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Non-viral nanovectors for gene delivery: factors that govern successful therapeutics

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Importance of the field: Gene therapy is regarded as one of the most promising therapeutic approaches, as it has the potential to treat disorders by correcting malformations at the nucleic acids.

Areas covered in this review: Some of the most recent developments in the process of plasmid DNA vector design and formulation are reviewed with a special focus on: different formulations of nanovectors and a summary of successful cases reported; requirements for systemic administration; and functionalization of the nanocarriers by use of targeting entities.

What the reader will gain: An understanding of the different physiological barriers and a comprehensive review of the recent strategies used to overcome these obstacles. Particular attention is given to formulations for intravenous administration, colloidal stability properties and different targeting entities used.

Take home message: Overall, vector formulation must take into account the administration route and inherent physiological barriers. Critical parameters for the success of pDNA nanovectors are: particles size, colloidal stability of the formulation and interaction between the carrier and plasmid DNA. Highly relevant is the fact that this interaction should be balanced to offer protection to degradation as well as allow dissociation of the therapeutic nucleic acid for obtaining maximal activity.

Keywords: *in vivo*, intravenous administration, nanoparticles, receptor-mediated uptake

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1. Introduction

Gene therapy is an emerging technology that aims for permanent, or transient, correction of a gene defect by intracellular delivery of nucleic acids. Provided that the carrier device is individually tailored with respect to targeting and pharmacokinetics, this tool holds the potential of having a significant impact on the treatment of a wide range of monogenic as well as acquired disorders. In fact, the concept of a 'personalized and targeted drug' dates back to the late nineteenth century and it is inherent to the concept of Paul Ehrlich's 'magic bullet' [1].

Conventionally, in the case of loss of function, this non-viral gene rectification is achieved by the introduction of a plasmid DNA (pDNA) vector encoding the native form of the gene (**Figure 1**). Conversely, in the case of gain-of-function mutations, therapies that reduce gene expression are of interest, such as RNA interference and microRNA approaches. MicroRNAs act as silencing agents of gene expression and, similar to siRNAs (small interfering RNAs), they can be used as therapeutics. However, several microRNAs are tissue-specific and this fact has been explored and developed further in Luigi Naldini's laboratory to direct gene expression (**Figure 1**) [2,3]. By introducing the corresponding targeting sequences of the tissue-specific microRNA into the pDNA construct, the vector is consequently equipped with an active

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Article highlights.

- Optimization of the pDNA vector regarding tissue specificity (e.g., promoter and enhancer sequences) and reduced immunostimulation (by removal of CpG motifs) are of great importance for the efficiency and general utility *in vivo*.
- The interaction between pDNA and delivery agent should be balanced in order to provide stability as well as allow for dissociation of the nucleic acid to exert its activity. This mild interaction might be easier to fine-tune with amphiphilic carriers.
- The use of stabilizers (glucose or dextrose) can support the colloidal stability of the formulation.
- *In vitro* characterization assays are well established but often do not correlate well with *in vivo* studies. Therefore, an early evaluation of the delivery system under *in vivo* conditions can be very informative for further developments of the carrier device.
- For *in vivo* applications less charged nanovectors are preferred to avoid: electrostatic interactions with proteins; and clearance mechanisms.
- Loco-regional and systemic administrations usually require vectors with different properties to overcome diverse physiological barriers; stabilization provided by steric hindrance and functionalization of the vector are probably required for systemic administration.

This box summarizes key points contained in the article.

'detargeting' component. Likewise, based on numerous steps of improvements by many researchers, Christine Wooddell *et al.* recently designed and optimized a pDNA vector for enhanced and long-term liver-specific gene expression (**Figure 1**) [4]. The overall implication of these reports is the relevance of the design of the plasmid DNA *per se*, that is, its individual components, for an optimal therapeutic effect. pDNA can be improved further by removal of bacterial sequences, such as unmethylated cytosine-guanine (CpG) motifs, that have been shown to be immunostimulatory [5]. On this note, the minicircle or miniplasmid [6], a small vector devoid of bacterial sequences including the antibiotic resistance gene marker, is an attractive alternative to normal-sized pDNA.

Owing to physiological barriers, however, naked pDNA is normally not capable of inducing relevant therapeutic effects when administered systemically *in vivo*, with the exception of hydrodynamic injections [7] wherein the conditions are very special. Hence, there is a need for a carrier system, which must first overcome the extracellular barriers (such as avoiding particle clearance mechanisms, targeting specific tissues or cells and protecting DNA from degradation) and, subsequently, the cellular barriers (cellular uptake, endosomal escape, nuclear entry and nucleic acid release). Although viral approaches, as opposed to non-viral, are at present more effective, their success has been hindered by safety concerns with respect to mutations, owing to random genomic integration and immune responses. Moreover, viral vector supernatants also contain numerous empty and defective particles, with intact particles making up merely a minor proportion. Attention has therefore turned to non-viral methods, in particular to chemical vectors, and great effort has been invested in the improvement of their major shortcoming – low efficiency. Chemical vectors refer to chemical-based delivery systems, where the nucleic acid is transported by natural and/or synthetic peptides, lipids or polymers. Alternative nano-strategies include inorganic particles such as gold [8,9] and silica-based systems [10].

This review focuses on chemical vectors that are characterized by an interaction, by means of either charge or hydrophobicity, between nucleic acids and the carrier polymer. Newly developed delivery systems are normally first characterized *in vitro*, and only a few thereafter *in vivo*. Importantly, a correlation between *in vitro* and *in vivo* rarely exists [11,12] and experimental conditions (buffers and concentrations), which are optimized for the given delivery vehicle, often vary among reports. Overall, despite the numerous studies in the field, it appears that relatively little is known about the properties of a successful *in vivo* gene delivery vector and only a few guiding principles are generally applicable. This results from the combination of extensive chemical possibilities together with the complexity of the living organism. Therefore, establishing a drug carrier is often a long and cumbersome process.

