# Nonviral Gene Therapy and its Delivery Systems

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**Abstract:** Nonviral gene therapy has significant clinical potential, yet its therapeutic utility has been hindered by low transfection efficiency due to a combination of extracellular and intracellular barriers. Recent developments in formulation and delivery methodology have allowed a number of advances toward high efficiency gene delivery to various cell types and organs. In particular, the extracellular and intracellular pharmacokinetics of plasmid DNA trafficking are better understood in a number of cell systems. Using cationic lipid or polymers (often with receptor targeting), more than $10^5$ plasmids can be delivered to a single cell. Endosomolytic agents promote endosome disruption, and include: weak bases, proton-sponge polymers, fusogenic peptides, viral particles, and photosensitizing compounds. Both classical and nonclassical nuclear localization signal (NLS) peptides have also been tested for enhancement of the probability of nuclear import events, a major rate-limiting step in DNA delivery to nondividing cells. For example, the M9 sequence from heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) protein, a non-classical NLS, has been found to increase gene expression level by more than 10 to 150-fold in a variety of cell types. This review will concentrate on the current understandings of the basic mechanisms of nonviral gene delivery and new approaches in the field.

## 1. INTRODUCTION

Nonviral gene therapy encompasses a range of strategies: therapeutic transgene expression via plasmid delivery, DNA vaccines, antisense oligos or ribozymes for targeting of mRNA, mRNA delivery or RNA polymerase/cDNA delivery for cytoplasmic transcription, and RNA-DNA chimerics for gene repair. Antisense reagents involve relatively short, rationally designed sequences of RNA, DNA, or peptide nucleic acid (PNA) to hybridize each target mRNA molecule to block expression. Recently, site-specific repair of genetic mutations has been achieved with the use of RNA-DNA conjugates (termed "chimeraplasts") that facilitate single base changes in DNA through the recruitment of cellular repair factors to a basepair mismatch [1,2,3]. Nucleic acid therapeutics can be delivered by *ex vivo* and *in vivo*. *Ex vivo* therapy requires removing cells from patients, modifying them *in vitro*, and introduction of a genetic drug can be achieved by various routes, such as aerosol inhalation to the lungs, arterial catheterization for cardiovascular delivery, topical delivery to follicles, or more commonly by injection (intravenous, intramuscular, subcutaneous, subdermal or intra-tumoral) [4,5,6]. The expression of therapeutic genes is dependent on both the efficiency of delivery to the cell surface and the efficiency of intracellular trafficking [7]. Intracellular barriers are especially challenging in nondividing cells, such as the vascular endothelium and smooth muscle cells, neurons, and epithelia.

An ideal nonviral vector should deliver a therapeutic gene into its target for sustained expression. The expression level should be high enough for therapeutic and potentially regulated. To some degree, nonviral approaches attempt to recreate these functionalities. To understand what

