

Review

WHAT CAN WE LEARN FROM VIRUS IN DESIGNING NONVIRAL GENE VECTORS*

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Abstract Gene therapy has emerged as a potential new approach to treat genetic disorders by delivering therapeutic genes to target diseased tissues. However, its clinical use has been impeded by gene delivery systems. The viral vectors are very efficient in delivering and expressing their carried genes, but they have safety issues in clinical use. While nonviral vectors are much safer with very low risks after careful material design, but their gene transcription efficiency is too low to be clinically used. Thus, rational design of nonviral vectors mimicking the viral vectors would be a way to break this bottleneck. This review compares side-by-side how viral/nonviral gene vectors transcend these biological barriers in terms of blood circulation, cellular uptake, endosome escape, nucleus import and gene transcription.

Keywords: Gene therapy; Gene delivery; Virus; Nonviral vectors; Cationic polymers.

INTRODUCTION

Gene Therapy

Genes are the base controlling all human body functions, and gene therapy has emerged as an exciting new approach to treat human diseases because of its versatility and functionality. Theoretically it is possible to treat a genetic disease by introducing therapeutic gene such as DNA or small interfering RNA (siRNA) into diseased cells to eliminate the underlying biological defects or promoting a protective immune response by administering genes encoded for specific antigens^[1]. Several devastating diseases were reported to be successfully treated by gene therapy^[2–4]. Therefore, gene therapy would be the most effective therapeutic method for treating gene-defective diseases at low cost if it is completely successful^[5].

Gene therapy consists of three key components, *i.e.*, the therapeutic gene, the target cells, and the delivery vehicle or the vector. The gene delivery system turns out to be the bottleneck of gene therapy because it has to be able to overcome many biological barriers including blood circulation, cellular uptake, endosome or lysosome escape, cytosolic trafficking, nuclear import and gene transcription to reach the targeted cells and promote efficient gene expression. At present, many types of vectors have been developed, among which viral and nonviral vectors are the two mostly studied in gene delivery.

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Viruses are naturally evolved parasites and have developed sophisticated mechanisms to target the host cells and control intracellular trafficking for efficient delivery and expression of their genomes. Consequently, viral vectors exhibit very high transcription efficiency. Various viral vectors have been designed in gene therapy such as retroviruses and adenoviruses^[6]. Many clinical trials using viral vectors have shown very exciting therapeutic results^[7], such as X-Linked Severe Combined Immunodeficiency^[3, 4], diabetes^[8], Alzheimer disease^[9, 10], human β -thalassaemia^[11], red-green colour blindness^[12], cancers including prostate^[13–15], kidney^[16], ovarian^[17] and various solid tumors^[18] and other devastating diseases^[19]. However, various safety problems associated with viral vectors have impeded their clinical applications including toxicity and immunogenicity, which may cause immediate death^[20, 21] and induce mutational hazards such as cancer^[22–24].

The safety concerns associated with viral vectors have encouraged the development of nonviral vectors^[17, 25]. Nonviral vectors, most cationic lipids, dendrimers, polymers and polypeptides, can package DNA into appropriate size, have good biocompatibility and capability for large-scale production, have attracted considerable attention^[25–29]. However, nonviral vectors constantly exhibit low transcription efficiency, at least 10^3 times less efficient than viral vectors, even though numerous types of differently structured polymers have been synthesized and tested.

Therefore, to further design nonviral vectors with transcription efficiency close to that of viral vectors, it is important to analyze the viral transfection process and learn from them for the nonviral vectors. Herein, we compare the commons and differences between virus and nonviral vectors from the aspects of DNA package, circulation, cellular uptake, endosome escape, cytosolic trafficking, nuclear import and gene expression.

Barriers for Gene Delivery

A successful gene delivery system requires a rational strategy to overcome many biological barriers, such as blood circulation, cellular uptake, endosome escape, cytosolic trafficking, nuclear import and efficient gene expression. The process of nonviral gene delivery is shown in Fig. 1. To achieve successful nonviral gene delivery, all the barriers during this process should be overcome. In the following part, we compare the process of viral and nonviral vectors.

