promising approach to the development of an efficient pharmaceutical carrier for systemic *in vivo* gene delivery. This strategy was shown to stabilise cationic liposome–DNA complexes for prolonged storage while maintaining their biological activity [19], and to result in complexes that are highly active *in vivo* [20]. Meyer *et al.* [40] have reported that incorporation of PEG–PE into cationic liposomes prevents lipoplex aggregation and increases their stability.

Alternative approaches include insertion of the polymer after the formation of cationic liposome–DNA complexes [41] and the use of PEG–lipid conjugates with cleavable bonds that would allow the shedding of PEG molecules under acidic conditions such as those found in the endosomal lumen. In this context, it should be mentioned that a promising strategy was reported involving the efficient entrapment of plasmid DNA into cationic liposomes containing a PEG–ceramide construct by employing a detergent dialysis procedure [42-44].

3. Mechanisms of interaction of cationic liposome–DNA complexes with cells

Despite the extensive use of cationic liposomes for gene delivery both *in vitro* and *in vivo*, the mechanisms by which DNA is delivered into cells are not yet fully understood.

Studies aiming at clarifying these mechanisms demonstrated that the efficiency of gene delivery mediated by cationic liposomes is strongly dependent on the physicochemical properties of the lipoplexes, particularly size, morphology and surface charge, which ultimately determine the processes of binding, cellular uptake and intracellular processing. **Figure 2** illustrates the key steps governing the process of intracellular gene delivery mediated by cationic liposome–DNA complexes, which are discussed in this section.

3.1 Cell association/binding of lipoplexes

Based on the observation that lipoplexes exhibiting a net positive charge frequently mediate high levels of transfection, a role for sulfated membrane-associated proteoglycans in the cell binding of cationic liposome–DNA complexes has been suggested [45]. Sulfated proteoglycans are highly negatively charged components of cell membranes, and consist of a group of proteins covalently linked to one or more polysulfated glicosaminoglycan (GAGs) polysaccharides [46].

Studies involving approaches that lead to the reduction of cell-associated proteoglycans, or approaches using mutant cells unable to synthesise proteoglycans, provided evidence that surface proteoglycans play a role in the binding of lipoplexes to cells, both *in vitro* [45] and *in vivo* [47]. In this context, it is interesting to note that, apart from being involved in a variety of other cellular processes, proteoglycans play an important role in the binding and entry of many viruses into cells [48], such as herpes simplex virus (HSV), human immunodeficiency virus-1