Over the past few years, however, knowledge has increased, and many advances have been made that have improved *in vivo* delivery, providing more information on common principles and general guidelines. Parameters inherent to any drug formulation that influence the activity of the cargo include: structure, composition and solubility of the polymer(s), and size and surface charge (referred to as zeta-potential; with particles with <-30 mV and >+30 mV being stable) of the nucleic acid complexes. Also, temperature, buffer composition and pH are likely to have an effect on the solubility, size and surface charge of the particles. For gene delivery in particular, intracellular barriers represent an extra concern when designing the formulation. Properties of the carrier have been suggested to have a major impact on cell internalization [13], endosomal escape [14,15] and nuclear uptake (**Figure 2**) [16-18]. Intracellular uptake and trafficking have been reviewed extensively elsewhere [16,19,20] and are therefore not the focus of this review. Instead, some of the most recent reports on plasmid delivery are discussed, addressing the effects of physicochemical properties of polymeric nanoparticles on their *in vivo* activity, biodistribution and clearance. Specifically, the composition of the nanoparticles concerning size, surface charge, PEGylation and functionalization is discussed.

2. Basic principles for formulation

2.1 Peptide/polymer–DNA interactions

Nucleic acids are negatively charged molecules owing to their phosphate backbone, which can mediate electrostatic interactions [21]. In 1997, Bloomfield defined DNA

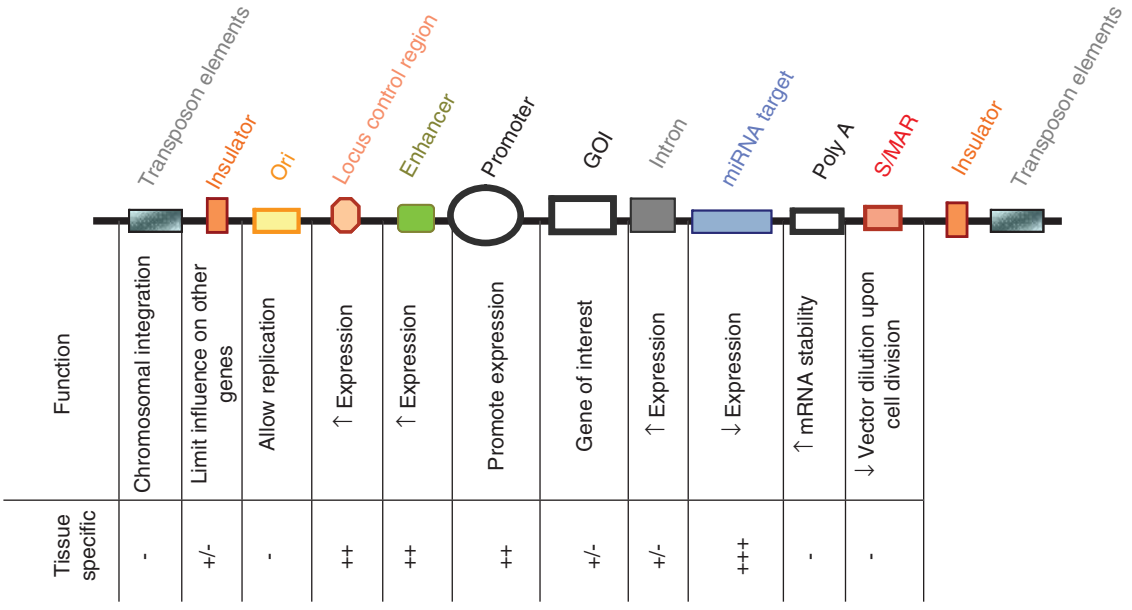

Figure 1. Possible components of a DNA vector. All vectors must contain a promoter (to initiate transcription of the gene), gene of interest (GOI) and a Poly A sequence (encoding for a poly-adenosine tail in the mRNA that is important for its nuclear export, translation and stability). Further optimization of the plasmid DNA vector is recommended and can be translated into increased and long-term gene expression. Enhancement of expression can be obtained by the inclusion of several other components: i) tissue-specific promoter and enhancer sequences; ii) the presence of splicing elements, or introns, that also contribute to higher gene expression of the correct protein; and iii) a locus control region (LCR) that can regulate the expression of linked genes through long-range interactions of various *cis* regulatory elements as well as chromatin modifications. Duration of gene expression may be increased with the inclusion of a scaffold/matrix attachment region (S/MAR). Repeated S/MAR sequences have been suggested to bind to matrix elements, thereby maintaining the episomal vector inside the cell [131]. Moreover, under certain circumstances, and in the presence of the origin of replication (Ori), they have been shown to be able to drive the replication of plasmids in a continually episomal state [132]. Also, transposons have been shown to integrate into the chromosomal DNA, therefore inducing long-term expression [39]. Although integration is random, it is dependent on an exogenous enzyme transposase, which must be provided. Insulators can be used to define domains of expression and block activation of nearby endogenous promoters. MicroRNA (mir) sequences, on the other hand, have been introduced as 'detargeting' agents to allow expression in selected target cells while silencing it in undesired tissues [2,3]. In addition, the innate immune system recognizes certain elements in a nucleic acid, such as unmethylated CpG motifs [5]. Mutating stimulatory sequences could be important for optimal performance [133].

condensation as the collapse of extended DNA chains into compact, ordered particles containing only one or a few molecules [22]. Typically, this process results from neutralization of negative charges of the DNA phosphate groups, with transition into the ordered phase occurring when 90% of the negative charges of the DNA phosphate groups are neutralized [22]. Classical DNA condensing strategies frequently rely on the use of cationic peptides or polymers, either natural or synthetic. Often DNA aggregates, large undefined structures containing several DNA molecules, are formed in addition to well-defined and ordered particles. This is a common phenomenon, and it highlights the delicate balance that this interaction requires, not only for structural but also for functional purposes. DNA aggregates are frequently formed when using highly cationic molecules such as polylysine or polyethylenimine (PEI) that can interact with different DNA chains thereby increasing the number of molecules per condensate and, consequently, the diameter. Moreover, highly cationic polymers will impair to a greater extent the DNA unpacking, which is critical for its activity.