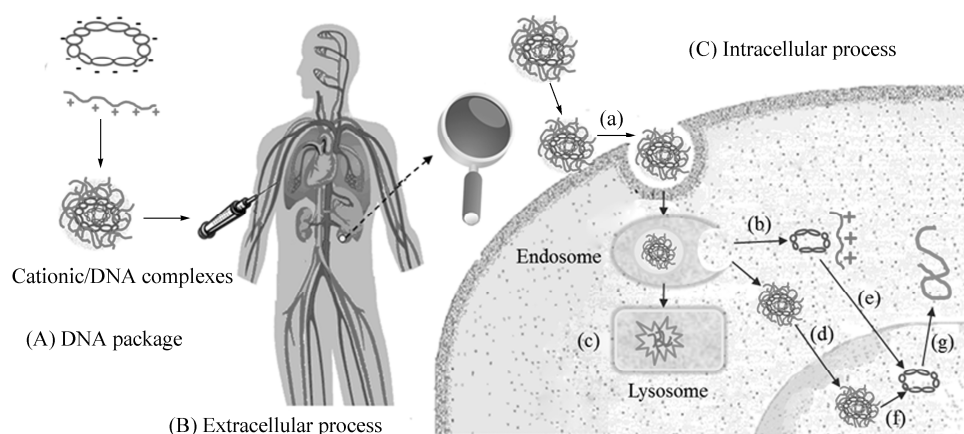


Fig. 1 Nonviral gene delivery process

(A) DNA package: Complexation of DNA is mediated by electrostatic interactions between the negatively charged phosphate backbone of DNA and cationic molecules; (B) Extracellular process: The complexes are injected into the bloodstream and may accumulate in tumor site by EPR effect and active targeting effect; (C) Intracellular process: The complexes enter endosomes through endocytosis (a), the complexes or released DNA may escape from the endosomes to cytoplasm (b) or are delivered to lysosomes and degraded (c), the complexes or DNA in the cytoplasm are transported to the periphery of the nucleus and internalized (d, e) into the nucleus, DNA is released from the complexes in the nucleus (f), and finally DNA is expressed and corresponding proteins are produced (g).

VIRUS AND NONVIRAL GENE VECTORS

DNA Package

Viruses have a large diversity of shapes and sizes, most of which have a diameter between 100–300 nm and a series of mechanisms have been developed to package their DNA to successfully replicate within living cells. Some virus particles consist of nucleic acid surrounded by a protective coat of protein called capsid. Proteins encoded by viral genome will self-assemble to form a capsid, which can protect nucleic acid of virus from degradation like a shell. Adenovirus is the most common vector applied in gene delivery and some recombinant adenoviruses have been designed to be efficient delivery vehicles for gene therapy^[30]. The schematic of the adenovirus is shown in Fig. 2. Each part of capsid proteins could interact with host proteins and elicit specific signaling for virus infection. The fiber is responsible for initial cell attachment and plays a critical role in endosomal release, while the penton proteins take charge of initiating endocytosis and causing endosomal lysis through conformational change at low pH. In intracellular trafficking, the penton and hexon proteins both play critical roles^[31, 32].

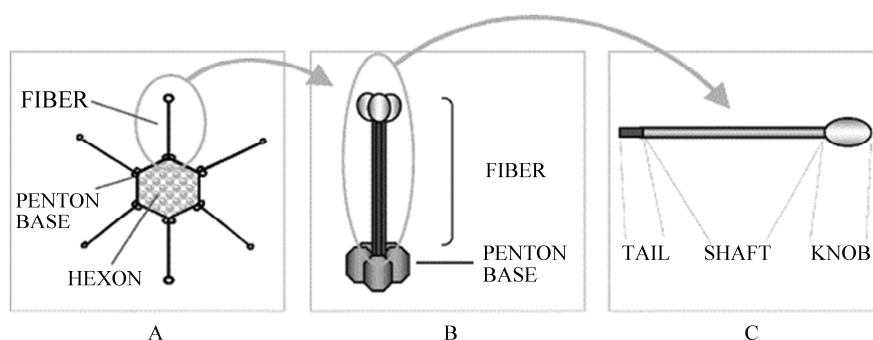


Fig. 2 Schematic of the adenovirus capsid (Reprinted from Ref. [32] with permission from Elsevier)