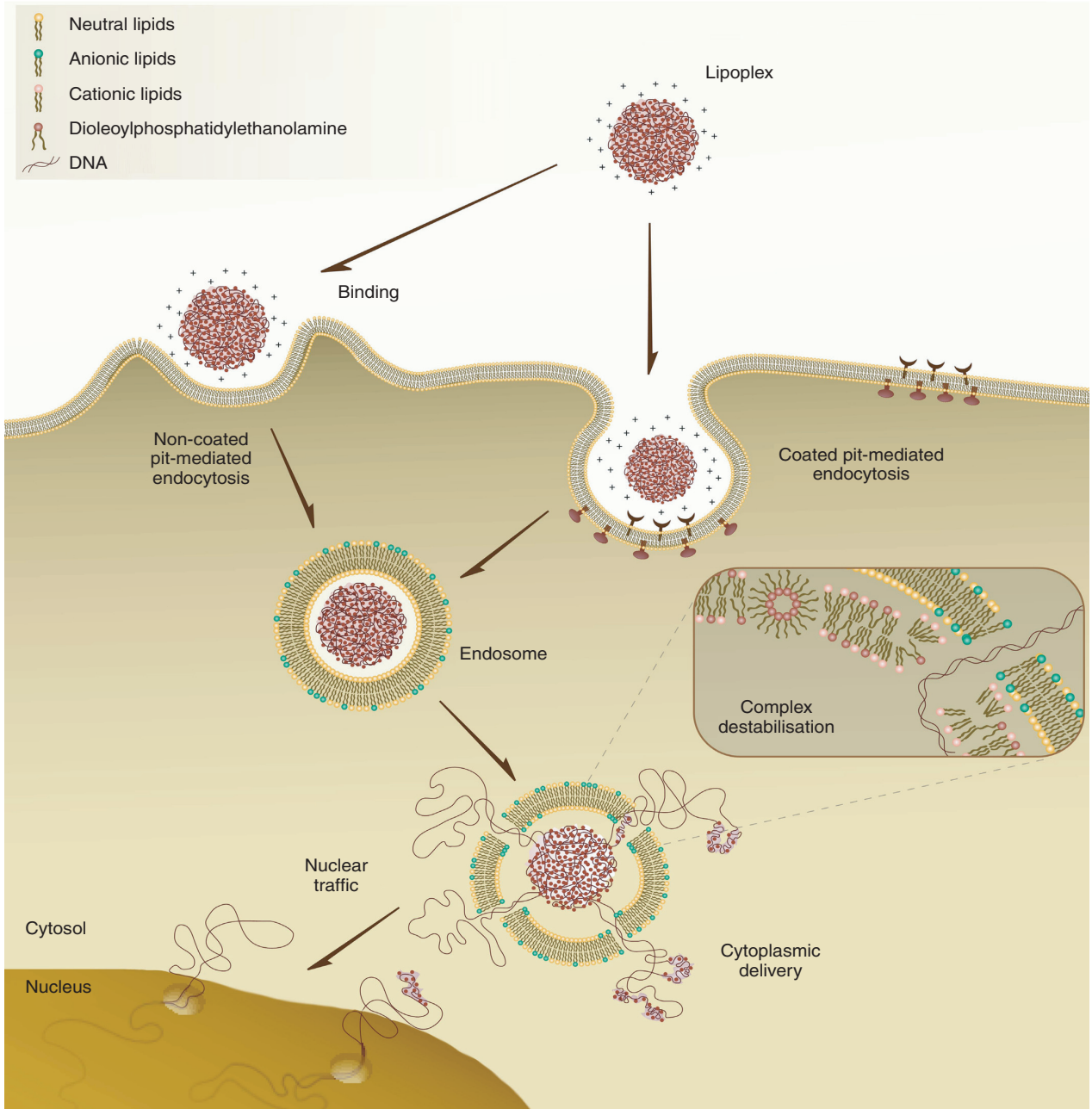

Figure 2. Overview of the main steps involved in the interaction of cationic liposome–DNA complexes with cells: binding and cellular uptake of lipoplexes, cytoplasmic release of DNA and nuclear traffic of DNA.

and adeno-associated viruses, all of which have been successfully used for gene therapy purposes.

Recent work by Kopatz *et al.* supports the involvement of proteoglycans in the internalisation of cationic liposome–DNA complexes into cells [49]. According to the proposed model, the authors show that binding of cationic particles to proteoglycans present at the cell surface is required for their internalisation. Clustering of proteoglycans bound to the particle triggers protein kinase C activity and the binding of actin to the cytoplasmic domain of proteoglycans, which then promotes the engulfment of the particle through a process similar to bacterial uptake.

Despite the evidence that proteoglycans potentiate transfection by acting as receptors for complex binding and uptake, additional studies on the interactions of GAGs with cationic liposome–DNA complexes suggested that extracellular GAGs may also have an inhibitory role in gene delivery, primarily by promoting destabilisation of complexes at the cell surface, thus leading to extracellular release of DNA [50-52]. In addition, coendocytosis of GAGs

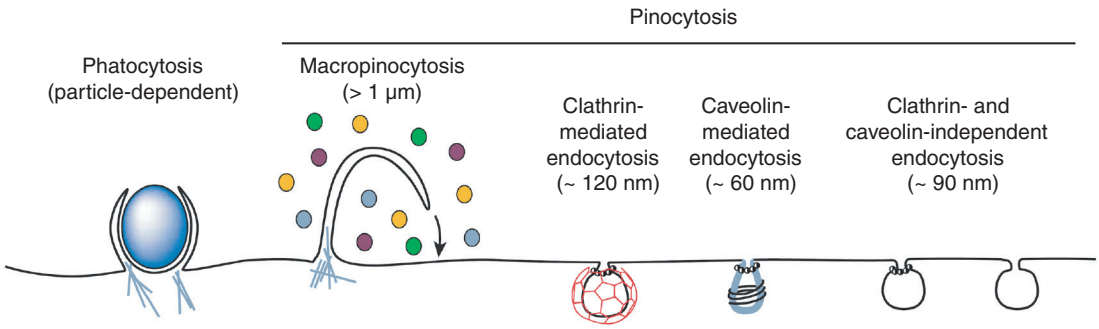

Figure 3. Schematic representation of different endocytic pathways. Endocytosis may occur through several distinct mechanisms, which are usually divided into two main categories: phagocytosis, a process restricted to specialised mammalian cells; and pinocytosis, which includes macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis and clathrin- and caveolae-independent endocytosis. The endocytic pathways differ in vesicle size and structure, nature of the cargo, as well as on the mechanism of vesicle formation. Reproduced with permission from CONNER SD, SCHMID SL: Regulated portals of entry into the cell. *Nature* (2003) **422**(6927):37-44.

with complexes has also been shown to modify the intracellular trafficking of complexes, leading to decreased transfection efficiency [51,53].

3.2 Cellular uptake of lipoplexes

Following binding to the cell surface, endocytosis has been recognised as the major pathway of internalisation of cationic liposome–DNA complexes [54,55]. Although fusion of these complexes with the plasma membrane was shown to occur concomitantly with endocytosis, this process is not correlated with efficient intracellular gene delivery [26,56,57].

Despite the fact that endocytosis of lipoplexes has been shown to be required for efficient transfection, the precise nature of the endocytic pathways involved in the internalisation of lipoplexes has been poorly investigated. Endocytosis may occur through several distinct mechanisms, usually divided into two main categories: phagocytosis, a process restricted to specialised mammalian cells; and pinocytosis, which occurs in all mammalian cells and encompasses macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis and clathrin- and caveolae-independent endocytosis (**Figure 3**) [58].

As indicated above, particle size has been shown to be a critical parameter governing cellular uptake and biological activity of lipoplexes. Nonetheless, it has been difficult to evaluate the exact effect of lipoplex size on transfection, given the size heterogeneity often associated with the colloidal instability of cationic liposome–DNA complexes (from 100 nm up to several micra).

