Expert Opinion

The biological routes of gene delivery mediated by lipid-based non-viral vectors

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Cationic lipid/DNA complexes (lipoplexes) represent an attractive alternative to viral vectors for cell transfection *in vitro* and *in vivo* but still suffer from relatively low efficiency. Comprehension of the interactions between vectors and DNA as well as cellular pathways and mechanisms in DNA entry into cells and ultimately nuclei will lead to the design of better adapted non-viral vectors for gene therapy applications. Here, some recent developments in the field on the pathways and mechanisms involved in lipoplex-mediated transfection are discussed. The techniques that are widely used to study the mechanism of gene delivery are also discussed.

Keywords: gene delivery, non-viral vectors, technology, trafficking

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1. Introduction

Gene therapy has become the focus of research for many laboratories for its therapeutic potential in clinical trials [1]. Over the last few decades, the principle of gene delivery for therapeutic purposes has been established. In the case of gene therapy, successful DNA transfer results in the production of therapeutic protein that is encoded by the transgene. Owing to safety concerns (immunogenicity, potential oncogenicity) and the difficulty of large-scale production, the usefulness of recombinant viral vectors is limited [2], so an easy-to-prepare non-viral delivery system without immunogenicity and other safety problems has been given more attention. Among the non-viral vectors, cationic polymers and cationic lipids are investigated most for gene delivery. Cationic lipids and cationic polymers were introduced in 1987 by Felgner *et al.* [1] and Wu and Wu [2], respectively, as new transfection vectors. However, non-viral gene delivery systems are less efficient than the viral systems [3]. There has been a lot of effort given to plasmid DNA delivery technology, such as developing more new cationic lipids. Understanding the mechanism may play a significant role in overcoming the hurdles in gene delivery. There are still many problems that have not been elucidated on the biological barriers and properties of gene delivery through non-viral vectors, although much effort has been expended on them. For example, it is still not clear whether the nuclear localization signal (NLS) peptides play important roles in nuclear trafficking because they have presented opposite results in recent research [4].

In this paper, some potentially rate-limiting steps in the process of gene delivery mediated by non-viral vectors are identified, which include cellular uptake, intracellular delivery and nuclear entry. Insight into the molecular features of each step is of great importance in order to determine the effectiveness as a barrier and to identify means of overcoming the hurdles. This review focuses on two steps that hinder successful gene therapy, which are endocytosis and nuclear entry, because they play very important roles in gene delivery and have been studied hotly and improved rapidly in recent years. Meanwhile, the advanced technologies that are

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used in the study of the transfection process are discussed, in order to provide more information and protocols to study the mechanism of gene delivery.

2. Cellular uptake and intracellular delivery of genes by cationic liposomes

Cationic lipids are amphiphilic molecules containing a positively charged polar headgroup linked to a hydrophobic domain by means of an anchor (**Figure 1**). There are many cationic lipids that are widely used as gene delivery vectors, such as *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTAP), *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) and N4-spermine cholesterylcarbamate (GL-67) (**Figures 1** and **2**). Cationic lipid/DNA complexes (lipoplexes) are mainly prepared by simply mixing cationic liposomes and DNA in an aqueous solution [4]. The driving force for their formation is the electrostatic interaction between the positive charges of the cationic lipid headgroups and the phosphate DNA backbones. In this step, the concentration, temperature, pH and kinetics of mixing are the factors that can influence the formation of lipoplexes [4,5].

