Enhancers in polymeric nonviral gene delivery systems

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\section*{Abstract}
Gene therapy is a promising therapeutic tool for cancers and inherited diseases. For successful gene therapy, gene delivery systems must be designed reasonably to allow DNA or other nuclear acids to be taken up by the cancer cells and then transported to target location for function. In the research of gene delivery systems, polymeric nonviral carriers for genes have attracted much attention. However, polymeric gene delivery systems suffer from low transfection efficiency. Various strategies have been developed for improving efficacy of polymeric gene delivery systems. These strategies can be categorized into covalent strategies and noncovalent strategies according to the way how the functional unit is combined with the carrier. Noncovalent strategies in improving gene transfection efficiency are simply mixing additional functional components with carriers. These relatively independent functional materials are named gene delivery enhancers here. In this review, we focus on enhancers in polymeric gene delivery systems. We discuss structures and enhancement mechanisms of enhancers in the order of the stages in which they function in gene delivery process. Barriers of in vitro gene delivery are also reviewed briefly.

\textbf{KEYWORDS}
enhancers, gene delivery, noncovalent strategies, polymeric carriers, transfection efficiency

\section*{1 | INTRODUCTION}

The delivery of genes allows target cells to be altered at the genetic level. Researchers can directly address the root cause of inherited disease or acquired genetic disease by manipulating the gene expression patterns of target cells.\textsuperscript{1,2} Gene therapy makes it possible to completely cure some intractable diseases. During the past decades, gene therapy has attracted increasing attention for cancer treatment.\textsuperscript{3-10} An efficient gene delivery system is of great significance for successful gene therapy.\textsuperscript{11,12} Constructing the appropriate gene delivery systems will allow genes to reach their site of action and enhance delivery efficiency. In general, a complete gene delivery system should include the two parts, such as a gene expression system, that controls a gene to achieve successful expression of specific protein products and a gene transfer tool that help the gene expression system reach specific site where functions in vivo.\textsuperscript{13}
Viral vectors are the earliest developed vectors due to their high transfection efficiency. However, viral vectors are limited by the challenges of immunogenicity, low loading, and scale-up. In view of this, nonviral gene delivery systems with reduced immunogenicity and enhanced biocompatibility have been largely developed. Polymeric gene carriers are one of the major types of nonviral gene delivery systems with the virtue of nonimmunogenicity, flexible properties, and high-packaging capacity. The prerequisite for successful gene therapy is high-efficiency transfection in vitro. How to achieve high-efficiency gene transfection in vitro is a basic and important scientific issue. Although a lot of efforts have been made to improve the delivery of genes, transfection efficiency of polymeric gene delivery systems still remains low level compared to viral delivery systems.

As usual, the process of gene delivery in vitro is mainly divided into cellular uptake, endosomal escape, and intracellular trafficking (Figure 1). Various transfection biological obstacles during gene delivery require differences on the rational design of polymer gene delivery systems, so designing and preparing efficient polymeric gene delivery systems remains a challenge. To some extent, the transfection ability of a gene delivery system means its ability to overcome delivery barriers. Enhancement of transfection efficiency can be divided into three levels, the carrier itself, the nanoparticle formed by the carrier binding nucleic acids, and the delivery process.

Introducing functional components into gene delivery systems is an effective strategy to improve transfection efficiency. For example, cationic polymers are often used to compress DNA to form nanoparticles to protect their integrity during delivery and promote their uptake by cells. Introducing antibody fragments or active targeting units on the carrier has been used to promote uptake in some specific cell lines. Cell penetrating peptides (CPPs) have been introduced into gene delivery systems to improve the efficiency of both internalization and endosome escape. Nuclear localization signals (NLSs) have been integrated into DNA or polymer carriers to facilitate intracellular targeted nuclear delivery. These multifunctional strategies can be divided into covalent conjugation and nonspecific adsorption. The additive functional materials which are introduced into the gene delivery system by noncovalent methods are called gene delivery enhancers here. In detail, the enhancer has two characteristics. First, it confers on the gene delivery vector the properties that are conducive to transfection. Second, it combines the vector in a noncovalent manner. Besides, some small molecule drugs that promote the gene delivery by pretreating cells are also regarded as enhancers here. Covalent conjugation is usually used for irreversible attachment of functional ligands. It can provide a more stable combination between