Condensing agent/DNA interactions are not only of electrostatic nature, as hydrophobic interactions also take place between the nucleobases of DNA and the polymer [23,24]. Combined with cationic contributions, these weaker bonds are probably tuning the interaction and can result in improved carriers [12,25,26]. However, the composition of both components, hydrophobic and cationic, plays an important role [12,25,26]. For example, with regard to fatty acid-modified spermine, longer fatty acid tails seem to result in enhanced DNA interaction, as reflected by greater retardation of the pDNA in agarose gel-shift assays. This apparent increased affinity was not, however, paralleled by improved *in vivo* activity [12]. In fact, in this report, the most active agent *in vivo* was composed of the shorter fatty acid that was

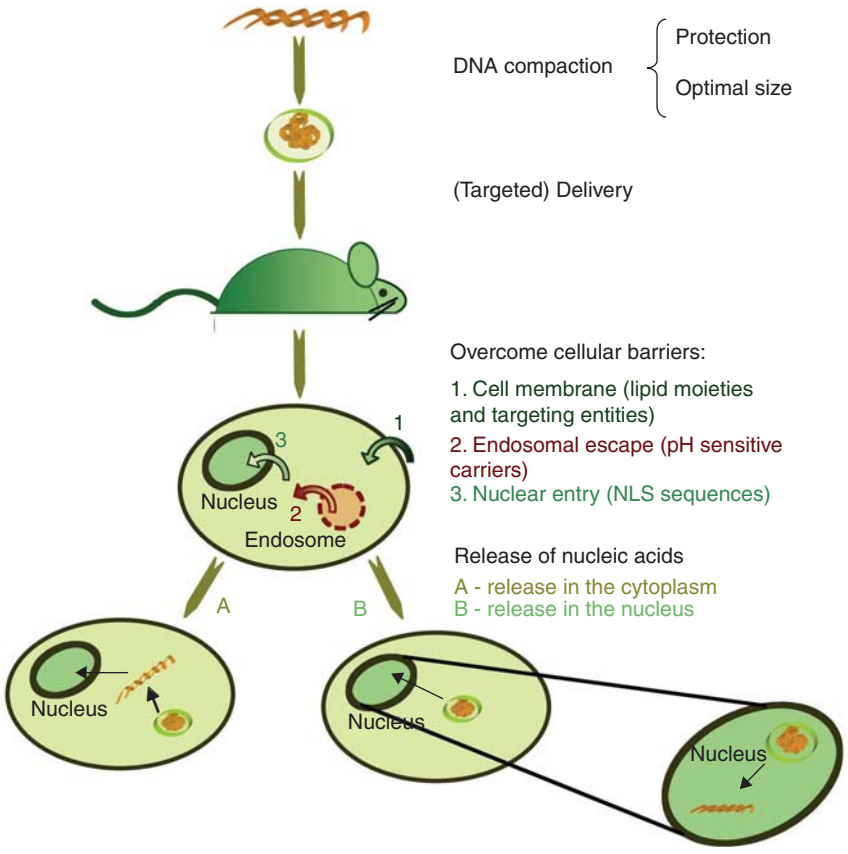

Figure 2. Schematic representation of the different stages in nanovector delivery. Plasmid DNA should first be compacted into well-defined particles of optimal size for organ delivery and capable of providing protection against enzymatic degradation and protein interaction. These are two processes that occur mainly in the bloodstream and result in clearance of the drug from the system. *In vivo* administration of DNA nanocarriers may be local or systemic (often by intravenous route). For systemic delivery, one way to reach tissue-specific administration is to include targeting ligands that confer receptor-mediated uptake. Once the vector reaches the target organ it must overcome certain cellular barriers (cell internalization, endosomal escape and nuclear entry) before reaching its active site. Release of the nucleic acids from the nanocomplexes is essential for their activity and very little is known about this process. It is hypothesized that it may take place in the nucleus or in the cytoplasm, in which case the uncondensed pDNA vector must then be transported into the nucleus.
NLS: Nuclear localization signal.

tested – butanoyl. Similarly, highlighting the relevance of the cationic component, Mäe *et al*. have shown that different cell-penetrating peptides (CPPs) when equally modified with stearyl moiety did not show the same increased efficiency [26]. Specifically, the modified stearoyl-TP10 had greatly increased activity in relation to its parental peptide as well as to the other modified peptides.

Together with the electrostatic binding, hydrophilic interactions (through hydrogen bonds) might also have relevant contributions for the resultant DNA condensates, as is the case for DNA/chitosan particles. The same importance applies for condensing agents with amphiphilic properties, as, for example, poloxamer block copolymers, often referred to as Pluronics® (BASF, Germany). Peptides or polymers that can interact with nucleic acids in a weaker fashion have lately become more popular for the reason that they are likely to be less toxic (highly positively charged particles can induce complement activation and binding of immunoglubulins, and subsequent cytokine release). More importantly, complexes assembled by weaker interactions facilitate the release of the nucleic acid, which is required for activity (**Figure 2**). Supporting this theory is the fact that spacing positively charged arginines in the CPP Arg9, by introduction of 6-aminohexanoic acid spacer, results in a more active peptide, named $(RxR)_4$ [25,27].

Polymers such as poloxamers, composed of sequential blocks and with amphiphilic characteristics, are attractive agents mainly for the reasons that: i) they present self-assembling properties [28,29] that allow for incorporation of drugs into the micelles; and ii) the wide range of possible combinations of their monomeric units (poly(propylene oxide) [PPO] and poly(ethylene oxide) [PEO]), which confer

significantly different characteristics from the polymers belonging to this class of molecules. More specifically, in the case of poloxamers, varying the number of the hydrophilic moiety in relation to its hydrophobic counterpart permits manipulation of properties such as solubility and critical micellar concentration (CMC). Depending on the temperature conditions and the concentration of the polymer, different types of micelle can be formed: spherical, cylindrical and lamellar [30]. Manipulation of such properties allows these polymers to be tailored according to specific applications [28,29,31-33].

Overall, independently of the type of interaction, the key factors for an efficient DNA delivery system are: i) to confer DNA protection; ii) to allow formation of small well-defined particles (preferably of ~ 100 nm) that are efficiently internalized by cells; and iii) to display a moderate binding affinity allowing liberation and subsequent activity of the nucleic acid inside cells (**Figure 2**).