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viruses are capable to transfect their host will have great impacts on the designs of nonviral transfection vectors. The adenovirus, in contrast to nonviral routes, displays high efficency DNA packaging (core proteins) and protection (capsid), receptor targeting (fiber), integrin-dependent internalization (penton base), acid-dependent endosome escape (penton base), cytoskeletal trafficking (hexon), nuclear pore docking (capsid), and nuclear import (terminal protein). Adenovirus (AdV) is widely used because it can transduce both dividing and non-dividing cells. AdV has an icosahedral capsid which is composed of hexons and pentons. Each penton is associated with a rod-like fiber protein to form the penton capsomere. Initial, high affinity binding of AdV to the surface receptors, coxsackie-adenovirus receptor (CAR) of target cells is mediated by the knob domain of the fiber capsid protein [8]. Following receptor binding, the internalization is mediated by the interaction of five Arg-Gly-Asp (RGD) sequences present within the penton base with its secondary targeting cell receptors, the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins which are required for efficient virus internalization [9]. The virus is first internalized in clathrin-coated vesicles which mature into early endosomes that acidify due to a proton pump activation. The low pH environment of the endosome causes a conformational change in the penton base allowing the virion to disrupt and escape into the cytoplasm [10]. Following endosome escape, the virus travels along microtubules toward the nucleus. One possible mechanism is that hexon protein associates with the HSP 70 protein after the virus penetrates into the cell and the HSP 70 is associated with microtubules [11,12]. The virus transport to the nuclear pore can be inhibited by using microtubule depolymerization agent, nocodazole. The capsid is generally found in the cytoplasm although hexon trimer can undergo some nuclear import [12,13]. At the nuclear pore, the virus undergoes a sequential disassembly through a protease-dependent pathway prior to nuclear import of the AdV DNA. The penton capsomeres are lost within 2-5 min after entering the cells and most other proteins are released within 15 minutes. The final dissociation of capsid is dependent on attachment of capsid at the nuclear pore complexes (NPCs) which can be inhibited by wheat germ agglutinin (WGA). By using cytosol-dependent in vitro assay, Saphire et al [14] revealed that the AdV DNA import could also be blocked by the competitive excess of classical protein nuclear localization sequences (cNLSs), likely through competition with cNLS on the terminal protein (TP) that caps the linear viral genome. The viral DNA transported into the nucleus is $Ca^{2+}$ and energy dependent [15].

## 2. EXTRACELLULAR PROCESSES

*In vivo* gene delivery faces a variety of physical and biochemical barriers such as anatomical size constraints, interactions with biological fluids and extracellular matrix, and binding to a broad variety of non-target cell types. The extracellular barriers are mainly dependent on the target tissue and delivery method. Steric barriers include mucus, glycoaminoglycan barriers in tissue interstitium and glycocalyx barriers at the cell plasmalemma. Steric barriers include interstitial matrix, pericardium, internal elastic lamina, and endothelial barriers as exemplified by the blood brain barrier. Extracellular barriers may also be biochemical and include nucleases, plasma coagulation, complement activation, and plasma proteins that bind charged components of a nonviral formulation. For example, deliver into the airway epithelia for cystic fibrosis gene therapy has many technical difficulties since the apical membrane provides a thick mucus layer that binds and eliminate vectors via mucus clearance mechanisms. Significant glycocalyx barriers can prevent access of plasmids to cell surface receptors [4]. Most *in vivo* clinical trial via nasal delivery or aerosol inhalation have observed beneficial effects, but they rarely meet the theoretical requirement to transfect more than 6-10% of surface epithelial cells to relive patients' symptoms [16].

### 2.1 Steric Barriers

Any gene therapy aimed at central neuron system disorders must bypass the blood-brain barrier (BBB). Tyler and coworkers [17] delivered

unmodified antisense PNA complementary to mRNA of the rat neurotensin receptor into brain by intraperitoneal injection. The antisense PNA was able to reduce the level of targeted mRNA. Shi and Pardridge [18] delivered plasmids into brain by a simple i.v. injection. To do this, they used neutral liposome instead of cationic lipid with incorporated polyethylene glycol (PEG) for circulatory stability and then conjugated with OX26 monoclonal antibody against rat transferrin receptor. These pegylated lipids were then used to encapsulate the plasmids, which carried the reporter genes and i.v. injected into rats. This study showed that plasmid gene therapy could be used for normal CNS targeting without disturbing the BBB. This work is also important in the selection of materials and techniques. The neutral liposome formula not only has great advantage in bypassing the BBB [19], but also was able to enhance expression in the liver [20,21] than that achieved with cationic lipids. Vascular disease gene therapy provides another example where significant extracellular barriers reduce gene delivery into the arterial wall. For example, it requires retroviral vector 15-30 min to transfect endothelial cells under no flow condition before any level of transgene expression can be observed [22]. Rome *et al.* [23] demonstrated that internal elastic lamina of intact arteries provides a steric barrier that prevents medial transduction with viral particles. A similar barrier would exist for a nonviral vector.