**FIGURE 1** Schematic diagram of polymer-based gene delivery process. Reproduced with permission. Copyright 2014, Ivyspring International Publisher
the functional units and the carriers. However, the feasibility of covalent conjugation strategies is often limited by system complexity and group restriction. Also, generation of these multifunctional carriers often requires tedious chemical synthesis and purification, which is likely to result in inefficiency and batch-to-batch differences. Noncovalent adsorption provides new ideas for functionalization. Functionalization of polymeric gene delivery systems through hydrophobic or electrostatic interactions can bypass complex chemical synthesis and modifications. The construction of nanostructures through self-assembly of multiple independent components and intermolecular interactions requires careful selection of the building blocks that make up the system.

In this review, we discuss noncovalent strategies enhancing in vitro gene transfection efficiency by simply adding enhancers. We focus on the materials used as enhancers in polymeric gene delivery systems to improve gene transfection efficiency in cellular level. First, we will discuss cellular barriers in gene delivery and some basic solutions will also be involved. Then, enhancers for different cellular obstacles will be discussed. In the end, we will discuss the prospects of gene delivery enhancers.

2 CELLULAR BARRIERS IN GENE DELIVERY

Plasmid DNA (pDNA) is the most commonly used DNA for upregulating gene expression levels. PDNA is a double-stranded circular plasmid which includes, at minimum, a promoter and a specific gene of interest. In general, pDNA itself is difficult to enter into cells because of the features including large size (10^3-10^5 bp), strong negative charge, and hydrophilic properties. To be successfully expressed, the DNA needs to pass through various membranes, such as the cell membrane and the nuclear envelope, but also overcome various biological barriers like DNase in cytoplasm. When the pDNA arrives to the nucleus, it can be transcribed into mRNA for transport into the cytoplasm and translation into the protein of interest.

The first barrier for in vitro gene delivery is the cell membrane because the electrostatic repulsion between pDNA and cell membrane is detrimental to internalization of pDNA. Cationic polymers (or polycations), such as polylysine (PLL), polyethyleneimine (PEI), and polyamidoamine (PAMAM), can bind and encapsulate pDNA into nanoparticles with cationic surface via electrostatic interactions with amines. After that, the positively charged nanoparticles are attracted by the negatively charged cell membrane and easily uptake by cells.

Once uptake, cationic nanoparticles tend to fall into the endosomal entrapment. It is necessary for pDNA to avoid digestion in the endosome and then escape into the cytoplasm for intracellular trafficking. Some reversibly protonated polycations, such as PEI, can function as a buffer of protons, which are pumped into endosomes, thereby preventing the endosome from becoming excessively acidified. Due to the formation of electrical gradient, this in turn cause chloride ions flow into the endosome. The subsequent influx of water caused by osmotic pressure eventually ruptures the endosome and releases the nanoparticles into the cytoplasm. This “proton sponge” effect is one of the important reasons why some cationic nanoparticles have endosome escape ability.

Inefficient nuclear delivery of pDNA is one of the important reasons that limit the transfection efficiency of nonviral gene delivery systems. After DNA-encapsulated nanoparticles escaped from the endosome into the cytoplasm, DNA may need to be released from the nanoparticles to enter the nucleus. When the DNA is free from the combination with the polymer carrier, it faces the dilemma of slow cytoplasmic diffusion and degradation by DNase, which limit the transfer efficiency of DNA in the nucleus. The process of DNA entering the nucleus is still not very clear. It is generally believed that the entry of DNA into the nucleus requires the disassembly of the nuclear membrane during cell division, or active nuclear transport through the nuclear pore complex.

3 POLYMERIC GENE DELIVERY ENHANCERS

As mentioned above, constructing functional polymer-based gene delivery systems is a promising strategy for enhanced transfection. Next, we will discuss the strategies promoting gene transfection by simply mixing functional units with existing polymeric gene delivery systems. According to the stage when the enhancers exert function, our discussion is divided into the following parts.