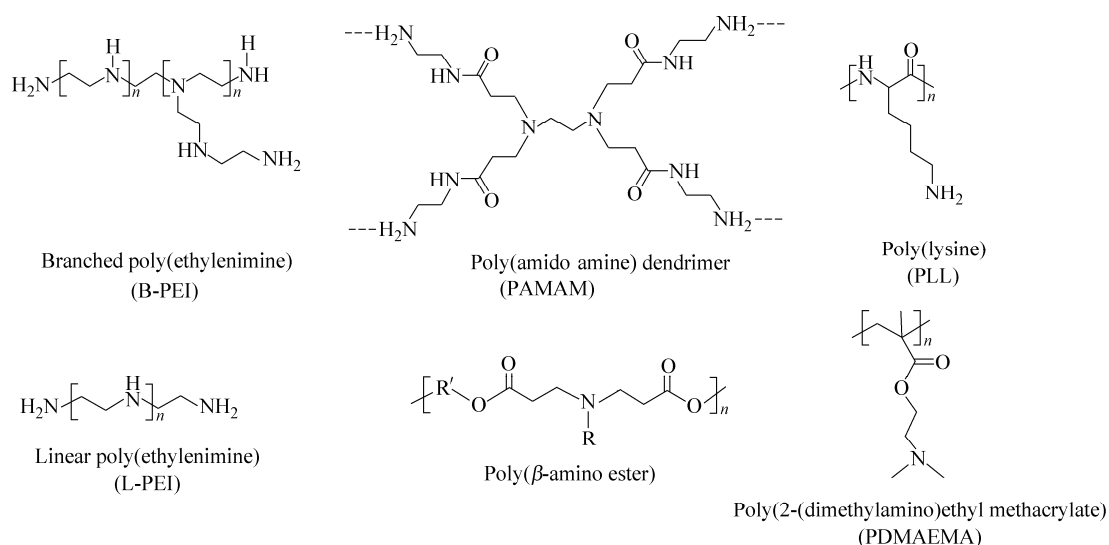


Fig. 3 Structures of several cationic polymers for DNA condensation

Being separated from living cells, the capsid is just like a macromolecule of protein. However, different from capsid, nonviral macromolecules do not have such a manner to compact nucleic acid so effectively. The most widely used nonviral vector is cationic macromolecules. Condensation of DNA with cationic macromolecules is through electrostatic interactions between the negatively charged phosphate backbone of

DNA and positively charged macromolecules, leading to charge neutralization and pack of the DNA, preventing the DNA from degradation. Commonly used positively charged molecules are PEI^[33], PLL^[34], PAMAM dendrimers^[35, 36] and degradable poly(β -amino ester)^[37, 38]. Structures of some types of polycations are shown in Fig. 3. These DNA condensing reagents could compact DNA to nanoscale particles like viruses. But the efficiency of this type of self-assembly by electrostatic interaction is much lower than capsid and it lacks multi-functions as capsid. In order to obtain multifunctional vectors like viruses, different polymers and functional groups may be combined to execute different functions. The hybrids of nonviral vectors, such as conjugates of peptides or targeting moieties or hybrids of cationic liposomes and polymers, are promising strategies for gene transfer^[39].

Circulation

As we know, hepatitis, caused by liver infection of Hepatitis B virus (HBV), is a severe disease harming human health. Our body has evolved many immune mechanisms to defend virus infection, but why HBV still can lurk without being eliminated by immune system? One reasonable explanation is that HBV has cultivated a possible immune escape mechanism as a result of an inadequate immune response towards this virus. It has been reported that hepatitis B virus surface antigen could make myeloid dendritic cells of patients with chronic HBV matured and impair their immune function, resulting in viral persistence^[40]. However, DNA/cationic nanoparticles will quickly be cleared once being injected into the blood due to positive charge-caused nonspecific interactions. To improve the serum stability of polymer/DNA complexes, many innovative ideas have been promoted. Surface modification of polymer/DNA complexes is one of the most efficient ways to achieve this goal.

Surface-modification with hydrophilic polymers, such as PEG, was proved to have the ability to extend the blood circulation lifetime of these complexes^[41, 42]. PEG is water-soluble and biocompatible. As a shielding tool, it can minimize the nonspecific interactions with serum proteins, and make polymer/DNA complexes more stable *in vivo*. PEGylation of cationic polymers and subsequent fabricating polyplexes have been used in gene delivery for this purpose^[43, 44]. However, PEGylated vector may reduce its buffering capacity and hinder its escaping ability from endosome which may reduce the gene delivery efficiency^[45]. To resolve this issue, linkages for dePEGylation have been introduced. For instance, acid-labile PEG-DOPE demonstrated a good ability of self-dePEGylation, recovery of an excellent fusogenic capacity of DOPE at low pH and efficiently escape from endosome^[46]. Bio-cleavable disulfide linkage between PEG and polycation segment has also been developed to trigger PEG detachment. PEG-detachable polyplex micelles based on disulfide-linked block cationomers showed both a 1–3 orders of magnitude higher gene transfection efficiency and a more rapid onset of gene expression than PEG-polyplex micelles without disulfide linkages^[47]. Besides dePEGylation, there is evidence that specific cationic charge-density-to-PEG-ratio is also important for efficient transfection and low cytotoxicity. Meanwhile, PEG molecular weight and PEGylation degree significantly affect not only physicochemical properties of the formed polyplexes but also their cytotoxicity and transfection efficiency^[48]. The cytotoxicity of PEGylated PEI is reduced and the transfection efficiency is decreased^[49].

Polyanion-coating by layer-by-layer deposition was also an effective approach to shield the cationic charges of the complexes. Some polyanion/polycation/DNA ternary complex systems were devised by using hyaluronic acid (HA)^[50], heparin^[51], oligonucleotides^[52] or PEG derivatives having carboxylic acid side chains^[53] as an anionic protective component. These resulting complexes have negative surface charges, and demonstrated high stability in the presence of serum proteins. Some of these polyanions are not only serving as a protective coating, but also a targeting ligand or transcriptional enhancer^[54, 55].

Although, many vectors claimed “intelligent” have been designed, their capability of escaping from immune elimination is low compared with HBV virus. It would be much efficient if the antigens involved in immune escape of the virus are introduced to the nonviral vectors. Furthermore, the gene delivery efficiency after the surface-shielding of the positive charges of the polyplexes *in vivo* applications is inevitably reduced due to the reduced interaction of the complexes with the cells and thereby slowed cellular uptake.