2.2 The tool box: *in vitro* characterization of DNA complexes

For characterization of DNA complexes there are several standard assays that allow for description of physicochemical and morphological properties. Some of the most common methods are illustrated in **Figure 3**. However, in the authors' opinion not all of these analyses are needed before proceeding to *in vivo* studies. Naturally, in a primary phase, the researcher is interested in obtaining general characteristics on the DNA interaction with the carrier. The simplest and quickest approach is to run a gel retardation assay or DNA-binding dye (such as ethidium bromide) exclusion assay. Alternatively, depending on the absorption spectrum of the complexing agent, circular dichroism studies can be more informative and detect interactions by analyzing the DNA structure and the angle of the DNA helix. These conformation changes allow for discrimination of minor and major groove binders. Second, assays that can provide information about the stability of formulations when subjected to conditions similar to those found *in vivo* can help to predict whether complexes can be administered intravenously or if further improvements are needed. Finally, evaluation of the particles' size is also relevant, as this might be limiting for the activity of the gene drug, depending on the target organ or route. Before continuing to *in vivo* characterization, cytotoxicity assays might also be required to determine the optimal concentration range to be used in order to avoid side effects. Provided that these studies are reproducible, these few assays grant the necessary information to carry on the *in vivo* evaluation of the carrier system. Owing to the lack of an *in vitro* system that is comparable to the *in vivo* settings, the authors believe that an evaluation of the delivery vector *in vivo*, at an early stage of development, is preferred.

Morphological studies, together with a wide set of *in vitro* cell work, may contribute to the understanding of the interaction mechanism as well as uptake, and possibly also the activity of the delivery vector. However, as the assessment is on an interaction-based system, few morphological methods are applicable that do not interfere with the original structure of the complexes. For example, negative staining electron microscopy requires ionic solutions that can interfere with DNA complexation, especially if the binding is relatively weak. Moreover, in atomic force microscopy (AFM), samples are often placed on mica surface, which may interact with lipidic components of the formulation, also disturbing the original structure of the DNA condensates.

3. Designing the formulation

Obvious relevant parameters for drug development are solubility and size. Physiological barriers such as endothelium and mononuclear phagocyte system might constitute impedance to the delivery of the cargo, because large-sized particles do not pass through the endothelium and/or might be opsonized and this way cleared from the body. Importantly, size must be considered in relation to the target organ and administration route. For example, the liver is an attractive organ owing to the wide variety of disorders that it is possible to target, but also because it is surrounded by discontinuous endothelium (with fenestrea of 50 – 100 nm). These fenestrea, similar to those found in bone marrow, might facilitate cell targeting and uptake, in contrast to continuous endothelium [34]. However, clearance by phagocytic uptake is a common fate, as larger particles will end up in Kupffer cells whereas smaller particles, ~ 100 nm, can be internalized by hepatocytes. As another example, on intravenous delivery the lung is probably the first possible target organ. Owing to the small diameter of capillaries and vascular beds, large particles can easily be retained in this organ, possibly inducing an immune response [35,36].

It has been reported previously that large positively charged particles resulted in lung embolism and consequent death of the treated mice [37,38]. On the other hand, retention of polyplexes in the lungs is not necessarily synonymous with toxicity but, perhaps, closely related to the properties of the delivery system. As an example, for pDNA delivery but also for quantum dots, it has recently been suggested that the particles first accumulate in the lungs but later continue into the bloodstream and locate to the liver when using conventional PEI-based delivery systems [36,39].

In the context of non-covalent systems that are based on charge interactions, buffer solution plays an essential role for size, stability and surface charge properties of the carrier. As reported by Brissault and collaborators, PEI/DNA particles present different sizes when formulated in the presence of salt (0.5 HBS, 150 mM NaCl) (visible formation of aggregates) and in salt-free glucose 5% (particles had an average diameter of 100 nm) [35]. The appropriate range of size and stability also varies depending on the administration route as an implication of the fact that the components of the bloodstream represent barriers to delivery that are not found on local application. Moreover, by loco-regional administration