The particular cationic lipid type and lipid content used are important factors influencing cellular interactions and formulation effects. Over the last few years, numerous cationic lipid formulations have been developed and shown to be efficient for *in vitro* transfection. Li *et al.* [6] designed and synthesized a cationic lipid–protamine-DNA (LPD) formulation composed of DOTAP liposome, which showed an efficient, systemic transgene expression in mice via intravenous administration. The well-known cholesterol-based cationic lipid 3β-(*N*-(*N*′,*N*′-dimethyla-minoethane)carbamoyl)cholesterol (DC-Chol) was mixed either with Tween 80 alone, or with extra lipid components including castor oil and phosphatidylcholine (PC) or dioleoylphosphatidylethanolamine (DOPE) to make different lipid formulations, which also had promising results [7]. Molinda Kearns *et al.* [8] synthesized four cationic cholesterol-based derivatives by systematically varying the methylation of the polar headgroup, after which the physicochemical properties were correlated with their transfection activity and interaction with cell membranes. The expression of acetylcholine cholesterol (AC-Chol) and 1,2-dimyristoyl-*sn*-glycero-phosphocholine monohydrate cholesterol (MC-Chol) illustrated that although cell surface binding occurred for all of the cationic lipids, only the active analogues were able to gain entry into the cytosol. Dabbas *et al.* [9,10] prepared different PEG (polyethylene glycol)-modified cationic liposomes (PCLs) with varying per cent cationic lipid content and lipid type. Liposome size and ζ-potential analysis of five different PCLs revealed significant differences in their physicochemical properties. It is assumed that the formulation of cationic lipid is an important factor to influence the interaction of lipoplexes with the target cells.

It was proposed that the size and the charges of lipoplexes play important roles in determining the transfection efficiency [5,11]. It is widely accepted that lipoplexes with a slight excess of positive charges confer higher transfection efficiency [12-14]. Relatively large lipoplexes (with a diameter ranging from 0.4 to 1.4 μm) showed a higher transfection efficiency in cell culture than smaller (< 0.4 μm) or larger (> 1.4 μm) ones [5], which was attributed to an enhanced sedimentation of the former onto cells. Meanwhile, larger aggregates and sometimes the precipitation of the lipoplexes have been reported at charge ratios close to neutrality, with a slight excess of positive charges [15-18].

Effective DNA compaction and the packaging of the cationic lipid are also important to achieve high transfection efficiency [19]. Eventually, the cationic lipids are wrapped entirely around the plasmids and the surfaces of the assembled complex appear smooth, implying proper DNA packaging [20,21]. In fact, it is most favorable to obtain high transfection efficiency if the amphiphiles are easily hydrated, forming fluid aggregates, and undergoing a transition to the inverted hexagonal phase in the presence of plasmid DNA at physiological ion strength [19,20]. The addition of DOPE greatly increases the transfection efficiency because it promotes the transition of lipoplexes from a lamellar to a hexagonal phase [21-23]. Koltover *et al.* [23] proposed that this inverted hexagonal structure was responsible for the fusogenic activity of the lipoplexes and represented a key feature in the transfection activity. It also causes neutralization of cationic charges by the negatively charged phosphodiester DOPE.

The entry of the DNA into the cells is the first step of transfection. First, the lipoplexes bind to the cell membrane (specifically or nonspecifically). It is generally accepted that nonspecific association involves mainly electrostatic interactions between the positively charged complexes and the negatively charged cell surface [24]. It was assumed that cationic lipid-mediated transfection resulted from fusion of the positively charged complex with the plasma membrane, resulting in direct entry of the lipoplexes into the cytoplasm [3,24,25]. However, in contrast to previous speculation, some results indicated that endocytosis was the major mechanism of entry. As revealed by electron and fluorescence microscopy, following incubation with cultured cells, lipoplexes could be detected in intracellular vesicles beneath the cell membrane, which suggested that lipoplexes entered cells by endocytosis [25,26]. After endocytosis, the lipid–DNA aggregated into large perinuclear complexes, which often showed a highly ordered tubular structure [25,27,28].

