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Review Article

Non-biological gene carriers designed for overcoming the major extra- and intracellular hurdles in gene delivery, an updated review

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Abstract

Gene therapy as a modern therapeutic approach has not yet advanced to a globally-approved therapeutic approach. Lack of adequate reliable gene delivery system seems to be one of the major reasons from the pharmaceutical biotechnology point of view. Main obstacles delaying successful application of human gene therapy are presented in this review. The unique advantages of non-biological gene carriers as compared to their biological counterparts make them ideal alternatives for overcoming extra- and intracellular barriers in a more safely manner. We, therefore, highlight the significant contributions in non-biological gene delivery and favorable characteristics of different design attitudes with focus on in vivo approaches. Bypassing the rapid extracellular enzymatic degradation of genetic materials is covered in extracellular segment of this review with emphasis on PEGylated and targeted formulations. The successful approaches to pave the rest of the way from cellular uptake to intracellular transfer and gene expression of unpacked DNA are also discussed. From these approaches, we emphasize more on optimization of cationic-based polymers and dendrimers, developing newly designed membrane-effective components, and adjusting the hydrophilic-hydrophobic balance of the synthesized vectors.

Keywords: Gene delivery, Non-viral carrier, Obstacle, Structural modification

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Introduction

A number of laboratories and companies are currently investing in gene therapy as a revolutionary approach to combat diseases. The US has conducted the greatest number of clinical trials worldwide (around 63.7 % of total cases) (1), however the Food and Drug Administration (FDA) has not yet approved any human gene therapy product for commercial application (2). Lack of adequate reliable gene carrier systems for delivering genes is generally assumed as one of the main reasons of lagging the fulfillment of the promise of gene therapy. Majority of efforts in the field is now going into carrier optimization to find a suitable system for human use. There is no doubt that biological vectors are considered as the most efficient available systems. Among biological vectors, viral-based carriers are still the most capable delivery machines to transfer the genetic material to its destination (3). Viral vectors can be classified as integrating, such as onco-retroviruses (also called gamma-retroviruses) and lentiviruses, or non-integrating, such as adenoviruses (AD), adeno-associated viruses (AAV), and HBV (4). Numerous biopharmaceutical companies are focusing on viral vector development (5). Different studies have used adenoviral vectors to treat diseases, such as arthritis, hemophilia, cystic fibrosis, and even cardiomyopathies **(**6, 7). Despite the high efficiency of viral vectors, some unsolved concerns exist regarding their carcinogenicity and immunogenicity (5, 8, 9). Other biological carriers are also not without safety or functional issues especially when considered for human use. Bacteria-based carriers provoke immune responses once applied into non-native tissues. These systems also induce a very low level of gene expression (10). Bacteriophage-based and virus-like particles were presented as efficient carriers, however, they suffer from rapid elimination by reticuloendothelial system (RES) (10) and eliciting the immune response, respectively (11).

Due to the abovementioned issues of biological carriers, designing non-biological vectors could increase the chances of developing the ideal vector without significant safety issues. To make the whole concept of gene therapy coming true with the non-biological alternatives, we need to design safe, efficient, and controllable systems for delivering the therapeutic genes (12). The synthetic gene delivery system should be able to overcome the potential extra- and intracellular hurdles while carrying the proper gene constructs, and to generate the desirable expression inside the cell. Although being different in either structure or gene delivery mechanism, all of non-biological systems are following one goal; helping the transgene to complete its journey to the site of action (13). The favorable characteristics of different non-biological carriers to bypass the major potential hurdles are briefly discussed in this review.

Extracellular obstacles

One challenge for improving gene delivery systems is how to resist the extracellular enzymatic degradation. This degradation has been at least partially dominated by condensing the negatively charged DNA molecules with positively charged synthetic vectors. However, the efficiency of gene transfer using non-biological methods is limited by some extracellular barriers which are explained below (14-17).

Positively charged cationic vector/nucleic acid complexes come into contact with serum proteins like albumin, complements, immunoglobulins, fibronectin and blood cells which have negative surface charges. These interactions could result in aggregation or dissociation of nanoparticles leading to rapid clearance of vector by reticuloendothelial systems (RES) (14-17).

Colloidal instability can be mentioned as the second extracellular obstacle. The difference between ionicity of carrier/DNA complexes and extracellular environment could cause colloidal instability followed by aggregation of the complexes (14-17). Vascular system is considered as the third obstacle. The size of nanoparticles for passage through or between vascular endothelial cells which are relatively small and have tight junctions is another issue to be concerned (14-17). Natural defense mechanism and activation of immune system would be another extracellular issue. Foreign hydrophobic particles may be eliminated by mononuclear phagocytic system through opsonization. Some synthetic vectors could also activate immune systems by inducing an inflammatory response or complement activation (14-17).

Structural modification of cationic synthetic vectors to overcome the extracellular barriers

Formulation of gene delivery vectors is an important factor in determining of their bio-distribution, circulation time and transfection efficiency *in vivo*. It is believed that physical, chemical and structural characteristics of gene carriers affect their ability to protect the nucleic acid from both degradation and non-specific binding (18). Many strategies have also been developed to improve the properties of nanoparticles in extracellular environment. Some of these methods are described as follows.