### 2.2 The In Vivo Metabolism and Pharmacokinetics of Plasmid DNA

In vivo studies have shown that cationic liposome/plasmid DNA or oligonucleotide complexes are rapidly cleared from circulation with the highest clearance by the "first pass" organs, such as the lungs, spleen and liver. By complexing reporter plasmid with DOTMA (N[1-(2,3-dioleyloxy)propyl [-N,N,N-triethyl-ammonium):DOPE (dioleoylphosphatidylethanolamine) (2:1, mol:mol), Mahato et al [24] reported that 15 min after tail vain injection of 60-90 μg DNA/mouse, the majority of recovered DNA was found in the lung (83%), liver (11.4%), kidney (2.2%) and spleen (1.9%). The DNA was also cleared quickly from blood (0.08%). In another study by using the HLA-B7 major histocompatibility related gene (MHC class I) complexed with cationic lipids, elimination of DNA by degradation in the blood after i.v. administration occurred within 30 min [25]. The circulation time can be increased with coating complexes with PEG [26]. Szoka and coworkers [27] have reported several serum proteins such as bovine serum albumin (BSA), HDL and LDL, and macroglobulin interact with cationic lipid/oligonucleotide complexes. These binding events alter the complex diameter and zeta potential (from positive to negative values). However, protein binding did not dissociate lipid from the complexes [27]. Also, certain tissues can maintain plasmid longer than others can. For example, plasmid (10 mg) for expression of serum protease-resistant porcine growth hormone-releasing hormone gene, when directly injected into pigs' semitendinosus muscle followed by electroporation, resulted in expression for several months [5,28].

Dosages of plasmid DNA used *in vivo* have varied in different studies. In the phase I clinical trial involving 17 HLA-B7-negative patients with metastatic melanoma, the intratumoral injection dose of cationic lipid plasmid was increased from 10 to 250 μg of DNA in single or multiple administration [29]. Studies of gene transfer to human coronary arteries with a perfusion-infusion catheter used 1 mg of plasmid complexed with 1 ml of DOTMA:DOPE liposomes for expression transgene VEGF or marker β-galactosidase [30]. No serious episodes have been reported with clinical use of plasmids. While cationic lipids used for lipofection are sometimes cytotoxic in tissue culture, clinical or histological changes that correlated with liposome/plasmid gene therapy are seldom observed [31].

## 3. INTRACELLULAR PROCESSES AND SUBCELLULAR TRAFFICKING

There are three major intracellular processes that affect nonviral gene delivery efficiency: endocytosis, endosome escape (endosomolysis) and nuclear targeting, in which the last step is

easily achieved in rapidly dividing cells and is often the rate-limiting step in nondividing cells [32,33,34] (Fig. **1**). All gene delivery vectors enter cells either by endocytosis or membrane fusion. While the detailed mechanisms of uptake for many nonviral delivery systems are incompletely resolved, the endocytosis pathway is considered the major route of plasmid internalization during lipofection. The subcellular trafficking process has been visualized in a recent *in vitro* transfection study carried by Godbey and coworkers [35]. They observed that it takes 30 minutes for the polyethylenimine (PEI) complexed DNA to attach to cell surfaces and form aggregates. Endocytosis occured 2 to 3 hr post-transfection, with nuclear import detected within single cells of the population occurring at 3.5 to 4.5 hr after plasmid/PEI delivery to the cells. After entering the nucleus, the earliest transgene expression was observed within 1 hr. In the context of most plasmid trafficking studies in individual cells in a population, the nucleus of individual cells entering and exiting G2/M may have altered plasmid transport properties and cell cycle remains an important variable in these types of studies.