There are various endocytic pathways in eukaryotic cells, including clathrin-mediated endocytosis by means of coated pits and endocytic internalization independent of clathrin, which include phagocytosis, macropinocytosis and caveolae-mediated endocytosis (shown in **Figures 3** and **4**) [4,29-31]. The clathrin-mediated endocytosis is commonly found in lipoplexes' uptake for several cationic lipid systems and for

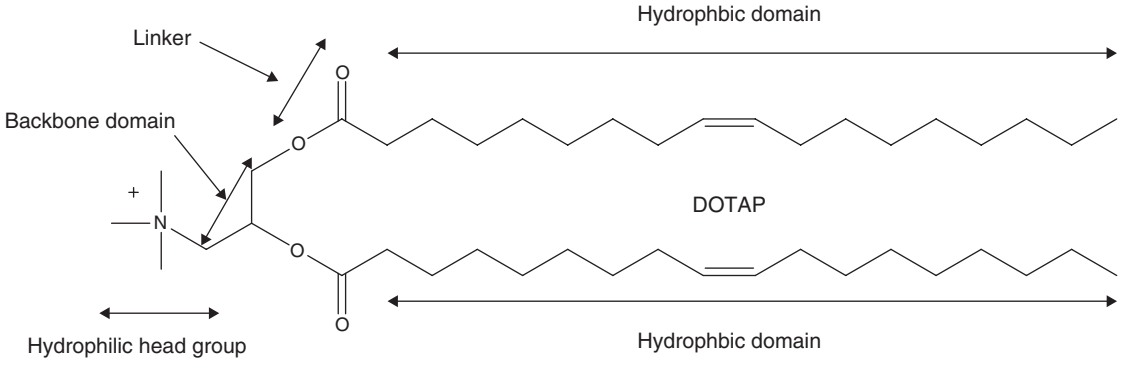

Figure 1. The three basic domains of cationic lipids: hydrophobic moiety, linker and headgroup.

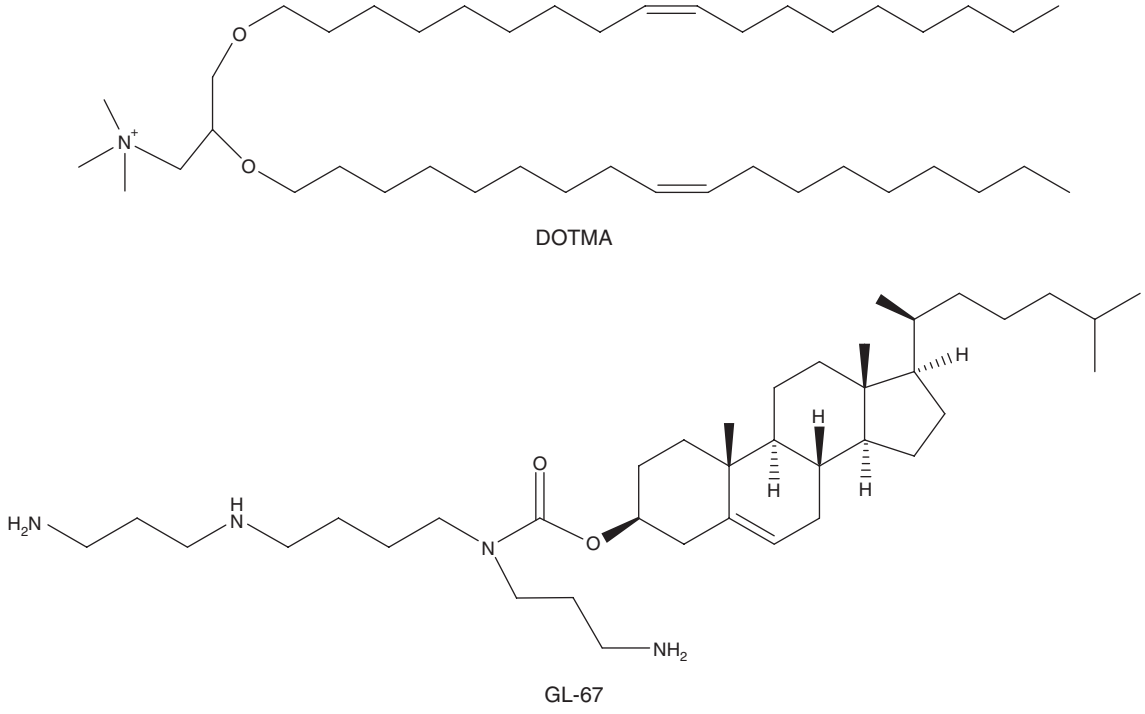

Figure 2. Structures of typical cationic lipids.