3.1 Materials enhancing cellular uptake

In general, the size and surface potential of nanoparticles have an important influence on cellular uptake. Considering the electrostatic binding during DNA condensation is key to constructing a highly efficient gene delivery system. Achieving DNA condensation while ensuring the rational size and surface potential of nanoparticles can promote gene transfection efficiency. Some rational designs have been developed. Chen’s group reported a charge/size dual rebound polymeric gene delivery system triggered by slight pH change (Figure 2). This system that was constructed through a convenient strategy met the requirements of
different stages in the transportation process. The introduction of PLG promoted gene transfection efficiency both in vitro and in vivo. Researchers prepared PEI/DNA (PD), PLG/(PEI/DNA) (G(PD)), and (PLG/PEI)/DNA ((GP)D) by mixing equal amounts of DNA, PEI, or PLG aqueous solutions in different orders. A total of 48 h in vitro transfection experiments in different groups showed that (GP)D has the most efficient gene delivery efficiency. Further research indicated that the optimal mass ratio of (GD)P is PLG:PEI:DNA = 1.25:2.5:1.42 Yin’s group developed hybrid nanoparticles (HNPs) which included a cationic polypeptide PPABLG with stable helical structure and TNF-α siRNA (Figure 3).43 HNPs achieved efficient delivery of siRNA for anti-inflammatory treatment. Considering that siRNA can’t generate a sufficiently strong electrostatic interaction with the cationic polymer carrier due to its short and linear characteristics,44 they introduced an anionic polypeptide PAOBLG-MPA,45 to enhance the interactions with the cationic polypeptides PPABLG. HNPs prepared in this way compressed TNF-α siRNA tightly, ensuring high-delivery efficiency.46

In addition to optimizing nucleic acids condensation and the surface potential of nanoparticles for enhanced uptake, it is also possible to increase the negative charge of the cell membrane through pretreatment to increase cellular uptake. Duvall’s group designed an anionic polymer poly(propylacrylic acid) (PPAA).47 Pretreatment of cells with PPAA can enhance cell uptake and endosome escape of cationic biomacromolecules and nanostructures (Figure 4). They proposed a mechanism by which PPAA enhanced cellular uptake. The hydrophobic propyl group contained in each repeat unit of PPAA can bind to the cell membrane through hydrophobic interaction, so pretreating the cell with PPAA is like creating a layer of PPAA coat to the cell. In each repeating unit, PPAA also contains a carboxylate anion. When the cell membrane was covered by PPAA, carboxylate anions will increase the net negative charge on the cell surface. The cell membrane with increased net negative charge enhances the electrostatic attraction to cationic cargo. The carboxyl groups of PPAA have an acid dissociation constant (pKₐ) of 6.7. As the pH changes from extracellular to the endosome, the hydrophobicity of PPAA will increase, thus, disturbing the endosomal membrane and enhancing the escape ability of the cation cargo. By constructing cationic polystyrene nanoparticles and polymeric micellar nanoparticles models, they verified PPAA can promote the transport of cationic nanostructures.
FIGURE 3  Composition and intracellular kinetics of HNPs. The anionic peptide PAOBLG-MPA played a role in optimizing the electrostatic interaction within the nanoparticles. Reproduced with permission. Copyright 2016, American Chemical Society

FIGURE 4  Schematic diagram of the mechanism of the anionic polymer PPAA-mediated cationic peptide uptake and intracellular delivery. Reproduced with permission. Copyright 2019, the Authors
It is possible to enhance cellular uptake by affecting the mechanism of nanoparticles uptake by cells. CPPs have unique membrane activities, which have been widely utilized to enhance cellular uptake and endosomal escape of nanoparticles. Well-known CPPs include penetratin, transportin, oligoarginine, melittin, and HIV-TAT. The helical structure is generally considered to be an important factor that CPPs have the ability to penetrate the membrane. This relatively rigid secondary structure can interfere with the integrity of the cell membrane, therefore, producing transient pathways to enhance internalization of exogenous substances. Some CPPs with DNA binding capacity and membrane perturbation properties have been developed to enhance the delivery of DNA or oligonucleotides and have been combined with not only polymeric gene delivery systems but also lipid-based gene delivery systems. Amphipathic CPPs, such as the fusion peptide of HA-2 subunit of influenza hemaglutinin, and its synthetic analogues JTS1, KALA, GALA, and histidine-rich peptides, have been applied to enhance transfection efficiency of polymeric delivery systems like PEI/DNA, PLL/DNA, and PAMAM/DNA. Similarly, the integration manners of the CPP into gene delivery systems are also divided into covalent conjunction and noncovalent adsorption. Numata’s group reported a peptide-based gene delivery system for DNA delivery targeting plastids (Figure 5). The pDNA/CTP/CPP complex formed when the N/P ratio is 1.0 and 2.5 maximized Rluc activity (2.1 times) compared with the pDNA/CTP complex alone. Reineke’s group found that adding heparin to the glycopolymer/pDNA polyplexes after complexation enhanced gene delivery. Heparin can induce negatively charged glycosaminoglycan to gather around the polyplexes, which leads to an increased dependence on macropinocytosis and does not
inhibit endocytosis of the particles.\textsuperscript{55} Nanoparticles entering the cell through macrocytosis may also affect the nuclear localization behavior of the DNA.