### 3.1 Receptor Targeting and Endocytosis: Lipofection

Mammalian endocytosis can involve phagocytosis, clathrin-dependent receptor mediated endocytosis (RME), and clathrin-independent endocytosis [36]. During plasmid endocytosis by the clathrin-dependent RME pathway, the plasmid containing complex binds surface receptors which then cluster to form clathrin coated vesicles (CCV). Plasmid degradation can occur within endosomes [37]. Within minutes in the early endosomes (pH 6.3-6.8), carrier vesicles transfer the plasmid to the late endosomes (pH 5 - 5.5) in which the plasmid resides for 10 to 25 min before arriving in the lysosomes. Lysosomes, with very low pH ($\Phi$5), accomplish intracellular digestion using a wide variety of hydrolytic enzymes [37] (Fig. **1**).

Cationic lipids spontaneously bind DNA, condense, and coat DNA [38,39,40]. These DNA/liposome complexes are aggregates with general sizes from 50 to 500 nm [26] and have weak DNA protection function against DNase. The earliest cationic lipid, DOTMA was developed in the late 80's [39]. A variety of formulations have been developed since [41], however, the experimental results with these lipids are dependent on cell types and individual laboratory protocols. One drawback of these lipid formulations is that the efficiency of transfection is very low with nondividing and differentiated cells. While lipofection efficiency can be higher than 80% for HeLa or COS cells, it can be less than 5% for confluent endothelial cells when a β-gal reporter gene is used [42,43] and essentially 0% with isolated neurons or macrophages.

During lipofection, the net charge and size of the lipid head group with the hydrophobicity and length of the tail group have impact on the transfection efficiency both *in vitro* and *in vivo*.

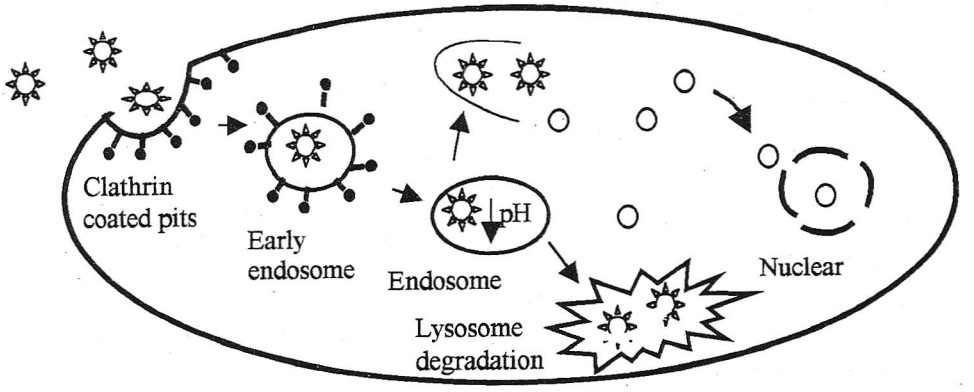


**Fig. (1).** Receptor mediated DNA/liposome uptake pathway with the three major intracellular barriers: endocytosis, endosomolysis and nuclear import.

Felgner *et al.* [40] showed that hydroxyethyl derivatives of the parent DOTMA, which contains a methyl group in the head group, improved transfection efficiency, but increasing polar hydroxy alkyl chains decreased transfection efficiency *in vitro*. The transfection efficiency is also decreased when the length of the saturated aliphatic chain increased from 14 to 18 carbon atoms with the transfection efficiency being lower than that achieved with their unsaturated counterparts. In a related *in vivo* study, Ren et al [44] showed that increasing the carbon numbers of the backbone that connects the cationic head group and the alkylchains in DOTMA could decrease the transgene expression levels up to 50-fold in mice lung, liver, heart and other organs. The *in vitro* transfection activity of lipid carriers can not be used to predict their in vivo activities. Floch and coworkers [45] compared a group cationic phosphonolipids' activities *in vitro* with cystic fibrosis airway epithelial cells (CFT1) and *in vivo* with tail vain injection of female Swiss mice by using luciferase as marker gene. They found that the lipid with oleyl acyl chains (GLB43) was very active *in vivo*, but far less active *in vitro*. However, its analogue with the myristyl acyl chains (GLB73) has reverse effect with high *in vitro* activity but almost no activity *in vivo*.