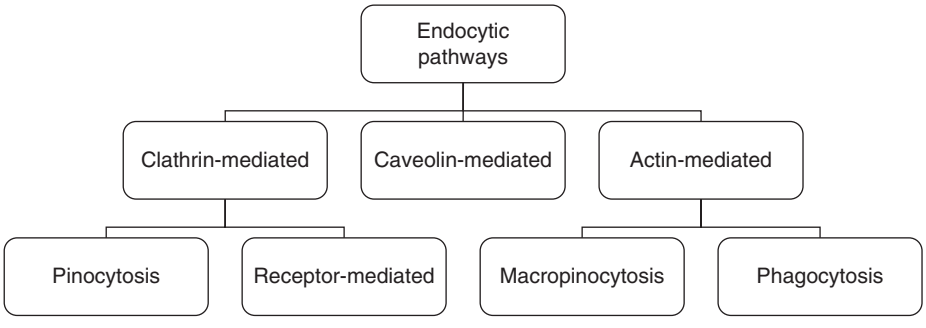

Figure 3. Proposed classification system for endocytic pathways.

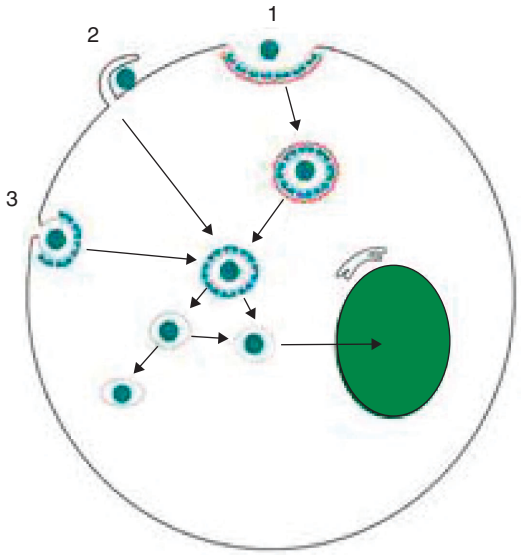

Figure 4. Internalization pathways of lipoplexes. Three endocytotic routes for internalization of lipoplexes are shown: (1) clathrin-mediated endocytosis; (2) macropinocytosis; and (3) caveolae-mediated endocytosis.

different cell types [32-35]. There has also been some progress in the importance of endocytosis independent of clathrin in recent years. Glebov *et al.* [36] proposed that flotillin-1 was one determinant of a clathrin-independent endocytic pathway in mammalian cells. Sigismund *et al.* found that an epidermal growth factor receptor (EGFR)/ubiquitin chimera, which can signal solely through its ubiquitin (Ub) moiety, was internalized exclusively by the non-clathrin pathway [37].

Some reagents can influence the efficiency of endocytosis. Thus, after treatment of the cells with specific inhibitors of the clathrin-mediated pathway such as chlorpromazine, transfection was suppressed [28,29,38]. However, overexpression of a dominant negative mutant of Eps15, which is a protein necessary for the formation of coated pits, led to an effective inhibition of internalization of the lipoplexes and hence transfection efficiency [36-38]. To mimic the *in vivo* situation, several authors have attempted to add serum to cells during incubation with lipoplexes [39-41]. Serum did not significantly inhibit the cell uptake of the lipoplexes and, interestingly, caused a decrease in the size of the lipoplexes in the studies as visualized by fluorescence microscopy [39-41]. However, the transfection efficiency of dioctadecylamidoglycylspermine (DOGS)/DNA lipoplexes was dramatically inhibited by serum [42,43]. From the confocal laser scanning microscopy (CLSM) images, the liposome/DNA complexes were transferred into the cytoplasm even in the presence of nigericin, which was able to dissipate the pH gradient across the endosome membrane. The images were quite different from those of the previous experiments where they used wortmannin, an inhibitor of PI-3 kinase. In the latter case the liposome/DNA complexes were hardly endocytosed into the target cells [44-47].