Cell Binding and Endocytosis

Viruses enter target cells mainly through receptor-mediated endocytosis^[56]. Different viruses can recognize distinct receptors at cell surface, including cellular proteins, carbohydrates and lipids, and then attach to it, helping virus concentrating on the cell surface. There are mainly three types of endocytosis routes for virus to be internalized into cells: clathrin-mediated endocytosis, caveolae-endocytosis and clathrin- and caveolae-independent endocytosis, in which clathrin-mediated endocytosis is the major cellular entry way and has been best characterized^[57]. How adenovirus (Ad), influenza virus and SV40 infect their host cells have been particularly elaborated before^[58]. Take Ad as an example. Ad is internalized through clathrin-mediated endocytosis. As shown in Fig. 4, the fibers of Ad first bind to coxsackievirus-adenovirus receptor (CAR) cell surface protein. Then the fibers begin to be released and the penton proteins are exposed to $\alpha_v\beta_3$ or $\alpha_v\beta_5$ through an Arg-Gly-Asp (RGD) motif. Such an interaction will induce intracellular pathway and local formation of clathrin-coated pits^[32, 59]. Once the clathrin-coated vesicles form, the Ad is encapsulated in this vesicle and internalized.

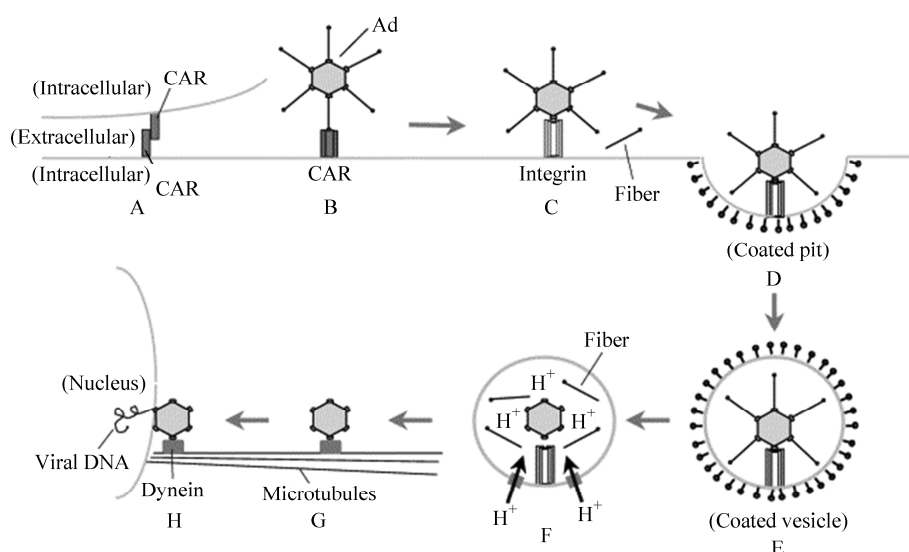


Fig. 4 Schematic of the Ad infection pathway (Reprinted from Ref. [32] with permission from Elsevier)

Clathrin-mediated endocytosis is also thought to be the most common and effective way for nonviral vectors. As shown in Fig. 5, the endocytosed cargo is called endosome. If the polymer is not capable of inducing the release of polymer/DNA complexes (polyplexes) from the endosome, the polyplexes will be delivered to lysosome and then degraded. However, the caveosome formed by caveolae-mediated endocytosis is neutral and will not develop to lysosome, which means a more efficient way for endocytosis^[60]. Therefore, the exact endocytosis mechanisms of different polymer-mediated gene delivery systems are critical for gene transfection. Enlightened by the manner of endocytosis of virus, a lot of receptors overexpressed at the surface of tumor cells have been applied in non-viral vectors, such as RGD peptide^[61], transferrin^[62], folate^[63], asialoglycoprotein^[64], galactose^[65], epidermal growth factor receptor (EGFR)^[66], integrin^[67] and mannose^[68]. Different ligands may introduce distinct endocytosis pathways. For well-known PEI polyplexes, caveolar pathway was discovered when introducing folic acid as a ligand, while clathrin pathway was discovered when introducing transferrin as a ligand^[69].

However, no matter what kind of endocytosis is involved, the efficiency of nonviral vectors is much lower than virus. Fortunately, more and more ligands/receptors are exploited for promoting endocytosis for gene delivery. Alpha-fetoprotein (AFP) is not expressed in normal human tissues and seems to be a potential ligand for receptor-mediated endocytosis to tumors^[70]. Glucocorticoid responsive element (GRE) targeting the

glucocorticoid receptor (GR), a transcriptional factor, can improve nonviral gene transfer efficiency in the lungs of mice^[71]. Triolein^[72], hyaluronic acid^[73], polymalic acid^[74], glucocorticoid hormone^[75], FGFR^[76] and Vitamin B12^[77] are also being exploited to implement receptor mediated endocytosis. It appears promising to introduce tumor cell specific targeting group to obtain only tumor targeting effect and reduce non-specific influence on normal cells of nonviral vectors.