Lipid formulations usually contain one cationic lipid and one helper neutral lipid such as DOPE and cholesterol. The neutral lipid is required for non-bilayer forming cationic lipids to form stable cationic bilayers. The ratio and combination of cationic lipid and helper are important factors for transfection efficiency and toxicity [41], both of which are cell type specific. For example, we noticed that with confluent bovine aortic endothelium cells (BAEC) transfection, lipofectamine [3:1 w:w DOSPA (2'-(1",2"-dioleoyloxypropyldimethyl-ammonium bromide)-N-ethyl-6-amidospermine tetra trifluoroacetic acid) and DOPE] provides better transfection than lipofectin (1:1 mole: mole, DOTMA:DOPE), but is similar to DOTAP(1,2-bis(oleoyloxy)-3-(trimethylammonio)propane):DOPE (1:1 mole: mole). In their *in vivo* studies, Mahato et al [46] found that the 4:1 and 2:1 molar ration of DOTAP:DOPE achieved higher transgene expression level in mice lung and liver that the 1:1 molar ration formula. Anwer et al [47] also indicated that 4:1 molar ratio of DOTMA:DOPE or DOTMA:Cholesterol had much higher transgene expression in both lung and tumor of mice than that with 1:1 molar ratio.

The neutral lipid DOPE, which is believed to disrupt the endosome membrane under acidic conditions due to a hexagonal phase transition [48] is not particularly stable *in vivo*. A DOTAP:cholesterol formulation provided a 40-fold increase in reporter gene expression in the mouse lung compared to DOTAP:DOPE after tail vein injection [49]. From *in vitro* studies with lipid/DNA complexes in mouse serum, Li and coworkers [50] showed a similar finding: serum-induced release of DNA from complexes is slower with cholesterol than with DOPE. The complexes with DOPE disintegrated within an hour, but with cholesterol the complexes lasted more than 5 hr.

DC-Chol (3β[N-(N',N'-dimethylaminoethane)-carbamoyl] cholesterol) was synthesized and tested as a transfection reagent by Huang and coworkers in 1991 [51]. Complexes formed between DNA and DC-chol/DOPE have an average diameter of 1.2 μm. When the polycations polylysine or protamine were incorporated into the complexes the average diameter was reduced to about 100 nm, a size that gives higher endocytosis efficiency. This liposome/polycation/DNA triplex (LPD) showed higher transfection efficiency than liposome/DNA complexes alone and also enhanced resistance to degradation by nucleases. Many other groups have tested DC-Chol in different cell types. Colosimo et al [52] reported that DC-Chol/DOPE liposome is more effective for transfecting human epithelial cells than other lipid types. In human bronchial epithelial cell lines, Peters et al [53] reported that both lipofectamine and DC-Chol/DOPE could only transfect a maximum of 3% of human bronchial epithelial cells (BEAS) expressing wild-type cystic fibrosis transmembrane conductance regulator, CFTR. However, lipofectamine could transfect a maximum of 6% of cells which has abnormal CFTR (2CF cells). DC-Chol/DOPE resulted in a maximum of 12% transfection of 2CF cells. The

DC-Chol/DOPE formulation is also reported more efficient for transfection of SKnSH (neuroblastoma) and the primary rat neuronal cells with less toxicity compared to the DOTAP/DOPE [54].