It has been suggested that in some cells lipid–DNA internalization may be an important barrier to transfection [5,7]. However, low transfection efficiency is not due to inability of the complex to enter the cells because most cells could take up the DNA [46,47]. It is likely that a very large number of plasmids are taken up by the cells, whereas < 50% of the cells express the transgene. So steps following uptake may be important impediments to transfection [48,49].

Evidence that the lipid–DNA complex enters the cell by means of endocytosis immediately raises questions about its intracellular fate. The mechanism by which the complexes enter the cytoplasm has been investigated. A model for the DNA delivery mediated by lipopoly (L-lysine)/DOPE liposomes was summarized by Zhou and Huang [44]. The DNA–liposome complexes either directly penetrate into the cytoplasm through the plasma membrane or are endocytosed into the endosomes. Disruption of endosomes is probably the major mechanism by which the complexes enter the cytoplasm, because this event was observed much more frequently than the penetration of the complexes through the plasma membrane in the electron microscopy (EM) observations [44]. Delivery from the endocytic vacuoles to the lysosomes results in degradation of the complexes. Alternatively, the complexes can escape into the cytoplasm by destabilizing the endosomal membrane. Curiel Kamiya *et al.* [50] have proposed that lipoplexes that have escaped from endosomes are released into the cell cytosol by a detergent-like destabilization mechanism of the endosomal membranes. Once released into the cytosol, the lipoplexes dissociate after interaction or fusion with the cytosolic membrane network, such as the endoplasmic reticulum, Golgi, mitochondria and nuclear membrane [50]. Incubation of cells with fluorescently labeled diC14-amidine/DNA lipoplexes led within 1 – 2 h to a diffusion of the fluorescence in the cytosolic membrane network [50,51]. Another mechanism of membrane disruption that has been proposed by Labat-Moleur *et al.* is a consequence of endosome swelling following endocytosis of lipopolyamine-formulated lipoplexes [51]. It is proposed that buffering resulting from endosomally localized lipopolyamine lipoplexes leads to osmotic swelling and subsequently membrane lysis [51,52].

After endosomal escape, the lipoplexes move to the nucleus [53]. Joseph *et al.* [53] labeled each component of the complex and evaluated the cellular location with confocal microscopy. In each case, by 24 h the fluorescence had coalesced and was observed predominantly in discrete foci in the perinuclear area. The results suggested that the lipid–DNA complexes were endocytosed and moved towards the nucleus where the endosomes fused, and coalesced into large membrane-bound vesicular complexes [53-55]. The observation plus results from direct injection of DNA and lipid–DNA into the nucleus and cytoplasm indicate that movement of DNA from the cytoplasm to the nucleus may be

Table 1. Adaptors of clathrin-dependent endocytosis [24-27].

Clathrin adaptor	Cargo motif recognized	Membrane component bound	Accessory protein motif or domain bound
AP2 complex	ΦxxYxxΦ [DE]xxxL[LI]	PtdIns(4,5)P_2	Dx[FW], FxDxF, WxxF
AP1 complex	ΦxxYxxΦ [DE]xxxL[LI]	PtdIns4P	$[DE]_n$ΦxxΦ
GGA	DxxLL	ArfGTP	$[DE]_n$ΦG[PDE]Φ
HRS/Vps27		PtdIns3P	STAM, ESCRT-1, SnX1
DAB2/ARH	[FY]xNPxY	PtdIns(4,5)P_2	AP2(α,β2)
Arrestin	GPCRs	PtdIns(4,5)P_2	AP2(β2)