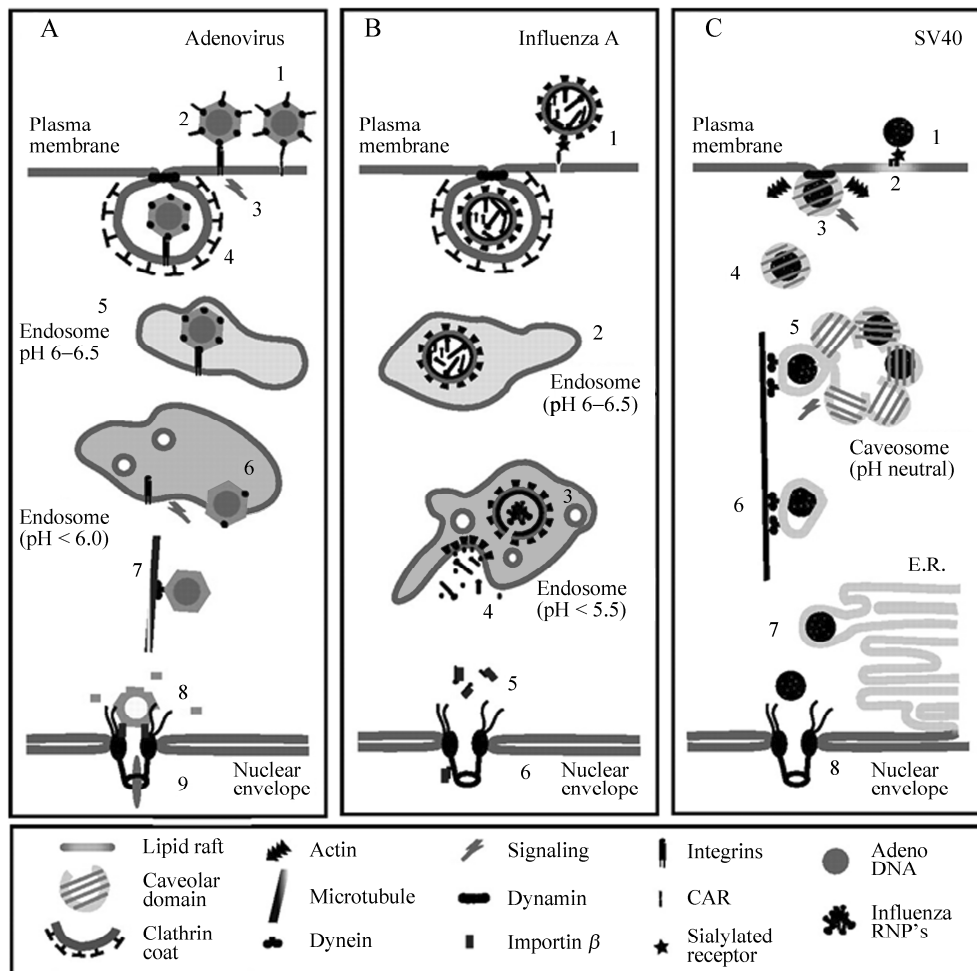


Fig. 5 Entry of adenovirus, influenza virus, and SV40 into their host cells (Reprinted from Ref. [58] with permission from Science)

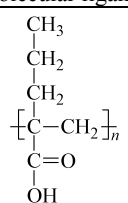
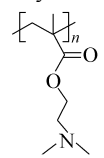
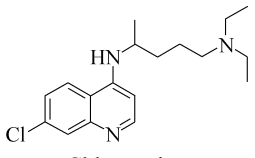
Endosome Escape

When exogenous materials enter cells *via* endocytic vesicles, majority of the vesicles are first delivered to endosomes, where the pH is 5.0–6.5. One risk after endocytosis is the delivery to the lysosome, where is rich in various kinds of enzymes causing degradation. In order to avoid being delivered to lysosomes, viruses have adjusted the threshold pH to match the early endosome (pH 6.0–6.5) or late endosomes (pH 5.0–6.0) to be released into cytoplasm. Different from envelope viruses, nonenveloped viruses lack such mechanisms as escaping from endosome using fusion-dependent pathway. Two mechanisms have been proposed for endosome escape of nonenveloped viruses: membrane disruption *via* a carpet-like mechanism and transmembrane pore formation *via* a barrel-stave mechanism. In this process, different viruses make use of distinct proteins to mediate the escape^[57]. Taking Ad as an example, membrane disruption mechanisms are involved and the penton base is responsible for membrane disruption. As shown in Fig. 5, during delivery to endosome, the low pH of

endosome will induce penton undergoing a conformational change, and exposing hydrophobic regions, which may insert into endosome membrane and induce the dissolution of endosome membrane and then membrane collapse^[58, 78].