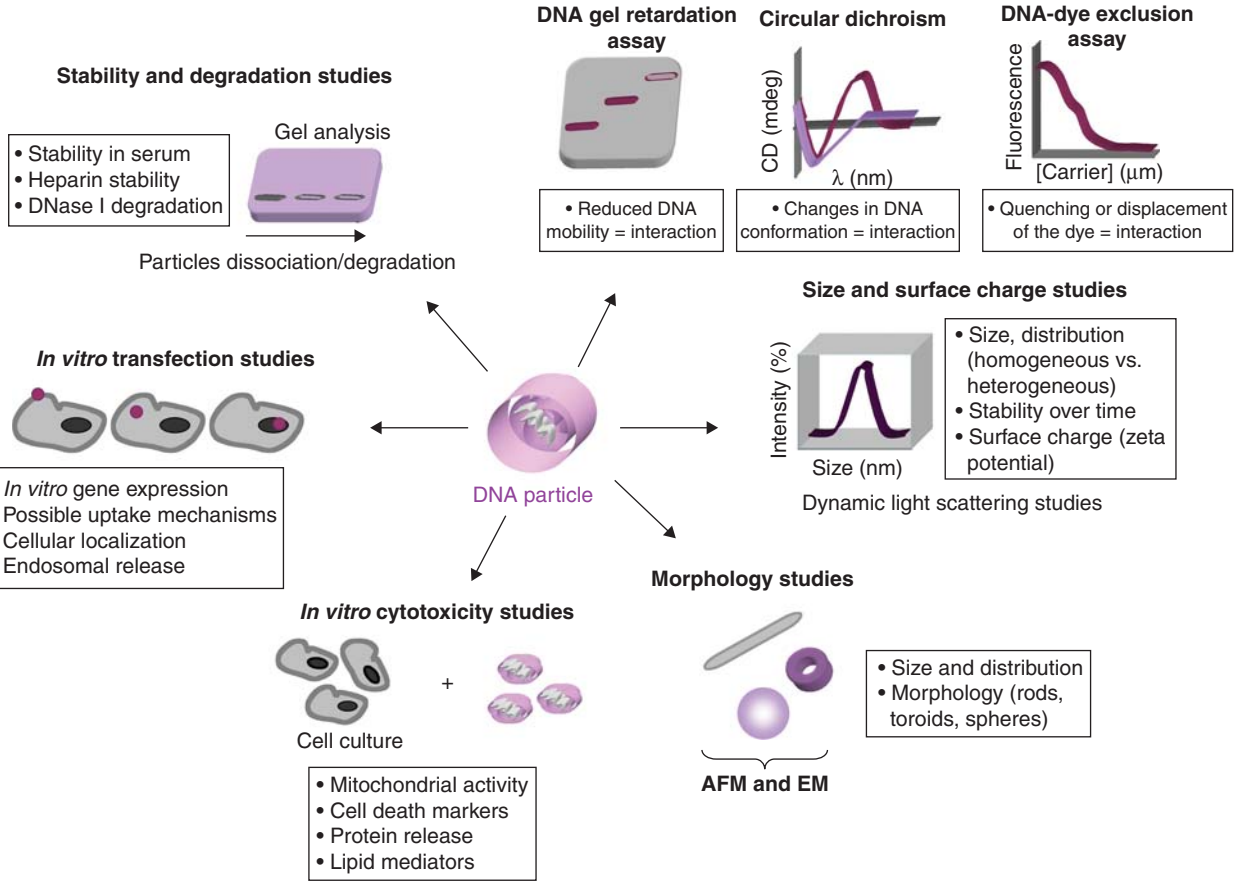

Figure 3. Pre-*in vivo* studies: physicochemical and morphological characterization of the nanovector. The figure illustrates the most commonly used methods for *in vitro* characterization of the delivery vector.

the active drug is often confined to a restricted region whereas in the blood it is subjected to dilution. Possible exceptions are subcutaneous and intramuscular injections that can function as a depot, and some particles may be able to diffuse slowly into the bloodstream. Accordingly, intravenous (i.v.) delivery often involves the use of larger doses, frequently prepared at higher drug concentrations. As a consequence, systems that proved valuable for local application may not suit intravenous delivery unless modified further. All together, these facts highlight the importance of optimizing a vector system in relation to the target organ and administration route. Also, size and surface charge might also play an important role in the route of cell internalization [19].

It is well established that on formation of nanoparticles those that are uncharged tend to aggregate in solution [40]. One possible way to circumvent this problem is by the inclusion of 'stabilizers' in the formulation [41]: amino acids, polyols (glycerol, mannitol [12]), chelating agents and sugars such as glucose [37,42-44] and dextrose [45,46]. Owing to their high content of hydroxyl groups, sugars help to stabilize the formulation by building a low-scale steric hindrance effect that prevents, to some extent, the flocculation of particles. This steric hindrance effect is probably the explanation for why PEI/DNA particles had an average size of 100 nm when formulated in 5% glucose [35]. On the other hand, the aggregation of particles in highly ionic-rich buffer was most probably a consequence of the small Debye length (the distance in which mobile charge carriers screen out the external electric field) resulting from the high concentration of salts. This aggregation was thereby favored by the screening of charge repulsion owing to this effect. Most probably, once in the bloodstream particles aggregate by binding to plasma proteins, but to what extent is unknown, therefore it is difficult to correlate size and activity. Only a few studies have reported on the size of the polyplexes in the presence of 10% serum [37], and one likely explanation for this is the inherent limitations of the dynamic light scattering technique. For the reason that serum proteins can aggregate into large particles, the actual population corresponding to the DNA complexes might be overlooked. Yet, it has been found that OptiMEM containing 10% fetal calf serum (FCS) presents a population distribution by intensity localized between 10 and 100 nm, with 38 nm as the average size [47]. When PEI-modified polyplexes formulated with

splice-correcting oligonucleotides were added to 10% FCS-containing medium, a second population distribution emerged, clearly distinct from that of the OptiMEM serum-containing solution. The authors, surprisingly, found that tyrosine-modified PEI (PEIY)/DNA particles had a smaller average size when formulated in serum-supplemented media as compared with in serum-free media. This effect is speculated to result from the stability provided by the amphiphilic nature of the polymer [48] and its self-assembly properties. Notably, these results suggest that polyplexes interact and behave differently depending on their surrounding media composition (e.g., salts or proteins).

4. *In vivo* studies

4.1 Intravenous administration

For intravenous administration an increased circulation time is important not only because it allows for an even distribution but also for optimal tissue targeting of the therapeutic agent. Once in the blood pDNA is immediately degraded by nucleases and hence DNA protection is required (**Figure 2**). Interaction with blood proteins is another physiological barrier that a drug carrier can encounter when in circulation (**Figure 2**). Positively charged nanoparticles will be rapidly cleared by the mononuclear phagocyte system, as they interact with proteins of opposite charge, thereby forming large aggregates [49]. pDNA formulation serves to provide stability and to prevent the nanoparticles from being recognized by the *in vivo* system as cell debris or pathogens and thus cleared. To avoid interactions the particles should be slightly negatively charged or neutral, such as the polylipid nanocomplexes developed by Jian Wu and co-workers for gene delivery to the liver [50-52]. If not neutral or with a discrete charge, nanoparticles can also interact with erythrocytes and induce hemagglutination [37,38,42,44].

As mentioned above, aggregation is a recurrent issue in vector formulation and one possible way to circumvent this problem is by the inclusion of 'stabilizers'. However, prolongation of the circulation time of the drug seems to require an extra element to provide for steric hindrance, that is, modification of the surface of polyplexes with hydrophilic polymers. Poly(ethylene glycol) (PEG) has been used extensively in drug delivery for stability purposes, in particular since 1990 when Klibanov and collaborators reported on the prolongation of the circulation time of liposomes using 'amphipathic poly(ethylene glycols)' [53]. At present, the selection of PEG molecules is rather wide – an assortment composed of different sizes and structures. Also, the PEGylation effect is dependent on the peptide/polymer/liposome, the density of PEG grafting, type of nucleic acid and environment [54]. The group of Thomas Kissel has done extensive work on the PEGylation of PEI, for delivery of siRNA as well as pDNA, and evaluated the biodistribution and toxicity [38,42,43]. In these studies they observed that particles composed of larger molecular mass PEGs were reduced in size as compared with those with smaller PEGs [55]. Regarding half-life in the blood, in general, most studies show enhanced circulation with PEG of molecular masses of 550, 2000 and 5000 [56-59]. Albeit being considered inert, repetitive administration of PEGylated carriers was found to elicit anti-PEG IgM antibodies, and hence accelerated blood clearance [60]. It is important to realize, nevertheless, that drug particles with prolonged half-life and negligible cell uptake are of limited interest, because they are inactive.