Recently, Rejman *et al.* investigated the effect of particle size on endocytosis, through systematic analysis of the mechanisms of internalisation and intracellular processing of fluorescent latex particles of defined sizes in nonphagocytic cells [59]. This very interesting analysis revealed that, in the absence of any ligand, size of particles may dictate *per se* the pathway of internalisation and the subsequent intracellular processing. Particles of small size (< 200 nm) were found to be internalised through clathrin-mediated endocytosis and to reach the lysosomal compartment rapidly, whereas particles of larger size (> 200 nm but < 1 µm) were internalised preferentially by caveolae-mediated endocytosis. The slow kinetics of caveolae-mediated internalisation may facilitate endosomal escape of DNA (either free or still complexed with cationic lipids) into the cytoplasm prior to reaching lysosomes, thus diminishing lysosomal degradation. These findings may help explain the higher transfection efficiency frequently observed for large lipoplexes (> 200 nm) [60,61].

In addition to clathrin- and caveolae-mediated endocytosis, the contribution of other processes, such as macropinocytosis, phagocytosis, or other less-described internalisation pathways, to the internalisation of cationic liposome–DNA complexes (particularly those exhibiting large sizes) resulting in efficient transfection should not be excluded, and requires further investigation.

Based on the evidence that endocytosis represents the major pathway of lipoplex internalisation, attempts have been made to enhance cell internalisation by specifically targeting cationic lipid-based systems to cells, through the association of protein or peptide ligands or antibodies directed toward receptors that mediate endocytosis, such as lectins and asialoglycoprotein, asialofetuin, integrin, folate, Her2/neu and low-density lipoprotein receptors [62]. In this regard, several groups have demonstrated the fact that association of transferrin to the lipoplexes enhanced transfection in a large variety of cells, including dividing and nondividing cells [12,63]. Moreover, studies have indicated that triggering internalisation of the lipoplexes through a nonspecific endocytic process (namely phagocytosis) can be

achieved by associating certain proteins (e.g., albumin) to the lipoplexes [39].

A better knowledge of the endocytic pathways involved in the internalisation of different complexes will thus be of crucial importance for the rational improvement of new gene delivery systems, namely for approaches aiming at targeting intracellular pathways more favourable to transfection.

However, it should be noted that promotion of the extent of binding and internalisation of the lipoplexes does not necessarily translate into a similar enhancement of transgene expression. Several studies from various laboratories have indicated that no correlation can be established between the extent of binding/cell association of the lipoplexes or the amount of DNA associated with the cell and the observed levels of transfection [57,62]. These observations reinforce that successful intracellular delivery of DNA is a complex multistage process that is also largely dependent on the capacity of lipoplexes to overcome the additional cellular barriers faced on cell entry, including the endosomal release and nuclear traffic of DNA [31,62,64].

3.3 Cytoplasmic delivery of DNA

Following internalisation, the release of the complexes from the endocytic compartments into the cytoplasm is of crucial importance to avoid DNA degradation at the lysosomal level. However, the mechanism by which the lipoplexes induce disruption of the endosomes in order to gain access to the cytoplasm is also a question that still needs to be fully resolved. According to a mechanistic model that has been largely accepted, the destabilisation of the endosomal membrane by the internalised complexes induces 'flip-flop' of anionic lipids from the cytoplasmic leaflet to the lumenal leaflet of the endosomal membrane. The subsequent formation of charge-neutral ion pairs between the cationic and anionic lipids is thought to result in the displacement of the DNA from the complexes, leading to the release of DNA into the cytoplasm [65].

In this context, it should be noted that the presence of DOPE in the liposome formulations plays an important role in mediating destabilisation of the endosomal membrane, as the acidification of the endosomal lumen activates the fusogenic properties of this lipid. It was demonstrated that following internalisation of the complexes via endocytosis, DOPE-containing cationic liposomes may promote fusion with the endosomal membrane under acidic conditions, thus allowing the release of DNA into the cytoplasm [66]. Moreover, as stated above, DOPE may be involved in helping the DNA dissociation from the lipoplexes due to the ability of its amine group to compete with cationic lipid for DNA phosphate groups, on lipoplex internalisation [15]. It is possible that pore formation at the endosomal membrane may also be involved in the escape of the complexes or of free DNA into the cytoplasm.

In an attempt to improve the endosomal release of DNA, pH-sensitive fusogenic peptides have been associated to lipoplexes. This association is expected to result in a triggered destabilisation of the endosomal membrane on acidification of its lumen, in a manner similar to that used by certain types of enveloped viruses to infect their target cells. By following this approach, it has been demonstrated that the association of either the Glu-Ala-Leu-Ala fusogenic peptide or the fusion peptide derived from the influenza virus haemagglutinin to lipoplexes results in a significant enhancement of transfection. This effect is particularly relevant for professional phagocytic cells (human macrophages) [63]. Nonetheless, it remains to be clarified whether or not the benefits of such a strategy are counteracted by potential immune responses elicited on *in vivo* application. Alternative approaches, which were also shown to enhance transfection by potentiating the endosomal escape of DNA, involve the use of lysosomotropic agents such as chloroquine, or of compounds that promote the osmotic swelling of endosomes (e.g., sucrose or lipopolyamines) [62].

3.4 Nuclear entry of DNA

Once in the cytoplasm, DNA has to reach the nucleus and surpass the nuclear membrane for transcription to occur. As with the other steps involved in the intracellular gene delivery mediated by lipoplexes, the knowledge of DNA trafficking into the nucleus is still scarce.