### 3.2 Materials enhancing endosomal escape

Materials that can disturb the endosomal membrane help endosome escape, including CPPs and PPAA, we mentioned above. CPP's promotion of endosome escape still account for its unique membrane disturbance property. It is worth mentioning that chloroquine also has the ability to enhance transfection.\textsuperscript{56,57} As a weak base, chloroquine can neutralize acidic endosomes and inhibit hydrolase, thereby avoiding degradation of nanocarriers. Besides, some substances containing rich hydrophobic domains also have ability to disturb the endosomal membrane, which can be utilized to enhance the gene delivery efficiency by promoting endosome escape. Oleyl-conjugated trimethyl chitosan (OTMC) is a hydrophobic derivative of trimethyl chitosan (TMC). TMC has good biocompatibility and biodegradability, so it is a promising candidate for gene delivery.\textsuperscript{58,59} Since its hydrophobic regions promote interaction with cellular membranes and endosomal membranes, OTMC is expected to show enhanced transfection efficiency by promoting cell uptake and endosomal escape. Cheng's group reported supramolecular self-assembled nanocomplexes (SSANs) which contained a cationic polypeptide (PVBLG-8) with stable α-helical structure, OTMC, a pDNA, and oleyl-PEG-mannose (Figure 6).\textsuperscript{21} Each component in SSANs can be regarded as an enhancer for gene delivery. SSANs maximized DNA transfection efficiency and minimized cytotoxicity by optimizing the elements including DNA condensation, membrane penetration, and targeting capacity.

Responsive gene delivery systems have also been developed to promote the endosome escape of genes under endogenous or exogenous stimulation. Physical energy, such as light, ultrasound or magnetism, can be used as a time-space controlled trigger to help nanocarriers overcome biological obstacles.\textsuperscript{60} Recently, photochemical internalization (PCI) has attracted much attention.\textsuperscript{61} PCI is a promising strategy based on the principle of photodynamic therapy (PDT), using light, oxygen, and photosensitizers to promote the endosome escape of nanoparticles.\textsuperscript{62} In PCI-mediated gene delivery, one of the most important challenges is to deliver the gene (pDNA) and photosensitizer (PS) in combination to target cells. The realization of codelivery requires that the carrier can load both the pDNA and the PS. At the meantime, pDNA and PS need to be compartmentalized because reactive oxygen species (ROS) produced by PS may damage pDNA. Kataoka's group reported a multicompartimentalized nanocarrier (DPc-TPMs) which realized the PCI-mediated gene transfection through codelivery of pDNA and PS.\textsuperscript{63} Here, dendrimeric PS (DPc) used as enhancer were separated from pDNA, so protecting encapsulated pDNA from photochemical damage. It is worth noting that the integration of carboxylic acid groups at the periphery of DPc makes its distribution in the intermediate cationic compartment tend to be stable, which is achieved by means of electrostatic interaction. These carboxyl groups restore neutrality in the acidic environment of the endosome, promoting the transfer of DPc from the intermediate cationic compartment of the micelle to the vicinity of the endosome membrane, where ROS generated by DPc exert. DPc-TPMs at optimized ratio displayed light-dependent transfection efficiency, achieving more than 100-fold increases in light-control transfection efficiency. Feng's group introduced cationic polyaniline to the star-shaped degradable polypeptide (SP)/pDNA polyplex as endosomal membrane destabilizer, achieving positive impact on gene delivery process by PCI (Figure 7).\textsuperscript{64}