Surface shielding with hydrophilic polymers

The most applied strategy to increase the stability of vector/nucleic acid complexes is shielding the outer surface of the complexes with poly(ethylene glycol) (PEG) (19, 20). The highly hydrophilic nature of PEG produces a steric barrier against nuclease degradation and aggregation of nanoparticles in bloodstream or in extracellular matrix. In addition, PEG is a biocompatible polymer and can also increase the solubility when it is incorporated into the structure of the vectors (19, 20).

However, it should be pointed out that the length of PEG moieties and the degree of PEGylation could affect the ability of DNA condensation and biodistribution of gene carriers *in vivo*. The optimal PEG length and content depends on gene carrier systems (21).

For example, grafting 8% of primary amines of poly(amido amine) (PAMAM) dendrimers with PEG (Mw=5000 Da) enhanced muscular gene expression efficiently when polyplexes were injected intramuscularly to the quadriceps of neonatal mice (22).

In another study, hydroxy-polyethyleneglycol-acid (Mw=2000 Da) was covalently coupled to the linear polyethylenimine (lPEI 22 kDa) through an amide bond (10% grafting). Nasal inhalation of PEI–PEG/DNA complexes in mice increased transgene expression significantly when compared to unmodified PEI (23).

Despite the promising results, some difficulties exist in conjugation of PEG to gene delivery systems. PEGylation could decrease binding ability of vectors to nucleic acids causing instability of lipo- or polyplexes in blood circulation. It may also affect the binding of nanoparticles to receptors on cell membrane (24).

Another problem with PEGylation is the accelerated blood clearance (ABC) due to activation of splenic synthesis of anti-PEG IgM antibody after first injection, resulting in the opsonization of the subsequent doses and uptake by liver kupffer cells (25, 26).

Some studies which have been developed to compensate the negative effects of PEGylation are presented in Table 1.

Table 1. Some strategies to improve PEGylated gene delivery systems.

Strategies for Improvement of PEG-vectors	**Examples**	**Properties of Modified Vectors (*in vitro*/*in vivo*)**	**Ref.**
Substitution of PEG with other non-ionic, hydrophilic polymers	*Polysaccharides such as chitosan and dextran*	PEI-chitosan conjugate showed maximum luciferase activity in spleen followed by heart and brain in the mice in comparison with branched PEI 25 kDa (*in vivo*)	(27)
		Dextran-spermine conjugate could partially protect pDNA from degradation by nuclease and exhibited optimal gene transfer efficiency in lung (*in vitro*, *in vivo*)	(28)
	poly(N-vinyl-2-pyrrolidone) (PVP)	PVP-coated nanoparticles instead of PEG-coated ones could evade the accelerated blood clearance phenomenon (*in vivo*)	(29)
	Polyoxazolines (POZ)	PLL-POZ could provide stability against serum compounds, enzymatic digestion and high temperatures (*in vitro*)	(30)
	N-(2-hydroxypropyl)methacrylamide (PHPMA)	The alpha-half-life for bloodstream clearance of PLL or PEI/DNA complexes could be extended using multivalent PHPMA coating (*in vivo*)	(31)
	PEG-acetal-MAL (MAL: maleimide moiety)	The PEG-acetal-PEI conjugate had a half-life of 3 min at endosomal pH 5.5 and 2 h at physiological pH 7.4 (*in vitro*)	(32)
pH-sensitive shielding of DNA polyplexes or lipoplexes	*(ω-2-pyridyldithio poly (ethylene) glycol α-(butyraldehyde) (N(1)-cholesteryloxycarbonyl-1, 2-diaminoethane amidocarboxy) pyridyl hydrazone) (OPSS-PEG-HZN-Chol micelles)*	At endosomal pH 5.4, OPSS-PEG-HZN-Chol micelles were destroyed within 30 min at 37 °C, while OPSS-PEG-Chol micelles remained stable (*in vitro*)	(33)
Use of enzymatically cleavable PEG linkers	*PEG-peptide-DOPE (PPD) that is cleaved in a matrix metalloproteinase (MMP)-rich environment*	A multifunctional envelope-type nano device (MEND) which was modified with PPD showed no hepatotoxicity and innate immune stimulation. Also less accumulation in liver and spleen was observed using PPD-MEND compared to the PEG-unmodified MEND (*in vivo*)	(34)
Production of reducible PEG nanoparticles	*PEG-ss-chitosan oligosaccharide-ss-polyethylenimine (PEG-ss-COS-ss-PEI)*	PEG-ss-COS-ss-PEI copolymers not only had much lower cytotoxicity, but also displayed high transfection efficiency as compared to the control branched PEI 25 kDa (*in vitro*)	(35)

Cell targeting

Most non-viral vectors possess cationic-charged surfaces. This could potentially enhance the cellular interaction of these particles with the anionic-charged proteoglycans at cell membranes. However, as stated above, it could also cause some unspecific interactions with other components lagging the DNA transfer to the specific cells. Therefore, the process of targeting comprises of two steps; modulating the positive charge of the complexes as well as incorporating some specific ligands into their structures (2). Different methods have been used for targeting purposes include conjugation of variety ligands such as transferrin (36), folic acid (37), growth factors (38, 39), monoclonal antibodies (40, 41), carbohydrates (42) and RGD (arginine-glycine-aspartic acid sequence) (43) to cationic lipids or polymers as well as covalent conjugation of targeting ligands directly to DNA (44) and the use of physical methods to concentrate complexes near the cell surface (45).