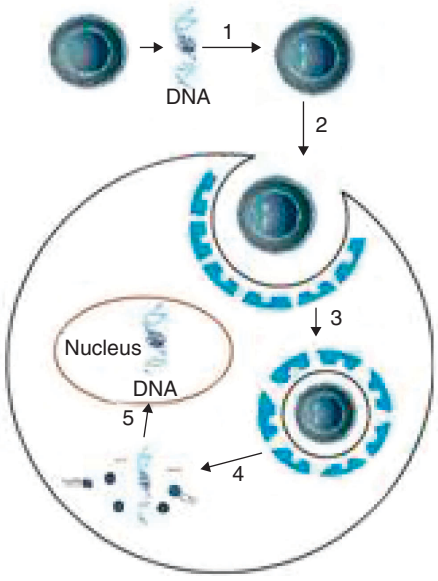

Figure 5. Summary of the steps involved in lipoplex-mediated gene delivery. (1) Formation of lipoplexes by interaction of DNA with cationic liposomes; (2) endocytosis of lipoplex; (3) intracellular delivery of lipoplex; (4) endosomal escape; and (5) nuclear entry.

one of the most important limitations to successful gene transfer [53,56,57].

There are several factors that are of great importance to get high transfection efficiency. For successful transfection, it is important that the internalized DNA be protected against enzymatic digestion before internalization. Another important parameter is the stability of the DNA within the endosomal compartment, as the lipid composition will play an important role in affecting the ability of the DNA to be processed following binding and entry into the cells. If endosomal escape is not achieved then the lipoplex, with its associate DNA, will eventually be degraded as it is transported from early to late endosomes, along the lysosomal degradation pathway [53]. It is suggested to enhance transfection by the addition of compounds (e.g., sucrose) to promote osmotic swelling or lysosomotropic drugs during the transfection step [58]. Therefore, lipids that are effective at mediating cytoplasmic delivery of DNA may have components (or properties) that have a propensity to lyse membranes. To improve the release of DNA to the cytoplasmic compartment, Wang and Huang [59] have demonstrated that pH-sensitive liposome can be used to enhance membrane lysis at the lower pH of the endosome. Others enhance release from the endosomal compartment by using lipoplex technology that incorporates peptides which can trigger endosome lysis or fusion [60]. Some of the peptides used to augment cell transfection are known to form transmembrane channels. The channels can disrupt the plasma membrane as well as the endosome and release the DNA into the cytoplasm [61].

The transfecting DNA has to move into the nucleus to be transcribed once it is released into the cytoplasm. There are a few factors that may influence this step, such as the diameter of nuclear pores, the cell cycle, and so on. The nuclear membrane represents a critical barrier for effective transfection, which contains highly regulated transport structures called nuclear pores. Nuclear translocation of plasmid DNA was limited because of the narrow functional diameter of the pores (~ 25 nm). Studies with lipoplex formulations have demonstrated that the efficiency of DNA transfer to the nucleus can be enhanced by the incorporation of nuclear proteins in the lipoplex [62-64]. The nuclear proteins are unique in that they bear nuclear localization signals, which are thought to enhance trafficking of the DNA into the nucleus. In non-viral vectors, many NLS peptides for efficient nuclear trafficking have been identified recently. For example, considering the hydrophobic nature of the nuclear pore complexes (NPCs), Vandenbroucke *et al.* suggested that *trans*-cyclohexane-1, 2-diol (TCHD), an amphipathic alcohol that reversibly collapsed the permeability barrier of the NPCs, enhanced the transfection efficacy of both naked DNA and lipoplexes [65]. After the NLS peptide derived from the SV40 large T antigen binds to importin α, which forms a complex with importin β, nuclear import of the peptide and its protein cargo are achieved [66-68]. However, Van der Aa Maem *et al.* [68] suggested that the SV40-derived

Table 2. Advanced technologies used to investigate the mechanism of gene delivery mediated by cationic lipids [81-102].

Technologies used	Aim	Technologies used	Aim
TEM	To characterize the lipoplex structures	SEM	To characterize the morphology of the lipoplexes
ELSD	To determine the concentration of liposomes	Zetasizer	To measure ζ-potentials of lipoplexes
FTIR spectroscopy	To measure the molar ratio of cationic lipid	CLSM	To trace the fluorescently labeled lipoplexes
CIDIQ	To quantify the distribution of DNAs	FM	To determine the expression of pGFP

CIDIQ: Confocal image-assisted three-dimensionally integrated quantification; CLSM: Confocal laser scanning microscopy; ELSD: Evaporative light scattering detector; FM: Fluorescence microscopy; FTIR: Fourier transform infrared; SEM: Scanning electron microscopy; TEM: Transmission electron microscopy.