### 3.3 Materials enhancing intracellular trafficking

PDNA that escaped from the endosomal chamber into cytoplasm will encounter cytoplasmic diffusion and metabolic barriers, therefore, the number of intact plasmids reaching the nuclear membrane is relatively low.\textsuperscript{19} Several results indicated that the transfer of pDNA in the cytoplasm to the nucleus may be one of the most important limitations for successful transfer of genes in vitro and in vivo.\textsuperscript{65} In recent years, the NLS pathway has been studied in detail, and some mechanism insights have been obtained. Some works have investigated the problem of nuclear entry and suggested that the use of NLS can enhance the intracellular transport of DNA from the cytoplasm to the nucleus. If a delivery system contains one or more NLS, whether covalently or noncovalently bound to DNA, the dissociation rate of the DNA-carrier complex and the loading rate of the complex into the NLS-mediated nuclear import may compete.\textsuperscript{66} The sequence of TAT peptide has nuclear localization function.\textsuperscript{67} In more detail, TAT peptides can bind nucleocytoplasmic shuttle protein importin β, which enters into nucleus through the nuclear pore. Rosennecker's group used TAT peptides to precompact DNA and PEI was added subsequently.\textsuperscript{58} They found that the transfection efficiency was improved by 390 times compared to the standard vector. Smith's group created a bifunctional PNA-NLS peptide by combining a peptide nucleic acid (PNA) with the SV40 core NLS.\textsuperscript{69} The PNA can interact with DNA specially. They
Figure 6 Schematic diagram showing the formation and intracellular kinetics of supramolecular SSANs. (A) SSANs are constructed through electrostatic interactions and hydrophobic interactions between components. SEM image of the SSANs. The scale bar indicates 200 nm. (B) Schematic representation showing that process of SSANs-mediated enhanced gene transfection. SSANs entered the cells through OPM-mediated mannose-receptor recognition endocytosis and PVBLG-8-mediated direct diffusion via pore formation. Thereafter, SSANs escaped from endosomes through PVBLG-8-triggered membrane destabilization. At last, DNA is transported to the nucleus for gene expression. Reproduced with permission. Copyright 2013, WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

verified the PNA-NLS peptide could work as a nuclear targeting signal when mixed to a fluorescence-labeled oligonucleotide or to a pDNA. Adding optimized amount of PNA-NLS can improve PEI-mediated transfection of lacZ or EGFP pDNA three- to eight-fold. Corticosteroid Dexamethasone can dilate the nuclear pores. Reineke’s group investigated the effect of dexamethasone on nuclear permeability of induced pluripotent stem cells (iPSCs) and primary human dermal fibroblasts (HDFs). They utilized dexamethasone to pretreat the cells in cultures before transfection and got a 16-fold increase in transfection efficiency compared to carrier alone for the large pDNA transfection.

4 CONCLUSIONS AND PERSPECTIVES

Gene therapy provides a promising method in curing cancers and other traditionally incurable diseases thoroughly. An efficient gene delivery system is vital to realize successful gene therapy. How to improve transfection efficiency of polymeric gene delivery systems remains a challenge.
FIGURE 7  (A) Schematic diagram showing the mechanism of cationic polythiophenes enhanced gene delivery by promoting endosome escape. (B) Chemical structures of cationic star-shaped polyaspartate (SP), hyperbranched polythiophene and linear polythiophene. (C) Molecular weights and PDI of polythiophenes measured by GPC. (D) Fluorescence quantum yields of polythiophenes and singlet oxygen quantum yields of cationic polythiophenes. Reproduced with permission. Copyright 2017, American Chemical Society

Compared with covalent strategies, noncovalent strategies provide more simple methods to integrate functional units into polymeric gene delivery systems. In this review, we summarized the materials used as enhancers in polymeric gene delivery systems. The wider application of gene therapy requires cost control of gene carriers. This facile functional strategy helps to reduce the cost of building a high-performance gene delivery system. Adding enhancers to polymeric gene delivery systems provides a promising method for efficient gene transfection. It is worth mentioning that researchers can preliminarily judge whether a certain material can be used as an enhancer according to the structure-activity relationship. For example, it is likely for materials with helical structure or partial hydrophobic structure to have the function of promoting endosomal escape. The biggest advantage of noncovalent strategies to build an efficient gene delivery system is more simple carriers preparation process. We think enhancers can help gene delivery systems to construct large-scale engineered cells to produce proteins of interest through in vitro transfection. We believe that polymeric gene delivery enhancers will attract more and more attention.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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