To address the problems associated with systemic administration of non-viral vectors, several groups have opted for approaches other than PEGylation. For example, to improve the *in vivo* delivery of PEI/DNA nanoparticles, Trubetskoy and co-workers developed ternary complexes, composed of DNA, polycations and synthetic polyanions. More specifically, polyacrylic acid was added to pre-formed DNA/PEI complexes. Studies performed in mice confirmed the necessity and function of the polyanion, as the survival rate of the animals increased with the charge ratio between the polyanion and the polycation (branched PEI) [44]. Similar, but more recent, is the work developed by Harris and collaborators [37]. These researchers used degradable poly(beta-amino ester) polymer instead of PEI, which has been shown to yield higher gene expression as compared with PEI [37]. The positive layer of the pDNA complexes was subsequently coated with poly-E, a peptidic sequence composed of polyglutamic acid attached to a linker, either containing or devoid of positively charged peptide. Coated particles were shown to be stable with a zeta-potential of ~ -10 mV that did not change in the presence of serum. Similar to Trubetskoy's work, Harris's uncoated nanoparticles also displayed some toxicity, which was, however, completely overcome with the surface coating.

Alternatively, David Oupicky and co-workers have developed the concept of 'lateral stabilization' according to which a modification of the surface of the polyplexes 'prevents the dissociation of complexes by molecules that otherwise penetrate through the polymer shielding layer' [61]. Interestingly, this effect is observed only when the polymer linked together with the molecules at the surface of the complexes is multivalent, as opposed to monovalent (like PEG). In fact, in this work the authors show that the blood circulation time of DNA/polylysine complexes is increased significantly when they covalently attach the polymer N-(2-hydroxypropyl) methacrylamide (PHPMA) as compared with PEG modification. Moreover, they have determined the minimum size of the PHPMA polymer to 30 kDa, in order to achieve the highest amount of DNA complex in the bloodstream.

Another attractive approach to stabilize the formulation is by the use of cholesterol [62,63]. Cholesterol commonly constitutes one of the lipidic components in liposomal formulations [45,50,51,64,65], whereas its use is not widespread in a non-liposome context. Interestingly, this lipid has been used as a stabilizing agent for systemic delivery of small oligonucleotides, known as 'antagomirs', first introduced by

Markus Stoffel *et al.* [66,67]. Cholesterol is also a component of the lipidoid-siRNA formulation, now being tested in clinical trials by Alnylam Pharmaceutics [63,68-70]. Aspects that support the use of cholesterol for delivery include the fact that these molecules are naturally occurring in the body and bind easily to serum components, thereby resulting in increased half-life of the cargo. Also, inclusion of lipid elements increases the membrane permeability of the nucleic acid, thus facilitating cell entry [13].

4.2 Functionalized carriers

Stable carriers lacking targeting functions frequently locate to the lungs, liver, spleen, bone marrow or kidney. Reasons for these preferences are related to the reticuloendothelial system (RES): liver, spleen, kidney and lungs are all RES-rich tissues [60]. In addition, the lungs are probably the first organs to be reached by the nanoparticles and adsorption to plasma proteins results in agglomeration, thereby trapping the particles in the vascular beds [49]. It is also known that liver, spleen and bone marrow are surrounded by a discontinuous endothelium that may facilitate uptake. To avoid destruction in the RES, targeted delivery to specific tissues is preferable. Size-wise DNA nanoparticles are comparable to viruses and their mechanism of cell internalization is likely to be common, by means of the endocytic pathway, such as macropinocytosis [71,72]. Hence, in addition to tissue-specific target entities, it might be of interest to consider elements that can actively trigger macropinocytosis and thereby enhance cell internalization (**Box 1**). Cell internalization of nanoparticles has been reviewed elsewhere [19].

Interestingly, several nanocarriers provided with stealth-like shield effects have been shown to accumulate preferably in highly vascularized tissues, a phenomenon referred to as enhanced permeability and retention (EPR) [64,73-75]. As a consequence, these nanovectors favor tumor targeting, where, in addition, blood vessels also often show increased permeability. Nevertheless, unambiguous targeting is still to be accomplished and, importantly, directed delivery reduces the dose requirements and hence possible side effects. Although most commonly used targeting entities consist of proteins and peptides, nucleic acids and carbohydrates have also been utilized (**Table 1**).

Tumors have been investigated extensively. In spite of their different origins and locations, the recurrent expression of certain genes and their corresponding proteins, such as various glycans or the epidermal growth factor receptor (EGFR), allows for tumor-specific targeting. The most common entities used include transferrin [76,77], epidermal growth factor, the integrin-binding tri-peptide Arginine-Glycine-Aspartate (RGD) [78-80] and, lately, aptamers (for the prostate tumor-associated antigen PSMA) [81,82]. Aptamers are described in more detail at the end of this section.

Brain targeting is one of the most intricate problems that researchers struggle with. Most current gene vectors do not cross the blood–brain barrier (BBB) after i.v. delivery and must be administered through intracerebral injection, which is highly invasive and does not allow for delivery of the genes to other areas of the brain. Alternatively, injections can be made into the cerebrospinal fluid. Intravenous delivery to the brain is greatly influenced by the size of the drug and its blood half-life. Formulations with a molecular mass >400 Da do not cross the BBB, and the addition of a targeting entity seems to be mandatory for successful delivery. Commonly used ligands for receptor-mediated uptake are insulin-like growth factors, transferrin or low-density lipid protein (LDL). Huang and co-workers have conducted several studies on brain uptake with the same basic vector, PEG-modified PAMAM, while varying the target entity [83-87]. These polymers conjugated to lactoferrin seemed to be the most efficient and have been further applied in models of Parkinson's disease. [83,85]. However, the levels of expression are therapeutically insufficient and this clearly highlights the present situation when targeting brain disorders. Targeting of the brain has been reviewed recently elsewhere [88,89].