In actively replicating cell lines, nuclear entry of DNA is thought to be extensively facilitated by the nuclear membrane disassembly that occurs during mitosis [67]. However, in the absence of cell division, how DNA gains access to the cell nucleus remains to be clarified. Passive diffusion of plasmid DNA into the nucleus is unlikely to occur, as pores act as a size exclusion sieve that avoids the free exchange of large macromolecules, such as proteins > 60 kDa and plasmid DNA molecules. Alternatively, DNA may be imported into the nucleus through an active process, most likely following its nonspecific association with proteins containing nuclear localisation signals (NLSs) or their receptors (karyopherin-α/β). Based on existing data, it cannot be excluded that trafficking of the complexes through the endosomal pathway may be involved in the import of DNA into the nucleus, through an unknown pathway.

A crucial question that is also tightly related to the nuclear traffic of DNA concerns the degree of condensation/compaction of the DNA during the process. Assuming that DNA is lipid-free, a rapid movement into the nucleus appears to be required in order to avoid its cytoplasmic degradation, as indicated by the finding that free DNA microinjected into the cytoplasm is degraded within a short time [68]. Therefore, it seems that partial coating of DNA with lipid would be advantageous at this stage, not only to ensure protection of DNA against cytoplasmic nucleases, but also to reduce the size of the plasmid. Moreover, it can be speculated that traces of cationic lipid still associated with DNA may play a role in the destabilisation of the nuclear membrane, thus facilitating DNA nuclear entry. In this context, studies by Zabner *et al.*

have shown that microinjection of free plasmids into the nucleus of oocytes results in gene expression, whereas microinjection of lipoplexes do not, suggesting that lipid coating of DNA inhibits transcription [69]. Nonetheless, it has been recently shown that microinjection of lipoplexes inside the nucleus of HeLa cells may also result in efficient transfection, clearly demonstrating that the dissociation of lipoplexes can also occur inside the nucleus [70].

In view of the very limited number of plasmid copies that are translocated into the nucleus when transfection is mediated by cationic liposomes, different strategies have been attempted to promote the nuclear entry of DNA. The majority of these strategies are based on the association of NLS peptides to DNA, and have been inspired by the observation that certain proteins bearing NLSs (e.g., histones, transcription factors, viral proteins etc.) have the ability to be actively translocated into the nucleus by a receptor-mediated process. Peptides derived from the well characterised NLS present in the SV40 large T antigen have been the most frequently used to enhance transfection of nonviral gene delivery vectors. Different versions of applying this strategy have been reported, including the simple association of NLS peptides with lipoplexes [71], their conjugation with polycations [72], or through coupling of NLS sequences to DNA, either by using a hairpin oligonucleotide enriched with amino groups [73] or a bifunctional peptide nucleic acid–NLS peptide [74]. Alternative strategies to promote nuclear delivery of DNA include its condensation, prior to the addition of cationic liposomes, with cationic peptides, such as the adenoviral mu peptide [75,76]. The karyophilic cell penetrating peptide derived from HIV-1 tat protein has also been shown to enhance transfection mediated by cationic liposomes [77,78], suggesting that cell penetrating peptides can also be of great value in the improvement of existing nonviral gene delivery vectors.

4. New trends in lipid-based gene delivery systems

Several advantages, including lack of immunogenicity, safety, ability to package large molecules of DNA and ease of preparation have been associated with cationic liposomes [79-81]. Although their ability to mediate efficient transfection in tissue culture has been largely demonstrated, it has been recognised that their *in vitro* efficiency does not correlate with their ability to deliver DNA after *in vivo* administration [18,19,82-85].

Decreased transfection efficiency *in vivo* is due in part to the interaction of the lipoplexes with blood components, such as serum proteins, which inhibit transfection [60,86-89]. *In vitro* studies indicated that the higher the serum (or plasma) content, the larger is the inhibition observed [90-92]. Evidence of the effect of serum components on the physicochemical properties of cationic liposome–nucleic acid complexes and of their interactions with cells has been reported. It was shown that serum components can decrease nucleic acid delivery into cells and promote dissociation of the complexes [93], which together with an increase of the size of lipoplexes in the plasma may explain their pattern of biodistribution, namely the high levels of transgene expression frequently observed in the lungs [94]. However, lung transfection mediated by cationic liposomes induces a strong cytokine response, which may be undesirable for some applications [95]. Furthermore, intravenously injected cationic liposome–DNA complexes unspecifically interact with blood cells such as macrophages, monocytes, neutrophils, platelets and erythrocytes, which often leads not only to disassembly and clearance of the complexes before they can reach target tissues [81,96,97], but also to haemagglutination, [98] potent inflammatory reaction and elevated serum levels of liver enzymes [99].

To overcome some of the referred limitations, different approaches have been taken to modulate the properties of lipoplexes *in vivo*, namely through the encapsulation of precondensed DNA into neutral or negatively charged liposomes.

Liposomes composed of neutral or zwitterionic lipids present longer circulation times and lower toxicities as compared with cationic liposome formulations due to the decrease of nonspecific interactions with serum negatively charged proteins and blood cells. Neutral liposomes have already been successfully applied as carriers for several drugs, improving their biodistribution profile, while decreasing drug-associated toxicity [100-102]. Small neutral liposomes have been shown to passively accumulate in tumours and sites of inflammation, where the vasculature is malformed or permeabilised [103], and avoid rapid clearance *in vivo*. In addition, active targeting strategies are more easily applied to neutral liposomes than to cationic liposomes given their increased circulation lifetimes [104] and the absence of nonspecific electrostatic interactions.