Other chemical and structural modifications

Other studies which have been done to overcome extracellular barriers were directed toward chemical or structural modifications of synthetic vectors. In the case of cationic lipids, incorporation of cholesterol could stabilize lipo-complexes against binding to red blood cells in comparison to (Dioleoyl phosphatidylethanolamine) DOPE-contain-ing lipoplexes (46). Wen *et al.* showed that modification of cationic dendrimer polyamidoamine generation 4 (PAMAM G4) with histidine moieties could increase transfection efficiency of PAMAM significantly in the presence of serum in the range of about 10% to 50% (47). Conjugation of lactose to chitosan as ligand for targeting the hepatocytes showed excellent DNA-binding ability, good protection of DNA from nuclease, and the suppression of self-aggregation and serum-induced aggregation (48). Novel conjugates of polyamidoamine (generation 1.5 and 2.5) and polyethylenimine (PEI); G2.5-PEI 423 or G1.5-PEI 423; showed a great ability to condense pDNA which protected the pDNA from nuclease degradation (49). Agarwal *et al.* synthetized novel cationic pentablock copolymers based on poly(2-diethylamino-ethylmethacrylate) (PDEAEM) and pluronic F127 which provided efficient resistance to its degradation by nucleases (50).

Cell membrane: interface of extra- and intracellular obstacles

Cytoplasmic membrane with the lipid-based composition is deemed as a natural protection for the cellular content and function. Therefore, crossing such a cellular defense could be a challenging issue in gene delivery (51). The rationale behind the majority of physical techniques applied to gene delivery systems is more or less overcoming the cytoplasmic barrier as the cellular gate. Furthermore, considering viruses as the most efficient gene carriers in a complex gene transfer pathway, most non-viral vectors are designed based on the viral cellular entry patterns. However, the simulation of the exact artificial structural copies of viral components in synthetic constructs has not yet been fully accomplished (13).

Disturbing membrane integrity by physical techniques

Physical methods apply physical forces to deliver the cargoes through the cellular membranes (Figure 1). These include microinjection, particle bombardment using gene gun, electroporation, sonoporation, laser-assisted transfer, and magnetofection (2). Microinjection is the most capable method to transfer pDNA directly across all of the membranes into the nucleus. It has been easily applied to different cell lines to transfer the recombinant DNA constructs; although it is really time-consuming (2, 52).

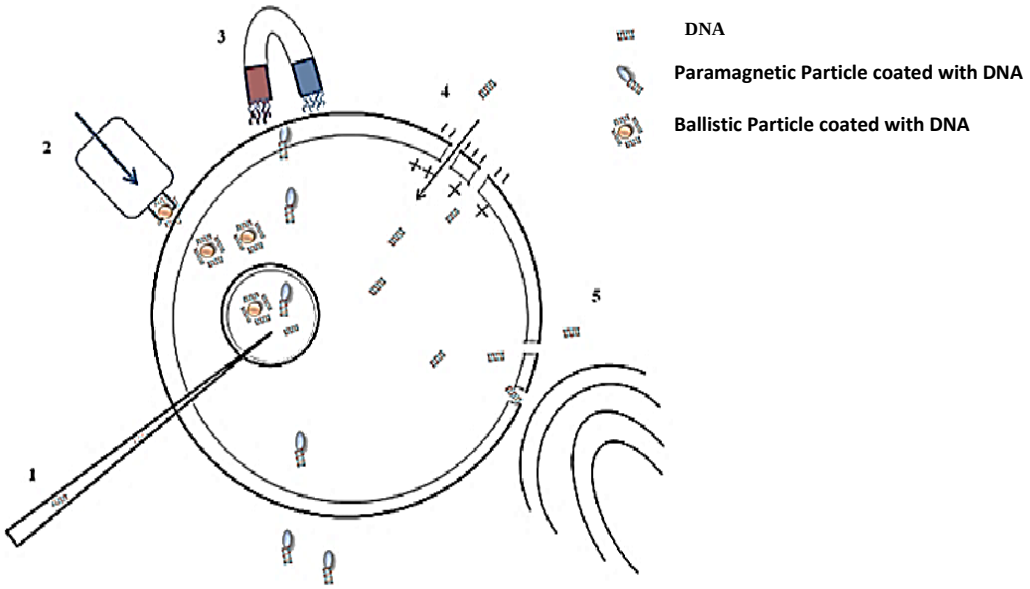

Figure 1. Major non-biological physical approaches used for gene delivery; 1) Microinjection, 2) Particle bombardment, 3) Magnetofection, 4) Electroporation, and 5) Sonoporation.