A relatively large amount of work has been devoted to liver targeting [60,90,91]. Certainly, the central role of the liver together with one very well-characterized hepatic receptor, the asialolycoprotein receptor (ASGPR), was a strong motivation for the development of systems for specific uptake. In fact, the first *in vivo* ASGPR-specific gene transfer was published >20 years ago, in 1988, by Wu and Wu, where DNA was delivered by a galactose-terminal (asialo-)glycoprotein covalently linked to poly-L-lysine [92]. Among the most commonly used liver-targeting entities are carbohydrate-related molecules (galactose, asialofetuin and N-acetylgactosamine) and folic acid [60,93-95]. Recently, James Ludtke and collaborators have reported on a new possible target receptor for hepatocytes, the low-density lipoprotein receptor-related protein (LPR) [96,97].

At present it is possible to tailor and chemically synthesize several non-naturally occurring targeting molecules. As an example, Mukthavaram and collaborators have developed open and cyclic galactose head groups, to be attached to glycolipids, for selective mouse liver targeting. Although they have performed a competition assay with asialofetuin for the ASGRP receptor, it was not shown clearly that specifically hepatocytes, not Kupffer cells, were being transfected [98]. Indeed, once in the liver, Kupffer cells are the next barrier for the nanovector. One possible approach to overcome these cells is by reducing the dimensions of the complexes, as Kupffer cells preferably take up larger particles as compared with endothelial cells and hepatocytes.

Distinct from the traditional approaches are the aptamers, an emerging class of targeting molecules with surprising potential for *in vivo* application. They are often referred to as 'nucleic acid versions of antibodies' but with the main advantage of combining high binding affinity with low immunogenicity [99]. Aptamers can be easily synthesized in large quantities and at a relatively low cost. Furthermore, there is a large library of chemical modifications available that confers resistance to degradation and improved

Box 1. Macropinocytosis: an induced internalization process.

The endocytic pathway is divided into two main classes: pinocytosis (for fluids, solutes and receptor-mediated endocytosis) and phagocytosis (for ingestion of large particles). Phagocytosis occurs mainly in cells of the immune system as a clearing mechanism to eliminate cell debris or pathogens, whereas pinocytosis includes clathrin-mediated endocytosis (CME), caveolin-mediated endocytosis (CvME), macropinocytosis as well as clathrin and caveolin-independent routes. Targeted delivery of gene therapy cargoes by ligands, such as transferrin, is through CME. However, heparin-sulfate binding particles, such as delivery mediated by cationic CPPs [71] and recently by lipid-like materials [72], have been suggested to occur by means of macropinocytosis. Macropinocytosis can be induced, initiated by external stimulation involving growth factors and receptor tyrosine kinases actively engaging a series of kinases, actin filaments, Ras GTPases and Na^+/H^+ (sodium/hydrogen) exchangers [134]. This suggests that inclusion of elements in the nanovector that can trigger this uptake mechanism, for example, by interference with receptor tyrosine kinase or Ras GTPases signaling pathways, could be beneficial, and improve efficiency of gene delivery

Table 1. Nature of functional entities used for cellular or intracellular targeting.

Nature of the functional entity	Examples
Protein	Transferrin [76,77] Lactoferrin [83-85,133]
Peptide	Integrin-binding peptide RGD* [78-80] SV40 virus NLS# [16,18]
Carbohydrate	Galactose [60,94,98] Asialofetuin [93,95]
Nucleic acid	Aptamer [81,106,108] m_3G-Cap NLS# [17]
Lipid	Cholesterol§ [62]

*Tripeptide Arginine-Glycine-Aspartate.
#NLS, Nuclear localization signal.
§Cholesterol can be used for liver targeting by binding to human serum albumin or high-density lipoprotein. Such approaches have been reported for delivery of siRNA [62] as well as antisense oligonucleotides [66] using chemically coupled cholesterol. Size limitations may apply.

pharmacokinetics *in vivo*. These characteristics have been confirmed by the clinical trials reported [100-104]. Interestingly, in the case of gene therapy they have been preferentially applied to the delivery of short oligonucleotides, in particular siRNA [105-108]. Most of this preference is due to the simplicity of the conjugation of aptamer-siRNA, resulting in an increased stability and circulation time of the active component in a non-immunogenic and low-cost manner. These are essential requirements for clinical trials, and further production and commercialization processes. Aptamers have also been used to deliver cisplatin targeting prostate cancer. Similar to approaches used for delivery of pDNA, the aptamer was attached to nanoparticles of poly(DL-lactic-*co*-glycolic acid) (PLGA)-PEG-functionalized [81]. Thus, these oligonucleotides materialize as very powerful tools to be applied to plasmid DNA technology.

4.3 Other administration routes

In most cases, on local-regional administration the dilution effect that is present in the bloodstream is either prevented or decreased by the microenvironment created by the injection volume, as is the case of intradermal or intramuscular injections. Through this type of approach the vector also avoids direct interactions with blood proteins, decreasing the probability of particle clearance. The most prevalent local application sites include the muscle, epithelium airways and intratumoral and ocular delivery. Muscular transgene expression may serve several purposes, such as DNA vaccination [31], production and secretion of an active protein with systemic effects [109,110] and treatment of muscle-specific diseases [7,111-113]. Pluronic block copolymers have been used extensively for *in vivo* plasmid delivery, particularly to the muscle tissue, and are reported to be one of the most effective systems, resulting in 10- to 100-fold enhancement in pDNA expression [110,113-117]. The specific mechanism of action of these polymers remains to be characterized, as well as their interaction with nucleic acids. However, it has been described that Pluronics do not promote DNA retardation in gel migration assays, nor do they offer protection against DNase I degradation *in vitro*, which indicates weak interactions [114]. It has been suggested that Pluronics confer efficient *in vivo* delivery by providing colloidal stability to the formulation and facilitating cell entry by increasing membrane permeability [29,33,118,119]. Pluronics have also been used in combination with other carriers, such as PEI or polylysine, to improve delivery of unmodified parental polymers [29,120,121]. As mentioned already in this review, these polymers benefit from their easy tailoring ability and, therefore, distinct copolymers have been used as delivery platforms to different organs (heart, skeletal muscle, lungs, tumors and eyes).