Anionic liposomes exhibiting pH-sensitivity have been shown to mediate gene transfer, but, similarly to other noncationic formulations, they present major disadvantages when compared with cationic liposomes:

- low DNA entrapment efficiency, which can be attributed on one hand to their low internal aqueous volume as a consequence of their small size (required for systemic administration) and on the other hand to the large DNA molecular weight [95,104]
- lower extent of cellular internalisation
- the fact that once in the cytoplasm, noncationic liposomes are not so efficient in protecting DNA against nucleases and in mediating its nuclear entry

Overall, this may justify the very low transfection efficiency achieved when this type of liposomes are used for *in vivo* gene delivery. In addition, it was shown that the passive encapsulation method of DNA into anionic liposomes requires the use of a high concentration of lipids, which leads to the generation of high amounts of empty liposomes. Procedures commonly used to improve liposome encapsulation such as repeated freeze–thawing cycles and sonication may also cause DNA damage.

Although several agents have been used to condense DNA prior to its complexation with cationic liposomes,

which led to promising results in terms of particle size, resistance to nuclease activity and transfection enhancement, this section will focus mainly on the encapsulation of condensing agent–DNA complexes into neutral or negatively charged formulations. Among the agents used for DNA condensation prior to its encapsulation or complexation with liposomes, polylysine and polyethylenimine (PEI) have been the most extensively used. Nevertheless, alternative methods for condensing DNA prior to its encapsulation into liposomes have been reported.

Efficient encapsulation of DNA into small and neutral liposomes was achieved by the addition of ethanol and calcium chloride to an aqueous mixture of small unilamellar vesicles and plasmid DNA, leading to the formation of liposomes with average diameters of < 200 nm and trapping efficiencies of ≤ 80% [95].

Another approach consisting of condensing plasmid DNA with spermine to reduce its size prior to encapsulation into liposomes was found to yield *in vitro* transfection efficiencies of the same order of magnitude as lipoplexes, but with lower cytotoxicity [105].

A novel type of liposomal vector for gene therapy, designated artificial virus-like particle, was proposed by Fahr *et al.* The lipid composition mimics that of retroviruses and the strategy consists of condensing DNA with low molecular weight branched PEI. The resulting particles are able to encapsulate condensed DNA, provide for endosomolytic properties, and exhibit small size (< 200 nm) and a negative surface charge. These features resulted in the absence of toxicity and reduced interactions with the biological environment, thus conferring serum resistance. Equipment of these particles with a cyclic Arg-Gly-Asp peptide ligand as a targeting device renders them selective for tumour endothelial and melanoma cells expressing high levels of $\alpha_v\beta_3$-integrins, and allows for an efficient delivery of the enclosed genetic material. The specificity of the vector system for melanoma cells could be further improved by using a melanocyte-specific tyrosinase promoter to drive transgene expression [106].

The work of Turner *et al.* emphasised that several aspects should be considered when designing strategies based on the liposome encapsulation of precondensed DNA. The authors demonstrated that the gene transfer activity of lipopolyplexes depends not only on the nature of the anionic lipids, but also on the mole ratio cationic polymer–DNA–lipid. Furthermore, it was shown that the observed transfection activity in Jurkat cells was significantly higher for targeted lipopolyplexes, through the coupling of anti-CD3 antibody to the distal end of distearoylphosphatidylethanolamine–PEG incorporated at the liposomal membrane, than for nontargeted formulations [107].

The inclusion of condensing agents bearing nuclear targeting properties is also a promising strategy. Plasmid vectors were complexed with the nuclear localising protein high mobility group-1, a nonhistone nuclear routing protein to target DNA to myocyte nuclei, prior to the encapsulation in liposomes coated with noninfectious haemagglutinating virus of Japan (HVJ). HVJ–liposome-mediated transfer is efficient for the transfection of both oligonucleotides and plasmids into cardiac myocytes *in vitro* without effect on cell morphology or viability at the concentrations used. This vector has also been shown to be efficient *in vivo* after intracoronary injection into the myocardium. Similar to adenovirus, HVJ-mediated transfection does not require cell replication and can be used to transfect terminally differentiated cells such as cardiac myocytes [108].

Murphy *et al.* reported a study of a model system that demonstrated an alternative approach to the compaction of DNA by cationic amphiphiles, in such a way that small and stable particles of condensed DNA are formed. The authors show that mixtures of a cationic peptide (acetyl-Cys-Trp-$[Lys]_3$-Pro-$[Lys]_2$-amide)–PE conjugate and anionic detergent form soluble complexes with plasmid DNA. Under appropriate conditions, these complexes are stable in solution, neither aggregate nor precipitate and contain DNA that appears fully condensed and compacted to a small hydrodynamic diameter. The same authors report that this compacted DNA can be subsequently combined with noncationic lipid to form small, homogeneous, nuclease-resistant liposome complexes [109].