Introducing robotics into the technique makes the follow-up analysis much faster and not limited to single cell level (53). Regarding the thickness of microneedles (~1 μm) compared with the size of small mammalian cells (spherical diameter = 2–15 μm), microinjection has always been difficult to achieve without damaging the cell (54).

Performing gene delivery and stem cell manipulation using a nanoneedle (diameter ~ 200 nm) and atomic force microscopy (AFM), caused much less damage to a cell than microinjection did (54). This technique also assures accurate three-dimensional control of the nanoneedle (54). Electroporation applies milder condition for transferring the genes by the exposure of cell membrane to high-voltage but short-time electric field, which forms pores throughout the membranes (2). The current limitations of the method, its efficiency in achieving *in vitro* gene expression in cells, and its potential use for *in vivo* gene delivery has been reviewed by Favard *et al* (55). Recent advances in the mechanism of *in vivo* gene transfer by electroporation, and the findings of significant preclinical and clinical studies using this technology is also reviewed elsewhere (56).

Huls *et al.* (57) took advantage of electroporation and two DNA plasmids consisting of a transposon coding for a gene of interest and a transposase which inserts the transgene into TA dinucleotide repeats to generate enough numbers of T cells applicable for human use.

Sonoporation uses low level ultrasound to enhance membrane penetration (2). Microbubble-enhanced ultrasound is mentioned as one of the most effective physical delivery methods which can be applied to a range of different cell types *in vitro* and a broad range of tissues *in vivo*. Compared to electroporation, microbubble-enhanced ultrasound is presented as the least damage-causing and least invasive method (58). The recent successes and challenges within the field of ultrasound-mediated gene and drug delivery have been reviewed by Castle *et al (*59).

Particle bombardment is a process in which the gene incorporating into ballistic particles can be accelerated by a high-intensity electrical spark or a helium

discharge into the target cell (2). Biolistic transfection has been used as an effective and straightforward technique to transfect the hard-to-transfect cells such as post-mitotic neurons (60).

It was also extensively tested for intradermal DNA delivery in DNA vaccine transfection and gene transfer to tumor tissue samples (61, 62). It is demonstrated that gene gun is the desirable device for delivering DNA vaccines in preclinical mouse models and possibly for future clinical development (62).

Magnetofection refers to transferring the paramagnetic particles across the membrane using the strong magnetic fields (2).

Both the principle and the efficiency of applying magnetofection in cell cultures and use of magnetic gene targeting for implementing minimally invasive gene therapy have been reviewed by Schwerdt *et al* (63).

Some of the physical methods were used successfully in local delivery of DNA to tissues such as skin and skeletal muscles (2). However, these techniques usually suffer from the low throughput and a limited application for a wide range of purposes (64).

Cellular uptake

Vesicular pathway

Endocytosis, the process of internalization of particles in vesicular compartments, has been shown to be the main uptake mechanism for non-biological vectors (13). Major types of endocytosis include clathrin-mediated endocytosis which is a well-recognized endocytotic pathway, and other less known routes such as caveolae-mediated endocytosis and macropinocytosis (13).

The role of endocytosis pathways in cellular uptake of nanomedicines and non-viral gene delivery vectors was extensively discussed in several review articles (65-67). Glycosaminoglycans (GAGs) on the plasma membrane are the major components of the cell surface with negative charge. Therefore, these molecules have been suggested to influence the delivery of gene delivery agents in various ways (68).

For example, the electrostatic interaction between cationic particles and GAGs on the cell surface leads to a much faster particle internalization compared to anionic particles (69). This is why cationization of different carriers appears to be one of the most well-known techniques to develop the transfection agents with enhanced cellular uptake (70). Cationization not only improves the cellular internalization by optimizing the charge density of the particle but also affects other major extra- and intracellular barriers as well (71).

Cationic-charged polymers and dendrimers such as polyethylenimine (PEI), poly(propylenimine) (PPI), poly(amido-amine) (PAMAM) as well as various designed cell penetrating peptides (CPPs) and cationic derivatives of lipids are presented as the major parts of different functional gene delivery systems (71). Polyethylenimine is a well-known cationic polymer which is believed to be able to condense DNA into positive-charged nanoparticles.

The positive charge of such nanoparticles would enhance non-specific electrostatic interactions with the cell surface proteoglycans and facilitate cellular uptake (72). Attachment to different targeting ligands is then necessary for PEI-mediated specific cellular uptake (72).

Different classes of CPPs, their major uptake mechanisms and recent CPP-modified nanocarriers were summarized by Koren *et al.* (73) and Bechara *et al* (74).

The basic amino acid residues such as arginine and lysine in the structure of CPPs have been proposed as the main parts responsible for their membrane translocation activity (75). One of the most investigated cell penetrating peptides is Tat peptide which is derived from the residues 48 to 60 of the original

transcription activating factor of human immunodeficiency virus (HIV-1) (75). Subrizi *et al*. showed that there was no difference between Tat sequence and its cationic analogs in cellular uptake, proposing cationic charge as the main factor responsible for Tat-mediated cellular uptake (75).