As for nonviral vectors, the acidic environment of endosome also has been fully utilized. Up to the present, endosomal disruption by a “proton sponge” mechanism has been proposed. Both small molecules and macromolecules have been developed possessing the capability to make use of “proton sponge” mechanism. Several conjugates that have been proved to promote endosome escape are shown in Table 1. PEI, which has been widely used in gene delivery both *in vitro* and *in vivo*, was reported providing an escape mechanism for PEI/DNA complexes to protect DNA from nuclease degradation due to such an effect^[79]. Primary, secondary, and tertiary amines of PEI could highly be protonated in acidic compartment, causing a substantial volume increase in response to increased osmotic pressure and eventually membrane disruption^[80]. Imidazole ring (pK_a 6.04), possessing the buffering capacity in endosomes, has been introduced into other gene delivery systems to promote endosome escape, thus increase gene transfection efficiency^[81, 82]. Poly(propylacrylic acid) (PPAAc) is a pH-responsive polymer, showing capability of disrupting cell membranes at low pH, but not at physiological pH, demonstrating endosomal releasing ability^[83, 84]. PDMAEMA, due to the ability of destabilizing endosome, has been successfully used in gene delivery and various modifications of PDMAEMA also have been developed^[85, 86]. Chloroquine is a small molecule and uncharged at the neutral pH, and is able to diffuse across lipid bilayers freely. Once entering endosomes of an acidic compartment, chloroquine becomes protonated and then incapable to diffuse across membranes, leading pH increase in endosome, osmotic swelling and degradation of endosomes^[87].

Table 1. Conjugates for promoting endosome escape

Molecular ligands	Reference	Molecular ligands	Reference
PEI	[80]		[83, 84]
 Imidazolyl	[81, 82]	 Poly(2-(dimethylamino)ethyl methacrylate) (PDMAEMA)	[85]
 Chloroquine	[87]		

Endosomal escape peptides (EEPs) with pH-dependent membrane lytic activity could destabilize the endosomal membrane and improve transfection efficiency of nonviral vehicles^[88, 89]. The diphtheria toxin (DT) has three functional domains, in which transmembrane domain (T domain) undergoes a conformational change at low pH and causes release of entrapped cargoes^[90]. When the T domain was chemically conjugated to poly-L-lysine, the gene transfer efficiency was significantly increased^[91]. Melittin, a 26 amino-acid cationic peptide of major component of bee venom, possesses membrane penetration properties and also enhances transportation into the nucleus possibly *via* the cationic cluster KRKR near the C terminus of the peptide^[92]. To convert melittin into gene transfer peptide, poly-melittin peptides were synthesized by the addition of Lys repeats at the C- or the N-subterminal end along with terminal Cys residues and the gene transfer efficiency of this poly-melittin analogues was equivalent to PEI in HepG2 cells^[93]. Several melittin analogs were designed and conjugated to PEI, getting up to 70-fold higher activity compared to the corresponding PEI polyplexes^[94].

However, inherent lytic activity at the neutral pH also provokes high cytotoxicity due to plasma membrane damage.

Unlike viruses that can regulate the timing of endosome escape, nonviral vectors can not be released at the most appropriate time and place. How to control the endosome escape of nonviral vectors may play a critical role to enhance gene delivery efficiency. Once in the cytoplasm, traversing toward the nucleus and accumulating at nuclear periphery are also of great significance.

Cytosolic Trafficking

Cytoplasm is another barrier for gene delivery. Cytoplasm is viscous, crowded and of no opportunities for free diffusion for macromolecules. Viruses employ the cytoskeleton move through cytoplasm to arrive at nucleus periphery. Cytoskeleton microtubules have inherent polarity. One end of microtubules exhibits rapid growth by addition of tubulin dimers (the “plus” end) while the other end exhibits slower dimer addition (the “minus” end). Plus ends of microtubules are usually found at the cell periphery while the minus ends are often found at the microtubule organization center near the nucleus. There also exist two types of motor proteins with specific directions of movement on microtubules in cytosol: dynein moving to the minus ends and kinesin moving to plus ends^[95].

As shown in Fig. 4, adenovirus has evolved to utilize microtubule-associated cellular motor proteins as a navigation to enter nucleus periphery. The capsid can interact with dynein and be driven rapidly moving along microtubules making use of minus end-directed microtubule motors^[96]. Besides this, some evidence also indicates direct electrostatic interactions between Ad and microtubules^[97], which assist such directional movement. SV40 is delivered to caveosome after caveolar-mediated endocytosis. Caveosome conducts as a cargo of dynamin motor to move along microtubules and is involved in the intracellular transport of SV40 from caveolae to the endoplasmic reticulum. Caveolar endocytosis is ligand-triggered and involves extensive rearrangement of the actin cytoskeleton that promotes cytoplasm trafficking^[98].