Other authors explored targeting of lipid-based particles (LPDII), prepared from pH-sensitive anionic liposomes composed of DOPE–cholesteryl hemisuccinate (CHEMS) and DNA–polylysine complexes, to specific cells through the coupling of folic acid to a PEG–lipid conjugate, which resulted in improved transfection efficiency. Depending on the lipid–DNA ratio, either positive (high lipid–DNA ratios) or negative (low lipid–DNA ratios) particles could be generated. However, cationic particles were highly active in transfection but were not tissue-specific. The efficient receptor-dependent transfection activity of the anionic LPDII particles makes them promising candidates for tissue-specific gene delivery [80]. In this regard, Reddy and Low demonstrated that the pH-dependent caged form of DOPE, C-DOPE (*N*-citraco-nyl-DOPE), when incorporated into pH-sensitive liposomes composed of cholesterol hemisuccinate and DOPE, significantly improved their transfection efficiency [110].

In contrast to LPDII formulations incorporating DOPE, the transfection efficiency of LPDII vectors composed of DNA–PEI complexed with anionic liposomes (diolein–CHEMS) was sustained in media containing ≤ 50% foetal bovine serum [111]. The authors have also found that crosslinking of PEI–DNA polyplexes with dithiobis(succicimidylpropionate) (DSP) or dimethyl 3,3′-dithiobispropionimidate 2HCl (DTBP) at molar ratios > 10:1 (DSP or DTPB–PEI) stabilised these complexes against polyanion disruption, and that this stabilising effect was reversible on reduction with 20 mM dithioerythritol (DTE). The combination of serum resistance conferred by the diolein/CHEMS liposomes and the increased stability owed to crosslinking may make LPDII vectors more stable in the systemic circulation after intravenous delivery [112].

As an alternative to liposome encapsulation, several studies have reported the complexation of polyplexes (polymer–DNA complexes) to ensure precondensation of DNA, with preformed negatively charged liposomes, namely pH-sensitive liposomes [80,106,107,110-115].

In comparison with conventional liposome formulations, the reported approach was shown to result in efficient condensation and protection of plasmid DNA. Furthermore, targeting of the formulations to a specific cell (through coupling of a PEG–lipid conjugate to a ligand) led to improved transfection efficiency.

Moreover, further improvements can be achieved by using different strategies, namely by incorporation of a nuclear targeting sequence into the plasmid DNA aiming at promoting its nuclear entry [114], or by the use of condensing agents containing nuclear localisation signals [116]. Through structural analysis of ternary complexes composed of DNA, cationic DNA binding protein and anionic liposomes, Hagstrom *et al.* reported that DNA was located on the outside of the liposomes, providing evidence that plasmid DNA encapsulation is not essential for transfection competency *in vitro* [116].

Another alternative approach to avoid liposome encapsulation of polyplexes was reported by Lee *et al.*, which consisted of generating water soluble lipopolymers (WSLP) on conjugation of a lipid (cholesterol) to a cationic polymer (PEI 1800 Da) for complexation with DNA. It was demonstrated that WSLP–DNA complexes undergo internalisation through the cellular cholesterol pathway, leading to high transfection efficiency of smooth muscle cells without causing cytotoxicity. The injection of WSLP–pDNA complexes into rabbit myocardium showed that WSLP mediated higher transfection efficiency than PEI (1800 Da) and led to longer gene expression than naked pDNA [117].

5. Perspectives for clinical application of cationic liposomes as gene carriers

The first gene therapy clinical trial was approved in 1989 and consisted of using tumour infiltrating lymphocytes, containing a marker gene (the gene coding for resistance to neomycin) delivered by a retroviral vector [118]. This represented the starting point for a period of great enthusiasm for clinical gene therapy. However, the great expectations associated with the application of this therapeutic approach have been hampered by the unsatisfactory results obtained [119]. Moreover, gene therapy faced a serious drawback in the late 1990s, when a patient, enrolled in Phase I clinical trials involving intravascular gene transfer mediated by an adenovirus type 5-based vector that contained the human ornithine transcarbamylase cDNA, experienced lethal complications [120,121]. This case raised serious safety and toxicity concerns regarding the use of viral vectors in clinical trials [119]. Nevertheless, sometime later Cavaszana-Calvo *et al.* published the first clinical success of gene therapy [122]. In a Phase I clinical trial, children suffering from severe combined immunodeficiency-X1 were submitted to an *ex vivo* gene therapy protocol involving autologous infusion of haematopoietic stem cells transduced with a Moloney retrovirus-derived vector containing the gene encoding the γ-c cytokine receptor subunit of IL-2, -4, -7, -9 and -15 receptors [122,123]. Unfortunately, the enthusiasm generated by the apparent cure of 9 out of the 10 infants turned to alarm when nearly 3 years after treatment T-cell leukaemia emerged in two of the children. This was attributed to retroviral vector integration in proximity of the LMO2 proto-oncogene promoter, leading to aberrant transcription and expression of LMO2 [124]. All these safety problems prompted the scientists to develop alternatives to viral vectors. Cationic liposomes have been one of the most used nonviral vectors in worldwide human clinical trials of gene therapy. **Table 2** summarises the main gene therapy clinical trials using cationic liposomes. So far, the clinical application of cationic liposomes has been essentially focused on cancer and monogenic diseases, cystic fibrosis (CF) being, among the latter, the most frequently addressed disease. CF is caused by mutation of the CF transmembrane conductance regulator (*CFTR*) gene. The normal CFTR protein is localised at the apical membrane of epithelial cells and plays a vital role in regulating transepithelial ion transport and water balance. In CF, the airway secretions dehydrate, mucociliary clearance, is impaired and opportunistic bacterial infections take hold. This causes inflammation and progressive lung damage, which is usually fatal by early adulthood [10]. Studies performed in transgenic CF mutant mice showed that the electrophysiological defect in the animal's trachea can be partially or wholly corrected by instillation or aerosolisation of human CFTR cDNA complexed with cationic liposomes [125,126]. However, in most of the performed human clinical trials, gene transfer efficiency was low, and most likely insufficient to achieve clinical benefit. This low gene transfer efficiency is probably due to the extracellular barriers such as mucus, the gycocalyx, tight junctions and mucociliary clearance, which limits the biological access of gene transfer agents [127]. Alton *et al.* showed that the administration, by nebulisation of patients' lungs, of complexes prepared from a plasmid containing the CFTR cDNA (pCF-1–CFTR) and cationic liposomes composed of GL-67–DOPE–dimyristoylphosphatidylethanolamine–PEG_{5000} (molar ratio 1:2:0.05), resulted in a significant degree of correction of the chloride abnormality in all the eight patients involved in the clinical trial [128].