In a more detailed study done by Gautam *et al.* (76), a prediction web tool was developed for the first time to assist in discriminating cell-penetrating peptides from non-cell penetrating peptides based on amino acid composition (76).

The authors used large dataset (708 CPPs) for training, testing and evaluating their support vector machine (SVM) models. Various features such as amino acid composition, dipeptide composition, binary profiles of pattern, and physicochemical properties have been used as input for developing the models. They concluded that certain amino acid residues such as arginine, lysine, proline, tryptophan, leucine, and alanine are preferred at specific locations for cell permeation ability (76).

Hydrophobic modification of non-viral carriers generally improves the cell membrane permeation of the polyplexes by mediating more favorable complex interactions with cells which can ultimately result in better cellular uptake and higher transfection (77).

In this regard, the optimum degree of hydrophobicity, the type of hydrophobic entity and the core structure could affect transfection efficiency of the transferred gene (78). Chitosan modifications with hydrophobic amino acid residues (79) and synthesis of poly(alkyl methacrylate) can be mentioned as examples of huge number of hydrophobic modifications which improved cellular uptake (80). Although N-alkylation of linear polyethylenimine has been shown as an effective strategy to enhance gene transfection, Klibanov group recently reported that this favored effect did not originate from enhanced cellular uptake and rather was influenced by the subsequent pathway (81).

Membrane fusion

Although facilitating the endocytotic pathway is assumed as the main mechanism of action of membrane-active carriers, there are also some studies introducing membrane fusion as an alternative theory of internalization for some of non-viral systems.

However, even in those types of carriers, cellular uptake by endocytosis rather than direct effect on membrane is weighed out in the streaming pile of data (73, 74, 82).

Lipofection, the use of lipids for DNA transfection, was first described in 1987 by Felgner *et al* (83).

Since then, several improvements in gene delivery with cationic lipids have been reported (84). Cationic lipids are amphiphilic structures with a cationic head group which is necessary for binding to the negative-charged nucleic acids (85).

They can be classified into monovalent aliphatic lipids, multivalent aliphatic lipids and cationic cholesterol derivatives. The head group of monovalent types contains just one functional amine while the multivalent lipids have several amine groups (85).

Generally, it is believed that lipidic structures internalize into the cells by direct fusion with the cell membrane or through endocytosis (82, 86).

Almofti *et al.* (86) suggested that the lipoplexes were internalized into the cell through the endocytic pathway but after being fused with the cell membrane as observed in their synthetic system.

The role of plasma membrane fusion was then proposed as triggering and/or facilitating the uptake of large lipoplex particles (86). One of the strategies to improve the transfection efficiency of lipid-mediated carriers is incorporating another class of lipids called co-lipids or helper lipids into the lipid formulations.

These so-called neutral lipids are supposed to increase the transfection efficiency of some lipid constructs by destabilizing the membrane through promoting conversion of the lamellar lipoplex phase into non-lamellar (87, 88).

Dioleoyl phosphatidylethanolamine (DOPE) has been used as a neutral lipid in a variety of effective formulations such as commercially available Lipofectamine™ Reagent (Invitrogen, Carlsbad, CA) (87). Cholesterol has also gained attention as helper ingredient in the cationic liposome formulations (89). Cholesterol-containing carriers synthesized by Yang *et al.* (89) obtained more stable particle size, lower turbidity, more steady transfection ability in presence of high concentration serum (50% FBS) compared to DOPE-containing formulation. This method for maintaining transfection activity under serum-containing condition seems to have potential for practical *in vivo* application (89).

Cell penetrating peptides (CPPs), also known as fusogenic peptides, are mainly found to enhance the transfection efficiency by interfering with the vesicular pathway and in a pH-sensitive manner (90, 91). However, the induction of membrane fusion by these peptides at cytoplasmic membrane cannot be excluded (92). In a study done by Tu and Kim (92), modifying cationic liposomes by WT peptide, a synthetic analog of a fusogenic peptide domain from herpes simplex virus, could increase the level of cellular uptake by mediating 80% membrane fusion at pH 7.4 at a 0.05:1 (peptide:lipid) mole ratio.

Intracellular obstacles

It is demonstrated that just around 50% of the cells which could uptake the vectors successfully, are able to express the delivered gene (93). This low yield can be a result of encountering intracellular barriers. Important approaches dealing with these types of obstacles will be conferred here.

Avoidance of endo-lysosomal degradation

Clathrin-mediated endocytosis (CME) as the main endocytic pathway normally results in the lysosomal destructive compartments (85).

Thus, the endosomal escape is recognized as a major rate-limiting factor with the highest impact on the efficiency of delivery systems (94). Crucial challenges for designing the best delivery agents and techniques to promote the endosomal release were reviewed by Varkouhi *et al* (90).