The ratio of DNA to lipid is an additional factor that influences the transfection efficiency. Theoretically, the optimal ratio should be the number of positive charges contributed by the lipid needed to exceed the number of negative charges on the DNA, so that the complex has a net positive charge to facilitate interaction with the net negatively charged cell surface [38]. In practice, charge ratios (+/-) considerably higher than 1 often provide the higher level of transgene expression *in vitro* [55]. Using TEM to study the cationic liposomes/plasmids complexes, Gustafsson et al [56] showed that excess of lipid can lead to the entrapment of plasmid between the lamellas in clusters of aggregated multilamellar structures. Similarly free or loosely bound plasmids were found in the vicinity of the complexes when excess plasmid was used. This effect is also generally true *in vivo*. By using DOTMA:DOPE (2:1, mol/mol) as carrier, Mahato et al [46] showed that 3:1 (+/-) charge ratio yield higher transgene expression than that of lower charge ratio both in the lung and liver of mice. However, this charge ratio has to be adjusted according to the targeted organ. By i.v. administrating DOTMA-based transfection complexes in mice, Anwer et al [47] demonstrated that the charge ratio from 5:1 to 0.8:1 (+/-) significantly reduced DNA levels in the lung, but did not change the DNA levels in the tumor.

Cationic lipids cause cytotoxic effects to varying degrees which are often due to lipid incorporation in the plasma membrane facilitated by lipid mixing or fusion [57,58,59]. This process has been shown to inhibit protein kinase C activity [60]. One strategy to reduce the cytotoxicity of lipids involved the synthesis of biodegradable cationic lipid with an amphiphile hydrophobic domain [61]. In this approach, Aberle et al [62] synthesized linchpin tetraesters by using ester linkage to tether both the polar DNA binding domain and the hydrophobic domain. The tetraesters showed comparable transfection efficiency to DOTAP and DC-cholesterol in NIH3T3 cells but with much lower cytotoxicity.

Transfection of DNA/lipid complexes can result in internalization of more than 100,000 plasmids per cell [38,39, SLD unpublished data]. By using thin section electron microscopy and peptide that inhibits membrane fusion, Zhou and Huang [63] directly demonstrated that in 98% cells the DNA/lipid complexes enter the cytoplasm mainly by destabilizing endosomes and with only occasional entering by penetrating plasma membrane. Using endosome disrupting agents, such as chloroquine, cytochalasin B or ammonium chloride, many have demonstrated that transfection efficiency in dividing cells can be increased [40,64]. In our works, after adding endosome disrupting fusogenic peptides or replication deficient adenovirus to DNA/lipid complexes with specific T7 promoter directed cytoplasm transcription assay [42,65], we also demonstrated that disrupting endosome membrane increased the level of transcribable plasmid in the cytoplasm by more that 20 to 120 fold [SLD unpublished data].

Various natural and synthetic polymers have been used for packaging DNA: poly-L-lysine (PLL), histones, chitosan, polyethyleneimine (PEI), protamine sulfate, polyethylene glycol (PEG), peptide nucleic acid (PNA), disulfide-linked detergents, amine terminated dendrimers, and biodegradable polymers such as polylacticglycolic acid (PLGA). These materials not only bind, but also condense plasmid. Commercial available PLL has a molecular range from 1 to 300 kDa, is convenient to use, however, can be toxic to the cells at nanomolar concentrations in some instances [66]. The benefit of histones likely involves packaging and DNase protection with little indication that the classical nuclear localization sequence facilitates nuclear targeting of plasmid [67]. Protein tetra-amine spermine was developed as a carrier by Gottschalk *et al* [68] and they found that peptide tyrosine-lysine-alanine-$(lysine)_8$-tryptophan-lysine was very effective for complexing DNA. DNA binding peptides can also be synthesized to couple with specific cell ligands, thereby allowing receptor

mediated targeting of the peptide/DNA complexes to specific cell types. One example is the RGD (arginine-glycine-aspartic acid) sequence. The RGD sequence is a common integrin attachment site in ligands such as fibronectin, laminin, and fibrinogen.