NLS peptide when attached to DNA was not able to improve either transfection efficiency or the nuclear import of the DNA constructs. Tanimoto *et al.* [69] also found no enhancement of nuclear entry by direct conjugation of an NLS peptide to linearized DNA.

One main mechanism that is proposed to explain how plasmid DNA (released from lipoplex) enters the nucleus is passive DNA entry into the nucleus during cell division when the nuclear membrane is temporarily disintegrated; the other mechanism is active transport of the DNA through the nuclear pores. The first mechanism was supported by the fact that nuclear import of DNA was facilitated during mitosis [70-72]. Non-mitosis-dependent entry of plasmid into the nucleus was also suggested because postmitotic cells such as neurons could be transfected. There are two distinguished mechanisms: a *cis*-acting transport and a *trans*-acting mechanism. The former one is DNA sequence-dependent, whereas in the latter addition of peptides carrying a nuclear localization signal is required. However, it is not clear how a plasmid DNA, which is larger in size than the nuclear pore, could transport across the pores [64,72,73].

Moreover, it has been proposed that transfection would be more efficient in rapidly dividing cells and transgene expression would be dependent on the cell cycle [71,74,75]. It has been proposed that PEI facilitated DNA translocation into the nucleus, as well as helped nuclear import of DNA by means of other indirect mechanisms, including better DNA protection or improved cytoplasmic mobility [76-78]. Coupling strategies involving electrostatic interactions [79] or peptide nucleic acid (PNA)-mediated hybridization [80] also increased nuclear entry efficiency. All of the steps of gene delivery mediated by cationic lipids are shown in **Figure 5**.

3. Technologies to study gene delivery

Many technologies and methods are applied to study the mechanism of gene transfection. The liposome and lipid/DNA complex structures were characterized by transmission electron microscopy (TEM) [81,82]. TEM indicated that the complexes correspond to aggregates containing globular substructures with liposome size [82]. The morphologies of the complexes of liposome with DNA could also be observed by scanning electron microscopy (SEM) [82,83]. The size and ζ-potentials of lipoplexes (1:6 wt ratio of DNA and lipid) were measured using a zetasizer. The lipoplex suspensions in Hepes buffer (pH 7.5) were loaded into the capillary cell mounted on the zetasizer, and their size and ζ-potentials were measured 5 times per sample at 25°C [84]. The ζ-potential of the cationic liposome and lipoplexes provides a measure of their net charge.

Enzymatic assays for cholesterol are commercially available and widely used to determine the concentration of liposome [84]. The results rely on hydrolysis of cholesterol esters with cholesterol ester hydrolase, followed by oxidation of the cholesterol by cholesterol oxidase and subsequent production of hydrogen peroxide [85,86]. Up to now, evaporative light scattering detector (ELSD) has been reported for determining the concentration of DMRIE-C/DOPE liposome [87]. Percot *et al.* [87,88] also determined the lipid concentration based on the colorimetric detection of the colipid DOPE and the measure of the molar ratio DOPE/cationic lipid in the liposome by Fourier transform infrared (FTIR) spectroscopy.

The interaction of liposome with DNA was characterized with dye intercalation assay and agarose gel electrophoresis. The dye-binding assays typically rely on the use of intercalating agents that fluoresce when bound to DNA [86-88]. It has been demonstrated that cationic lipid-mediated particle formation in the presence of detergent can yield a macromolecular structure that permits dye binding yet still maintains DNA in a form that is protected from Dnase I degradation [89,90]. Meanwhile, DNA intercalating dye-binding assays can also be used to measure dissociation of lipids from DNA [90-93].