To avoid degradation, vectors should be able to escape from the endosomes before they fuse into lysosomes or to bypass this step by internalizing through other pathways like caveolae-mediated endocytosis or macropinocytosis. DNA nanoparticles formulated with polyethylene glycol (PEG)-substituted cysteine–polylysine peptides (PEG-CK30) have shown promising *in vivo* gene delivery activity into different tissues (95-97). These highly compacted nano-particles are composed of plasmid DNA and a 30-mer lysine peptide with an N-terminal cysteine conjugated to PEG. It is found that their high *in vivo* gene transfection could be due to the fact that they may not employ the traditional clathrin-mediated endocytosis to enter the cells and rather use non-traditional pathways such as caveolae-mediated internalization in some cells (98, 99).

In another study, Nathan *et al.* (100) showed the importance of the caveolar uptake pathway and potentially avoidance the endosomolysis route in providing high gene transfer efficiency of folate-targeted PEI in HeLa cells. Despite the abovementioned favorable results, there are also some evidence showing that even the pathways believed to bypass lysosomes such as caveolae-mediated route can end up in lysosomes in certain cases (101).

Development of pH-sensitive vectors with the ability of membrane-disruption limited to acidic pH can be considered as

another pursuing strategy in this field (72, 102).

These pH-sensitive formulations could destabilize the endosomal membrane upon change in pH via mechanisms such as osmotic burst (72, 103) or direct fusion (104) resulting in release of DNA/carrier complexes before degradation.

Polyethyelenimine was proposed as a sponge attracting significant amount of protons in the acidic environment of endosomes; leading to osmotic burst which is known as the major mechanism for PEI-based vectors' high gene transfection (72, 103). The proton sponge effect of PEI triggered by its amine content is strongly dependent on its molecular weight as small PEIs do not provide an effective buffering capacity to induce efficient endosomal escape (72). The pH-dependent protonation of histidine residues at endosomal pHs of around 6, grant histidine-containing peptides similar buffering capacity feature to PEI, especially when the number of histidine residues is high enough in the constructs (105). However, for both PEI and histidine-rich peptides, the direct interaction with endosomal membrane was also suggested by some investigators even as the more prominent mechanism of these vectors efficacy (106, 107). KALA sequence, a condensing and fusogenic peptide, undergoes a pH-dependent conformational change from random coil to amphipathic alpha-helical as the pH increased from 5.0 to 7.5 (91). This conversion enables the peptide to interact with the membrane more effectively (91). It also happens for other fusogenic peptides such as conventional Haemagglutinin peptide, a peptide derived from the influenza virus coat, which could be converted from an anionic, hydrophilic coil at pH 7.4 to a hydrophobic helical conformation at the acidic endosomal pH (90). Shaheen *et al.* successfully employed KALA sequence on the endosome fusogenic (outer) and nuclear membrane-fusogenic (inner) envelopes of a multi-functional envelope-type nano-device (108). Their liposomal delivery system exhibited higher gene transfection than that of Lipofectamine PLUS (108). Soltani *et al.* (109) recently reported encouraging results by incorporating KALA sequence as a fusogenic segment into a multi-functional recombinant vector backbone in order to facilitate disruption of endosome membranes.

Hydrophobic modification is also presented as an attractive strategy to possibly enhance the endosomal release by interfering with the organization of lipid bilayers, destabilizing the membrane integrity and enhancing the vector efficacy.

Our lab, therefore, took advantage of hydrophobic modification in enhancing the transfection efficiency of different PEI-based vectors by assuming that alkylcarboxylation of PEI would have a synergistic effect on its endosomal release capability (110-112).

The role of helper lipids such as DOPE on lipoplex assembly and the importance of earlier-mentioned conversion of the lamellar lipoplex phase into a non-lamellar structure in destabilizing endosomal membranes and mediating endosomal escape of DNA have also been discussed by Wasungu *et al* (87).

Considering the entrapment of CPPs such as TP10 in the endosomal compartments, Arukuusk *et al*. synthesized novel stearylated TP10 analogs, named NickFects, and applied them for transfecting a large variety of cell lines (113).

Their novel system was not only efficient in gene transfection but also suggested to be useful for mammalian protein production system to express and produce recombinant proteins in hard-to-transfect suspension cells (113).

Facilitating cytoplasmic transport

The cytosol of the eukaryotic cells is extremely crowded by nucleases, the cytoskeletal meshwork and other

organelles which can degrade DNA or slow down its transfer to the nucleus (13). Although it has been shown that the diffusion of macromolecules through the cytoplasm is size-dependent (59), but endo-lysosomes can take advantage of a natural transport along the cytoskeleton to the nuclear periphery.

Moreover, in some cases, the electrostatic interaction between cationic complexes and anionic microtubules or motor proteins could enhance the mobility of vector/DNA complexes (13).

Similar to viruses, plasmid DNA-lipid complexes were found to interact with microtubules and move along the microtubule network (114). They become first immobilized in peripheral actin cytoskeleton immediately after cellular internalization, and their aggregates form smaller ones, which are probably moved to the adjacent microtubules and used them cargo to move towards the cell nucleus (114).