The commonly used PEI (at 2, 25, or 800 kDa) condense plasmid and increase their stability inside of the endosomes potentially by interference of acid-dependent DNase [35,69,70]. Recently by using a linear-PEI (22 kDa) as carrier for *in vivo* plasmid delivery, Zou et al [71] were able to transfect 1-5% of mouse lung cells (2 ng luciferase/mg protein), with other organs remaining refractory to transfection (1-10 pg luciferase/mg protein). Alveolar cells, including pneumocytes, were the main targets when β-galactosidase histochemistry was used to monitor marker transgene expression. A polymer system, named PINC™ (protective, interactive, non-condensing system) has also been developed for plasmid delivery [72,73]. One example is polyvinyl polymers (PVP), which might form hydrogen bound with DNA to reduce their negative charge, protect them from DNase, and facilitate the uptake in tumors [73] or muscle tissue [74]. Chitosan, a natural polycationic bio-compatible polysaccharide, is also extensively used by the laboratory of Leong and others [75,76,77]. One advantage is that chitosan is less immunogenic, nontoxic, and potentially useful for oral gene delivery carrier [75]. Chitosan and plasmid DNA produce stable complexes with diameters of approximately 50-100 nm. With HeLa cells, chitosan/plasmid had higher transgene expression level than using both lipofectamine and PEI [76]. However, the poor dissociation of chitosan from plasmid complexes may inhibit early stage transcription reaction [76,77].

### 3.2 DNA Condensation, Protection, Receptor Targeting, and Endosome Escape

Kircheis et al [78] reported that gene transfer after intratumoral application was 10-100 fold more efficient with transferrin-polyethylenimine (Tf-PEI)/DNA or adenovirus-enhanced transferr-infection (AVET) complexes in comparison to naked DNA. Shi & Pardridge [18] delivered plasmid through BBB by selectively used materials like neutral liposome, PEG and antibodies. By conjugating PEI to the integrin-binding peptide CYGGRGDTP (PEI-RGD), Erbacher and coworkers [79] condensed plasmid into a homogeneous population of 30-100 nm toroidal particles. In comparison to PEI, PEI-RGD increased transfection efficiency 10 to 100-fold in integrin-expressing epithelial (HeLa) and fibroblast (MRC5). With lactosylated poly-L-lysine and endosome disrupting reagents, such as, chloroquine, combined with E5CA peptide (GLFEAIAEFIEGGWEGLIEGCA) as a fusogenic agent, Kollen et al [80] was able to obtain 10,000-fold increase in transfer of pCMV*Luc*/ lactosylated poly-L-lysine complex than conventional transfection in the immortalized cell line CF/T43, a nasal airway epithelial cells from a cystic fibrosis patient. Folate receptor has been targeted for plasmid delivery also since it is a highly selective tumor marker, such as it is over expressed in more than 90% of ovarian carcinomas [81,82].

To increase the gene delivery efficiency of lipid by enhancing endosome escape, inactivated virus has also been used. We have found that replication deficient adenovirus can enhance endosome escape by 20-fold at a multiplicity of infection of 100. This only led to an increased gene expression level about 8-fold in confluent BAEC [SLD unpublished data]. Hemagglutinating virus of Japan (HVJ) was also complexed with liposomes [83] for plasmid delivery. Kaneda and coworkers developed a hybrid-type liposome (HVJ-liposome), in which DNA is packaged in a liposome that comprises phospholipids and cholesterol. The liposome is fused with UV-inactivated HVJ, which is able to fuse with the cell surface to deliver the DNA directly to the cytoplasm. In some cases, nuclear proteins, such as high mobility group 1 (HMG-1) have been co-introduced with the DNA to enhance transgene expression [84,85]. By adding anionic lipids into the HVJ-liposome, Saeki et al [86] were able to increase gene expression level by 5 to 40-fold, both *in vivo* and *in vitro* in comparison to the early version HVJ-liposome or Lipofectamine.