DNA-induced liposome–liposome fusion was monitored by observing the dequenching of the fluorescent probe *N*-4-nitrobenzo-2-oxa-1,3-diazole phosphatidylethanolamine (NBD-PE) incorporated along with rhodamine-phosphatidylethanolamine (Rh-PE) into the liposome membranes [94,95]. To investigate the mechanism of lipoplex entry into cytoplasm, the efficiency of liposome/DNA complexes was studied

in the presence of endocytosis and fusion inhibitors [94,96]. To inhibit endocytosis pathway or fusion, cells were pretreated with a mixture of metabolic inhibitors or fusion-inhibiting peptides, respectively [96,97]. Localization of fluorescently labeled lipoplexes and the intracellular distribution of lipoplexes were examined by CLSM [94].

Furthermore, many other apparatuses and strategies are also used to study the process of gene transfection. Fluorescence microscopy (FM) was widely used to determine the transfection results of green fluorescence protein (GFP), which was encoded by pGFP. Flow cytometry studies were conducted to investigate the impact of cationic liposome on nucleic acid cellular uptake [98]. The oligonucleotide labeling material FITC, a pH-sensitive fluorophore, demonstrated much higher fluorescence intensity in basic solutions than in acidic ones [99]. The method of immuno-gold labeling was used to detect plasmid in the complex and revealed the presence of DNA inside the aggregates [100]. Moreover, the intracellular trafficking of the complex was monitored with TEM [101].

Recently, Kentaro *et al.* [65,102] proposed a new strategy to quantify the distribution of pDNA in the cytosol, endosomes and the nucleus simultaneously, with sequential Z-series images captured by confocal laser scanning microscopy, which was referred to as confocal image-assisted three-dimensionally integrated quantification (CIDIQ). Early after the transfection, pDNA was detected in clusters [65,102]. Meanwhile, the pixel areas of the clusters were used as an index for the amount of pDNA for quantification. In this way, the fraction of pDNA in each compartment relative to the total pDNA per cell can be calculated [102].

4. Expert opinion

This article has focused mainly on cellular uptake, intracellular delivery and nuclear entry of genes by cationic liposome. Over the past decade, significant progress has been made in the understanding of the cellular pathways and mechanisms involved in lipoplex-mediated gene transfection, but the interaction of cationic lipids with cell components and the consequences of such an interaction on cell physiology remain poorly described. Numerous challenging areas of investigation lie ahead for those studying the pathways of gene delivery. As an important rate-limiting step, it will be important to obtain a better understanding how the different endocytic mechanisms such as clathrin-dependent and clathrin-independent endocytosis regulate in different pathways. It is possible that there are new endocytic mechanisms to be discovered. Another major obstacle in non-viral gene delivery is the nuclear membrane. Although it is unclear how the NLS peptides play their roles in nuclear trafficking and there has been limited success in improving the transport of DNA to the nucleus through the NLS, it is highly unlikely that this is a random event. It has been proposed that the nuclear pore complexes contain a hydrophobic phase [65], where hydrophobic importins can dissolve. To this point, using importins with a hydrophobic group, more probably than not, may facilitate nuclear entry and enhance non-viral gene delivery significantly. Meanwhile, the molecular mechanisms involved in some steps still need to be elucidated. For example, it is still not clear where the liposomes go after releasing DNA in the step of endosomal escape and whether they will lead to immune responses. Although it is proposed that the liposomes degrade ultimately, there is limited evidence to support the assumption. Different cationic lipids showed different transfection efficiencies depending on the cell type and the condition of transfection, so it is unlikely that a universal vector will emerge. Using better designed lipid-based carriers to deliver optimized expression vectors will probably arise as a consequence of improvement in lipoplex technology, such as synthesizing more and more cationic lipids that have low cytotocixity and high cell targetability. The ultimate goal of gene delivery is to target the DNA to the immortalized cells and get enough expression for the therapeutic purpose. Some biological hurdles can be overcome by understanding the mechanism of gene delivery through lipid-based non-viral vectors and increasing transfection efficiency, even achieving gene therapy.

Declaration of interest

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