However, the authors detected no alterations in the sodium transport abnormality and observed influenza-like symptoms in seven out of the eight treated patients enrolled in the clinical trial [128]. Using complexes prepared from *p*-ethyl-dimyristoylphosphadityl choline (EDMPC)–Chol cationic liposomes and a plasmid expressing hCFTR, Noone *et al.* concluded that the cationic liposome/DNA complexes are safe but were not capable of mediating consistent evidence of gene transfer to the nasal epithelium [129]. Nevertheless, Hyde *et al.* were able to demonstrate that liposome–DNA complexes (DC-Chol–DOPE–CFTR cDNA) could be successfully readministered to the nose of

Table 2. Cationic liposome formulations applied in gene therapy clinical trials.

Disease	Clinical trial	Vector	Clinical Phase	Ref.
Cystic fibrosis	Delivery of the cystic fibrosis transmembrane conductance regulator gene to nasal epithelium and lung by nebulisation of cationic liposomes/DNA complexes	Cationic liposomes (GL-67–DOPE–DMPE–PEG_{5000})	I	[128]
Cystic fibrosis	Delivery of the cystic fibrosis transmembrane conductance regulator gene to nasal epithelium by cationic liposomes/DNA complexes	Cationic liposomes (EDMPC–Chol)	I	[129]
Cystic fibrosis	Repeated delivery of the cystic fibrosis transmembrane conductance regulator gene to nasal epithelium by cationic liposomes/DNA complexes	Cationic liposomes (DC-Chol–DOPE)	I	[130]
Glioblastoma multiforme	Delivery of *IFN-β* gene transfer via cationic liposomes	Cationic liposomes	I	[131]
Glioblastoma multiforme	Administration of genetically modified replication-disabled Semliki Forest virus vector carrying the human *IL-12* gene and encapsulated in cationic liposomes	Cationic liposome/Semliki Forest virus	I/II	[133]
Metastatic melanoma	Direct gene transfer of DNA plasmid containing the *HLA-B7* gene complexed with cationic liposomes (Allovectin-7) used as an immunotherapeutic agent	Cationic liposomes (DMRIE–DOPE)	II	[201]
Metastatic melanoma	Comparing the response to dacarbazine with and without Allovectin-7 (HLA-B7 plasmid complexed with cationic liposomes) as an immunotherapeutic agent	Cationic liposomes (DMRIE–DOPE)	III	[201]
Breast and ovarian cancer	Intracavitary injection of *E1A* gene complexed with cationic liposomes	Cationic liposomes (DC-Chol)	I	[136]
Glioblastoma multiforme	Stereotactically guided intratumoural delivery of an *HSV-tk* gene by cationic liposomal vector and systemic ganciclovir	Cationic liposomes	I/II	[137]

DC-Chol: 3 β(*N*-[*N'*,*N'*-dimethyl amino ethane]-carbamoyl)cholesterol; DMPE: Dimyristoylphosphatidylethanolamine; DMRIE: *N*-(2-hydroxyethyl)-*N*,*N*-dimethyl-2,3-*bis*(tetradecyloxy)-1-propanaminium bromide; DOPE: Dioleoylphosphatidylethanolamine; EDMPC: *p*-Ethyl-dimyristoylphosphadityl choline; HLA: Human leukocyte antigen; HSV-tk: Herpes simplex virus-thymidine kinase; PEG: Polyethylene glycol.

CF patients. The authors showed that repeated administration of these complexes was safe, with no activation of the immune system, and resulted in efficient gene transfer. Changes were detected in CFTR-dependent cellular function, following each of the three doses (administered 4 weeks apart) in 6 out of 10 treated patients [130]. All these studies show that in order to improve the clinical prognosis of CF patients, current cationic lipid-based formulations will need to be optimised, aiming at increasing the level and persistence of transgene expression.