Nonviral vectors share the same way for cytosolic trafficking and perinuclear accumulation. In order to find out the behavior of nonviral vectors after being released into cytoplasm, fluorescence microscopy and confocal microscopy are used to track the polymer/DNA complexes. PEI/DNA complexes were reported to transport through the cytoplasm toward the nucleus on microtubule^[99]. By Live-Cell Imaging, the mechanism for PEI/DNA complexes employing microtubule associated trafficking was further proved^[100]. Gene transfection mediated by liposome also demonstrated the same mechanism. Nocodazole or taxol, disrupting or stabilizing microtubules, apparently increased the efficiency of gene transfection by preventing transport of liposome-DNA complexes from endosome vesicles to lysosomes^[101].

All these findings confirm that cytoplasm trafficking is not the limited step for gene delivery. Other steps following perinuclear accumulation like nuclear entry and polymer/DNA unpacking maybe the possible rate-limiting processes for gene transfection.

Nuclear Localization

Gene transcription happens in the nucleus, so no matter viral or nonviral gene delivery systems should gain access to the nucleus. Trafficking between the cytoplasm and the nucleus takes place through pore complexes within the nuclear envelope called nuclear pore complexes (NPC). The nuclear membrane pore has a diameter of *ca.* 25 nm and molecules > 45 kDa must possess a specific targeting signal, known as a nuclear localization signal (NLS), or they will be excluded from the nucleus^[102, 103]. The best understanding classic NLS sequence is from SV40 large tumor antigen (TAG) containing a single stretch of basic amino acids (PKKKRKV)^[104–106].

Virus nuclear import has been particularly elaborated in other reviews^[107]. They employ specialized proteins with unique amino acid sequences to recognize cellular nuclear transport machine to obtain nucleus localization. Small viruses, like parvovirus of only 18–24 nm, can directly pass through the nuclear pore without capsid disassembly in intact form. While adenoviruses are large non-enveloped viruses, which can not penetrate the nucleus membrane, should exclude affiliated elements except DNA. A series of proteins are demonstrated to be involved in the process of capsid disassembly. Ad capsid contains conserved specific domains that mediate binding to the nuclear envelope^[108]. When entering nucleus periphery through microtubule, this binding might

cause its capsid dissembled, and release its genomes into nucleus.

However, for nonviral vectors aggregating at nucleus periphery, nonviral DNA exists either in free or carrier-bound forms. No matter in which form, they lack nuclear targeting proteins to facilitate nuclear transport. Nuclear import is still an unmet challenge in gene delivery. As being mentioned above, NLS plays an important role in nuclear import and has been widely used to promote nuclear uptake of nonviral DNA^[109]. Several factors have been identified that have direct influences on gene transfer when utilizing NLS, including the type of NLS peptide, morphology of DNA, and method used to incorporate NLS peptides into nonviral vectors^[109]. Viral proteins normally mediating nuclear delivery of viral DNA also have been incorporated into nonviral vectors to facilitate nuclear import. A lipopolyplex containing polyoma virus VP1 protein increased gene transfer efficiency due to NLS signal sequence in the peptide^[110]. It has been recognized that majority of polymer/DNA complexes are localized in nucleus during cell division when the nucleus membranes disappear. For non-dividing cells, this kind of localization seems useless. Furthermore, this type of nucleus entry is of potential damage, for it is not tumor cell selective and may cause harmful side effect on the surrounding healthy normal cells. Therefore, tumor cell-specific nuclear targeting is of great importance. Several proteins targeting tumor cells selectively have been summarized^[111]. Using cell-type-specific nuclear signals seems promising.

Gene Transcription

Besides nuclear import, efficient transgene transcription is also required to achieve maximal transcription efficiency of gene delivery vectors. It is of great importance for DNA dissociating from its vectors. For adenovirus, DNA is released outside the nuclear membrane and imported into nucleus individually. But for nonviral vectors, DNA dissociation can not be controlled. It is unclear how and when DNA delivered by nonviral vectors is released. If DNA is released near nucleus periphery or at nucleus, gene transfer efficiency would be highly improved.

Some types of gene vectors have been designed to promote DNA dissociation from its vector, such as polymers containing photo or thermo-responsive unit, or reductive environment sensitive disulfide. Complex formation-dissociation was also said controlled by thermo-responsive polymers. This polymeric carrier is composed of three components: a temperature-responsive unit, a cationic unit and a hydrophobic unit. The cationic unit is used to interact with DNA and forms a condensed DNA/vector complex, while the hydrophobic can adjust the transition state of the delivery system^[112]. Thermo-responsive copolymer had a lower critical solution temperature (LCST), above which the DNA and carrier polymers formed, while below which DNA dissociated from its carrier^[113].

The other technique promoting DNA release makes use of the reducing intracellular compartmental environment, where disulfide bonds can be reduced and cleaved by intracellular glutathione (GSH), and then cause DNA releasing^[114]. A redox-triggered destabilization mechanism could be a promising strategy to design synthetic non-viral vectors with engineered intracellular release characteristic in respect that cancer cells seem to show a higher activity of glutathione-S-transferase (GST) and hence higher GSH levels^[115, 116], thus showing enhanced reduction capacity. It is hypothesized that once gene vectors cross-linked by disulfide bond gain access to the nucleus, it would be reduced by GSH, inducing dissociation of polymer/DNA complexes and releasing DNA to exhibit its function. Responsive polyelectrolyte complexes for triggered release of nucleic acid therapeutics was particularly reviewed^[117]. We have further showed that the nanocapsules releasing free DNA had higher gene expression efficiency once been internalized^[118].