Many chemicals, such as cytochalasin B, bafilomycin A and nocodazole have been used to study or alter plasmid processing within the endosome/lysosome pathway. Cytochalasin B blocks phagocytosis and pinocytosis by inhibiting actin polarization, but not the process of RME. When 5 μg/ml of cytochalasin B was preincubated with rat tracheal epithelial cells, plasmid uptake through pinocytosis and phagocytosis were inhibited by 65% and 93% respectively, but not the uptake through RME. The uptake of lipid/plasmid complexes was also decreased about 50%, indicating acting polymerization is involved in endocytic mechanism of lipid/plasmid entry [87]. The relative reporter gene expression level was decreased in a dose dependent manner with treatment of cytochalasin B [88]. Bafilomycin A, inhibits the vacuolar ATPase endosomal proton pump [89] and is also used for detecting endosomolysis activity of materials. Bafilomycin A blocked the endosomolysis activity of histidylated polylysine and decreased reporter gene expression [90]. Nocodazole increased reporter gene expression in both cytoplasm and nuclear expression assays by blocking maturation of transport vesicle to late endosome/lysosome and increasing plasmid releasing from early endosomes [88].

Chloroquine is a weak base which can reduce acidification of endosomes and prelysosomes [91] with the potential for enhancement of endosome disruption. Results are conflicting in different studies and likely cell type specific due to sensitivity to chloroquine toxicity. For examples, Erbacher et al [92] reported that 100 μM chloroquine is needed to yield the maximal transfection efficiency in HepG2 cells with glycosylated-PLL /plasmid complex. However, Cho et al, [93] reported that chloroquine had no effect in HepG2 cells transfection when asialo-oroso mucoid-PLL (ASOR-PL) was used. Ogris et al [64] reported that chloroquine increased gene expression approximately 10-fold with PEI (800 kDa) in K562 or Neuro2A cells. However, Huang and coworkers [88] reported that chloroquine actually decreased gene expression levels in both cytoplasm and nuclear expression. Kollen and coworkers [80] found that chloroquine used with pCMV*Luc*/ lactosylated poly-L-lysine complex produced 20% transfection efficiency while chloroquine with glycerol produced 90% transfection.

Berg and coworkers developed a new technique named photochemical internalization (PCI) to increase endosome escape [94,95]. Photo-sensitizing compounds, such as $AlPcS_{2a}$ (aluminum phthalocyanine with two sulfonate groups on adjacent rings), endocytosized along with proteins or plasmids, become localized in the membranes of endosomes and lysosomes. After exposure to light, these membrane are destroyed, and the endocytosed DNA released into the cytoplasm. Using this method, they delivered type I ribosome-inactivating proteins, horseradish peroxidase, a p21ras-derived peptide, and a plasmid encoding green fluorescent protein into cytosol in a light-dependent manner. In a human melanoma cell line the light treatment increased the transfection efficiency more than 20-fold, reaching transfection levels of about 50% of the surviving cells.

Membrane fusion and disruption are common biological processes during viral infection. The haemagglutinin (HA) peptide from influenza virus is one of the best characterized pH-dependent fusion protein. The HA is a trimeric glycoprotein embedded in the viral membrane, and responsible for the specific binding to the cell surface sialic acid-coating receptors and for the fusion of two membranes. The HA trimer has three identical monomers and each monomer is composed of two subunits, the HA1 and HA2 linked together by a disulfide bond. Uptake by the host cell is facilitated by HA1 subunit binding to sialic acid-coated receptors. In the low pH environment, conformational changes in the trimer. Expose the hydrophobic residues in the HA2 subunit towards to the targeting membrane and this hydrophobicity is the driving force for the disruption of the endosome. The N-terminal of HA2 subunit with sequence of GLFGAIAGFIENGWEGMIDG-WYG, includes three negatively charged residues, Glu (11, 15) and Asp (19), which prevent the fusion peptide from forming an α-helix at neutral pH. At low pH, HA2 will form an α-helix after the

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