Suh *et al*. suggested the involvement of microtubule-associated motor proteins in the active transport of PEI/DNA nano-complexes (115). On the other hand, Doyle *et al.* hypothesized PEI-encapsulated endosomal transport facilitated by microtubules rather than a direct interaction between PEI and microtubules as the mechanism of cytoplasmic transfer (116). However, both of these studies, emphasized on the accumulation of PEI/DNA polyplexes at nuclear boundary area which could possibly enhance the chance of nuclear uptake during mitosis and/or direct association of polyplexes with the nuclear membrane (115, 116).

Furthermore, Doyle *et al.* (116) reported no specific localization at adjacency of the nucleus by incorporation of arginine residues into PEI structure, which could be probably due to the absence of microtubule trafficking for polyplexes prepared from modified PEI constructs such as PEI-Arg polyplexes.

The importance of microtubules in nuclear import of DNA nanoparticles (DNPs) self-assembled from polyethylene glycolated cysteine–lysine 30mer (PEG-CK30) and plasmid DNA, was also discovered (117). These potentially therapeutic DNA nanoparticles which have been evaluated for phase I clinical trial in treatment of cystic fibrosis, were found to interact with the cell surface receptor called nucleolin. Nucleolin further associates with glucocorticoid receptor (GCR).

The aforementioned nanoparticles then transfer through the cytoplasm in association with GCR and dynein complexes along microtubules (117). Considering the involvement of GCR in the transfer complex, cortisone and dexamethasone could enhance transfection by DNPs in both HeLa and polarized 16HBEo− cells, a model system for airway epithelium (117).

Therefore, authors proposed that similar approaches may have potential in gene transfer to non-dividing airway epithelial cells *in vivo* and in humans as well (117).

Barua *et al.* (118) reported the use of tubacin, a selective small-molecule inhibitor of cytoplasmic histone deacetylase 6 (HDAC6) which acts in cytoplasm, to increase transgene expression in prostate cell lines.

The enhanced acetylation/stabilization of microtubules following tubacin treatment, and enhanced recruitment of dynein and kinesin motor proteins by acetylated microtubules were suggested to facilitate the transport of pDNA towards the nucleus and consequently increase transgene expression (118).

Yet the effect of parameters such as polyplex size, intracellular localization patterns, and primary levels of transgene expression on the overall observed improvement have not been excluded (118).

Not only the type of carrier, but also the sequence of the gene transferred seems to play role in cytoplasmic trafficking.

Using a cell-free microtubule-binding assay, plasmid microinjections and real-time particle tracking, Badding *et al.* showed the sequence-specific plasmid-microtubules interactions (119).

Plasmids containing binding sites for cyclic AMP response-element binding protein (CREB) exhibited strong microtubule binding while those lacking CREB-binding sites could not interact properly (119). It was therefore proposed that inclusion of transcription factor binding sites such as CREB within plasmids could enhance movement to the nucleus and increase transfection efficiency (119).

Unpackaging and nuclear transport

To be effective, the nucleic acid should be separated from the carrier preferably at the site of action. Early release of DNA into the cytoplasm could end in degradation by cytoplasmic nucleases (120).

On the other hand, late release of DNA could result in non-efficient expression. In this regard, an optimum association with cationic components can keep the nucleic acid safe as well as providing a free status for the nucleic acid to perform its duty (13). Any therapeutic gene should be able to cross the nuclear envelope to be expressed by the natural expression machinery of the nucleus.

Non-viral transfections seem to be cell cycle dependent and the nuclear envelope is especially considered as a significant barrier in non-dividing or slow-dividing cells (121).

For transfecting these kinds of cells, gene delivery systems need effective components to pass through the fine pores of the nuclear membrane. Significant approaches to improve nuclear targeting of plasmids and the prospects in the field were highlighted by Lam and Dean (121).

In general, any membrane permeability enhancer would be able to affect almost all of the physiological membranes such as cytoplasmic, endosomal and nuclear membranes resulting in total enhancement of gene expression.

From this point of view, various chemically membrane interfering methods using cationic polymers or dendrimers, lipid-based formulations and peptides containing basic residues of lysine or arginine as well as physical techniques disturbing the membranes can be categorized as non-specific nuclear delivery promoters. However, probably the most common strategy which has been used specifically to increase nuclear localization of DNA was the inclusion of synthetic or naturallyoccurring nuclear localization signal (NLS) peptides in DNA carrier formulations (121).

Numerous studies have investigated the effect of different types of NLSs such as classical, bipartite and non-canonical NLS peptides (121, 122) in their systems. Ma *et al.* (123) used the NLS glucocorticoid triamcinolone acetonide (TA) to synthesize polyethylenimine-grafted triamcinolone acetonides (PEI-TAs) as nucleus-targeting gene carriers. Both *in vitro* and *in vivo* studies revealed that TA moieties could more effectively translocate low molecular weight PEI 1800 Da into the nucleus than high molecular weight 25 kDa PEI (123). We also recently reported the enhanced transfection efficiencies by coupling peptide nuclear localization signals (SV40 large T antigen NLS or C-terminus of histone H1) to PEI (10 kDa) (122).