However, the ideal timing of DNA unloading remains unclear and challenging. The DNA molecules delivered by 11 kDa PEI to the nucleus do not exist in the form of PEI/DNA complexes, indicating DNA dissociation from PEI prior to the nucleus entry^[60]. The site (cytosol or nucleus) for DNA release needs further investigation in order to develop more appropriate gene delivery vector.

After DNA being dissociated from delivery system, viruses evolved different mechanisms for gene transcription. Retrovirus inserts genes into host's genome, resulting in permanent gene expression and providing full correction of disease phenotype^[4]. But this kind of insertion is non-selective, and the insertion to the critical genes may cause carcinogenesis.

For adenovirus, the possibility of integrating into host's genome is low. From this point it is similar to nonviral vectors. It is commonly thought that the condensed plasmid DNA could be decondensed by the substitution of negatively charged components such as endogenesis DNA or RNA. Gene replication and transcription are along with cell division of host. This kind of gene transcription is transient and especially appropriate for cancer treatment, because exogenous gene can be eliminated once the treatment completes.

Gene transcription is a rate-limiting process in gene transfection. A research on quantitative comparison of intracellular trafficking and nuclear transcription between Adenoviral and Lipoplex Systems demonstrated that differences of transfection efficiency mainly arise from nuclear gene transcription, not from intracellular trafficking^[119].

Gene transcription is regulated by many factors. A remarkable positive nonlinear relationship has been discovered between dose and transfection activities in nonviral gene delivery systems^[120, 121]. This suggests that even if DNA gets access to and is released in the nucleus, gene expression is still cumbered by various factors. For instance, the released DNA could be bounded and inactivated by histone as heterochromatine, and methylation of the DNA can be another inactivation mechanism. In order to gain high gene expression level to implement gene therapy level, further study will be required to identify the factors responsible for the positive non-linearity between dose and transfection efficiency.

PESPECTIVES

The past decades have witnessed the tremendous progress of polymeric systems for gene delivery. From the perspective of virology, many innovative notions have been contrived in designing nonviral vectors to obtain as high transfer efficiency as viral vectors. Multifunctional genes delivery systems have been brought forward to overcome various barriers both *in vitro* and *in vivo*, such as cellular uptake, endosome escape, nuclear targeting and gene expression. For instance, a unique pH-responsive terpolymer brush has been developed. This polymer can compact DNA into a virion-like nanocapsule and encapsulate DNA in its core surrounded by a tight hydrophobic membrane like the capsid of virus at the neutral pH^[118]. Most importantly, the DNA in the particle is in free form, not complexed by cationic charges. Therefore, the DNA is also released as free form for easy transcription. How this multifunctional nanocapsule executes its function is shown in Fig. 6. Kogure and coworkers have devised a multifunctional envelope-type nanodevice as a non-viral gene delivery system which makes gene delivery to nucleus possible^[122]. In future, much more functional devices like virus should be equipped into gene delivery systems to overcome various biological obstacles and enhance gene expression level.

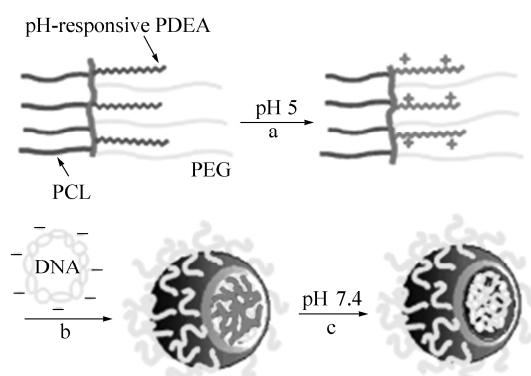


Fig. 6 Nanocapsule formation by a pH-controlled self-assembly of PCL/PDEA/PEG and DNA (Reprinted from Ref. [118] with permission from Wiley)

The PDEA chains are positively charged at pH 5 (a) and then self-assemble with DNA to form a hydrophilic core. The hydrophobic PCL chains collapse and form a membrane surrounding the core, the hydrophilic PEG chains are incompatible with hydrophobic PCL layer and extend into the aqueous solution as a PEG outer layer (b). After the solution pH is raised to 7.4, the PDEA chains are deprotonated, become insoluble and dissociated from the DNA, leaving free DNA in the core (c).

From the perspectives of nonviral vectors for gene therapy in clinical use, there still needs to substantially enhance their gene transcription efficiency. Challenges still stand before us and deeper insight in how to surmount biological and cellular boundaries should be highlighted. If nonviral vectors can be designed as intelligent as virus, efficient gene delivery would be achieved.

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