Most of the approved gene therapy clinical trials address cancer, in which protocols involving the use of cationic liposomes are included. These protocols include the transfer of various types of genes for the application of different antitumour strategies, such as immunopotentiation, oncogene inactivation and 'suicide' gene therapy. Recently, Yoshida *et al.*

published a clinical study involving five patients with malignant glioma (glioblastoma multiforme or anaplastic astrocytoma), in which the safety and effectiveness of IFN-β gene transfer via cationic liposomes were evaluated. Transgene expression and antitumour activity were detected in four out of five treated patients. Two patients showed a partial response (> 50% tumour reduction) and two others had stable disease 10 weeks after beginning therapy [131], thus suggesting the feasibility and safety of IFN-β gene therapy mediated by cationic liposomes. These clinical trials were based on a preclinical study on the antitumour effect and mechanism of action of cationic liposome-mediated murine IFN-β gene therapy in mouse B16F1 melanoma. The authors observed that intratumour administration of cationic liposome–DNA complexes resulted in a 5.5-fold reduction in the mean volume of subcutaneous melanoma and eradication of the tumour in 18% of the treated mice, by directly inducing cell death and stimulating natural killer cells [132]. In addition, Ren *et al.* tested a novel therapy strategy in adult patients with recurrent glioblastoma multiforme, which was aimed at evaluating biological safety, maximum tolerated dose and antitumour efficacy of a genetically modified replication-disabled Semliki Forest virus vector carrying the human *IL-12* gene and encapsulated in cationic liposomes. Preclinical work with these systems in breast and prostate cancer animal models demonstrated their biosafety and antitumour efficacy [133]. Several clinical trials in Phase II and one in Phase III have been performed using Allovectin-7® formulation (Vical Inc., San Diego, CA, USA), which consists of a DNA plasmid containing the human leukocyte antigen-B7 gene complexed with DMRIE–DOPE cationic liposomes [201].

The HER-2/neu oncogene encodes an epidermal growth factor receptor-related transmembrane protein and is overexpressed in many types of human cancers. Therefore, this oncogene represents an excellent molecular target for antitumour strategies, namely in HER-2/neu-overexpressing human cancers, such as human breast and ovarian cancers [134]. Preclinical studies have demonstrated that the adenovirus type 5 *E1A* gene is associated with antitumour activities by transcriptional repression of HER-2/neu and induction of apoptosis [135]. In this context, Hortobagyi *et al.* evaluated the feasibility of intracavitary injection of *E1A* gene complexed with DC-Chol cationic liposomes in patients with breast and ovarian cancers in a Phase I clinical trial. The authors observed *E1A* gene expression that was accompanied by HER-2/neu downregulation, increased apoptosis and reduced proliferation [136]. In a prospective Phase I/II clinical study, Voges *et al.* treated eight patients suffering from recurrent glioblastoma multiforme with stereotactically guided intratumour convection-enhanced delivery of an HSV-1 thymidine kinase gene-bearing cationic liposomal vector and systemic ganciclovir. The treatment was well tolerated without major side effects in two out of eight patients. The authors observed a > 50% reduction of tumour volume and focal treatment effects in six out of eight patients [137].

The main objective in gene therapy is to achieve successful *in vivo* gene transfer into target tissues. The choice of the gene delivery system varies according to the application. For example, prolonged and sustained expression is needed for treating monogenic hereditary diseases, whereas short duration of gene expression may be sufficient for most cancer treatments [138].

6. Expert opinion and conclusion

Due to their safety and versatility, cationic liposomes have emerged as promising alternatives to viral vectors for the development of gene therapy approaches. Numerous *in vivo* applications have been reported in the literature, focusing not only on aspects related to their pharmacokinetics and biodistribution, but also on their toxicity and immunogenicity. The demonstrated efficacy of such systems in mediating delivery of polynucleotides, including therapeutic genes and antisense oligonucleotides, have made them promising candidates for the treatment of several diseases, which has been reflected in their extensive and increasing application in a large number of clinical trials. However, it is generally recognised that the efficacy of lipid-based gene delivery systems is still far from that observed for viral vectors, especially when high levels and long-term transgene expression are required.

Aiming at circumventing these limitations, much work has been devoted to the synthesis of new cationic lipids and the design of new plasmid constructs with more efficient promoters/enhancers. However, the progress achieved is still far from being satisfactory. This has prompted investigators to focus their research activities on not only the nature of the lipoplex components, but also on the variables affecting their formation, mode of interaction with cells and *in vivo* behaviour (e.g., colloidal stability). The aim is to generate complexes of small size with a narrow distribution, while presenting a neutral or negatively charged surface (to prevent nonspecific interactions with blood components) that ensure complete protection of DNA, exhibit specific targeting and have the ability to promote efficient intracellular delivery of carried material and to facilitate its translocation into the nucleus, thus leading to high and sustained levels of transgene expression without causing cytotoxicity. In this regard, it will be rather laborious and difficult to design a nonviral vector capable of fulfilling the conflicting requirements imposed by each of the different stages involved in the gene delivery process. Nevertheless, it is also clear that governing formulation variables may allow the tailoring of lipoplexes to particular transfection applications. Therefore, the biophysical properties of the complexes such as size, charge, stability and the extent of interaction with cells should be controlled in such a way that their application to different protocols (*in vitro*, *ex vivo* and *in vivo*) and routes of administration can be optimised.

Recently, different formulation strategies have been attempted to confer viral attributes to lipoplexes, namely through the association of certain proteins or peptides.

Whether these improvements result in a system that, while exhibiting satisfactory ability to mediate *in vivo* transfection, would lead to such a complexity that could endanger its versatilily and large scale production or could limit extended/repeated *in vivo* use due to immunogenicity, are important questions that remain to be addressed.

Thus, before embarking on extensive and expensive animal experiments and clinical trials, it is advisable to pursue fundamental research focused on the mechanisms by which lipoplexes are formed and deliver their DNA, as well as on methods by which the different biological barriers they face can be overcome.

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