However, it still remains to be evaluated if enhancing nuclear transport holds a crucial role in the eventual improvements by our multifunctional vectors (122). Furthermore, it should be also mentioned that not all studies report the promising transfection results by incorporating NLSs in their systems and there are also some instances of non-effectiveness of NLSs in the whole scenario (121).Considering nuclear membrane with its nuclear pore complex (NPC) as a selective barrier, Liashkovich *et al.* (124) emphasized on the

decisive effect of NPC on limiting the efficiency of gene therapy. They showed the unequal contribution of nucleoporins, the building blocks of NPC, in formation and maintenance of the permeability barrier with a given molecular weight cut-off value. Based on the outcome of their study, they proposed the surface modification of the nano-carries with both trans-1,2-cyclohexanediol (CHD) (as effective modulator in breaking down the NPC permeability barrier), and importin β. Importin β would guide the imagined nano-carrier specifically and quickly to the NPC while CHD would induce the leakiness of NPC thereby enabling nuclear transfer of the nano-carrier. Thus, a targeted disruption of the NPC permeability barrier through dissociation of the most effective barrier-forming nucleoporins has been proposed as new approach resulting in significant improvement of gene therapy potential (124).

Treatment with mediators of intracellular trafficking such as the class I and II histone deacetylase inhibitor trichostatin A (TSA) indicated that HDAC inhibitors with both nuclear and cytoplasmic activity could also be employed as enhancers of transgene expression (118).

There is always a potential risk for vectors acting as membrane permeabilizers causing cell damages such as apoptosis (125).

Hence, a delicate balance between cytotoxicity and high expression efficiency is needed. It will also be useful to allow the effective transcription happens by helping the unpackaging of carrier/DNA properly rather than by using membrane-effective components to improve the efficacy of delivery. In an investigation done by Yamada *et al.*, (126) a close relationship between the efficiency of DNA release and transcription efficiency has been demonstrated. They proposed the cationic density as the factor governing the release of enough amount of supercoiled pDNA, from pDNA particles condensed with biocleavable polyrotaxanes (DMAE-ss-PRX).

Increasing the cationic density above a certain value however, could hamper the transfection efficiency by assumingly disturbing the post-transcription process; transcription, nuclear mRNA export, translation and related processes (126). The free cations released from the condensed pDNA particles were presented as one of the reasons for the obtained lower transgene expression (126). Providing the suitable charge density and hydrophobic-hydrophilic balance in polymeric carriers has been also excessively studied in our laboratory (110, 112, 122, 127).

Although seemed to be more effective on cellular uptake and endosomal release, incorporating the hydrophobic groups into the cationic polymers could probably assist in unpackaging DNA from the tightly bound polymer/DNA complexes as well (110).

On the other hand, modification of poly-L-lysine with serine residues enhanced the gene expression probably due to the hydrophilic nature of the final compound which may facilitate the recognition of polypeptide/DNA complexes by the transcriptional factors and result in subsequent high efficiency of the vectors (128).

Apart from carrier type, the presence of specific sequences in the structure of DNA plasmids termed DNA nuclear Targeting Sequences (DTSs) has been shown to increase nuclear delivery of plasmids (121).

However, by performing quantitative PCR on isolated nuclei, van Gaal *et al.* (129) claimed no remarkable benefits of DTS on overall transgene expression if combined with strong promoters. They did not exclude the probable effect of DTS at nuclear entry, but hypothesized that the

value of DTS would be obvious when using weak promoters. Assuming the post-transcriptional level as the bottleneck in cases using a strong promoter, the advancements in pre-transcriptional processes such as nuclear uptake may not affect overall gene expression levels (129). Since weak promoters can be used as alternatives to the strong promoters for minimizing immunological responses and promoter shutdown *in vivo* and for transcriptional targeting (129), the use of DTSs may still considered as effective *in vivo* strategy.

Conclusion

Despite achieving few regional approvals and the great potential expected for gene therapy, a gene-based therapeutic has not yet reached the global market. Not only the inadequate knowledge of the potential delivery hurdles especially in mechanistic manner, but also lacking of a well-designed safe vector can be stated as main reasons.

Obviously, it is hard to imagine that only one ideal vector could be the best option for all of gene delivery applications. However, overcoming the major extra- and intracellular obstacles by designing more efficient and safe vectors is considered as one of main objectives to improve the gene therapy status especially for human use. In recent years, remarkable progress has been made in both characterizing the obstacles in a mechanism-oriented manner and developing delicate and rational-based gene delivery constructs.

A brief on each assumed barrier and the applicable strategies to conquer such barrier have been discussed here.

The examples highlighted in this review provide an overall optimism on ultimately paving the way to clinic by the current wave of barrier-defeating approaches. However, in order to introduce an efficient vector for *in vivo* use, more detailed studies are required to address the issues of *in vivo* barriers.

Moreover, standardization of the current methods for evaluation the efficacy of the new vectors can be proposed as an approach for deciding which vectors can be selected and used for *in vivo* studies.

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