Although local injections do not present the same type of barriers as the intravenous administration, most of the systems that have been used are either PEG-modified or contain similar large polymers that can provide steric hindrance of some sort [11,122]. Contrarily to this trend, the authors have recently shown that the conjugation of short fatty acids (from 4 to 18 carbons long) to spermine significantly enhanced plasmid delivery into skeletal muscle [12]. Interestingly, spermines modified with the shorter lipids, butanoyl and decanoyl, were the most promising and butanoylspermine resulted in enhancement of transgene expression comparable to that shown by some Pluronics. Similar to Pluronics, the activity of these fatty acid-spermines depends on the hydrophobic–hydrophilic balance. In addition, both carriers interact in a weak manner with nucleic acids, perhaps reflecting

the importance of efficient pDNA unpacking. Comparable tendencies were seen with deacetylated chitosan and the unmodified molecule [123]. In fact, chitosan has also been used for delivery of several types of drug [123] to different targeted tissues and, owing to numerous modifications, altering their molecular mass and degree of deacetylation, these agents can also be tailored [124]. Other advantages of this 'bioadhesive material' include the ability to extend the period of contact between the formulation and the targeted tissues and to increase cell uptake by facilitating the opening of tight junctions and the permeabilization of cell membranes [124].

Targeting the lungs, in particular the respiratory epithelial cells lining this organ, is still a challenge and many efforts are focused on improving the efficiency of vectors, especially owing to the high frequency of cystic fibrosis (CF) in Caucasian populations. Recently reviewed by Pringle *et al.* [125], two different systems have been proposed as promising for this purpose (apart from the cationic lipid GL67A, already tested and now undergoing a second round of clinical trials, and the PEG-modified 30mer-lysine peptide, also previously tested in CF patients). Unmodified-PEI 25 kDa, but not 22 kDa, was one of the candidates with high expectations. It was shown that on aerosol delivery these formulations resulted in efficient airway gene expression with no significant signs of inflammation [126]. Noticeably, these studies stress the relevance of the composition of the carrier and the method of administration for successful gene delivery with reduced side effects.

5. Conclusions

Overall, published studies have shown that for the development of plasmid-based technologies it is important to consider peptides/polymers that can confer protection and stability to the pDNA but that also allow for pDNA unpacking, necessary for gene expression. Recent developments suggest that this balance is easier to tune in a vector with amphiphilic properties that binds the nucleic acid in a moderate manner. Such properties can be achieved by modifying cationic peptides/polymers with hydrophobic amino acids or short fatty acids or, as in the case of chitosans and similar polymers, by manipulation of the degree of deacetylation. Properties such as size, stability and solubility of the formulation must be determined and demonstrated to be consistent in early stages of the vector development. For this purpose the use of glucose or dextrose at iso-osmotic concentrations (5%) is recommended. Subsequently, owing to the essential lack of correlation between *in vitro* and *in vivo* transfections, the authors believe that an evaluation of the *in vivo* effects of the carrier is beneficial for further advances. Differences between loco-regional and systemic administration cannot be ignored and properties for adequate behavior in the bloodstream are essential for efficient delivery. For systemic use further modifications might need to be considered to provide the carrier system with the appropriate stability. PEG is, without doubt, the best well-characterized polymer for this purpose, which may contribute to a quicker development of the delivery vehicle. However, a drug with a very long half-life in the circulation combined with a modest cellular uptake is of little interest and herein functionalization may allow for proper localization and desired activity.

Current knowledge suggests a balanced and moderate interaction between the carrier and the pDNA as one of the key factors to successful therapeutics. Further essential requirements are: i) the formation of particles with an optimal size of ~ 100 nm (considerably smaller sizes may be indicative of strong interactions and thereby hinder pDNA unpacking, whereas larger particles hamper tissue targeting); and ii) a stable formulation (resistant to external electrostatic influences). Finally, it is important to recognize that pDNA has properties very distinctive from RNA (single or double stranded) and short oligonucleotides. Not only the length but also the composition and three-dimensional structure of the nucleic acids are key aspects for the interaction between a carrier and the nucleic acid. For this reason these guidelines are not directly applicable to other nucleic acids.

6. Expert opinion

Characterization of the *in vitro* physicochemical properties is essential for the development of nanovectors in order to ascertain consistent formulation. Methods such as circular dichroism, DNA stability and cytotoxicity assays are straightforward. Less standardized are morphological studies as well as the detailed description of the nature of the peptide or polymer/DNA interaction. As opposed to liposome-based vectors, which have been characterized extensively (single or multilayered, with the DNA located inside the micellar core or in between the lipid layers), the structure of most DNA particles that do not result in micelle formation is generally unknown. Simulation studies could potentially provide some answers in this respect.

Mechanisms of cell internalization and intracellular localization of nanovectors are now better understood. However, cell and *in vivo* imaging systems accessible at present often require the use of fluorescent dyes that are likely to influence the results to an unknown extent. These limitations are also reflected in the lack of correlation between the *in vitro* and the *in vivo* studies. In fact, this deficiency largely contributes to the current impasse in non-viral gene delivery. As a consequence, the *in vivo* system remains a 'black box' in many respects – what happens to the delivery vehicle once it enters the bloodstream and why receptor-mediated uptake is often not very efficient remain essential, unanswered questions. A clarification of the *in vivo* processes could allow for a prediction of cause–effect events, thereby leading to improved design and formulation of the nanovector. In this regard, two-photon microscopy [127] is a recently developed imaging system that appears very promising for addressing some of these questions. Particularly interesting with this technology is the possibility to

monitor events in real time. Present limitations of this technique relate to the restricted width of the tissue sample to be analyzed and the available dyes that translate into restrictions in the type of tissue and organ that can be investigated. As an alternative to labeled nucleic acids or delivery vectors, *in vivo* gene expression can be monitored by bioluminescence when using reporter genes such as Luciferase [12]. The activity of the protein can be detected after administration of the substrate, allowing for: determination of the tissue targeted by the carrier; and an over time assessment of gene expression.

A stage in the process of pDNA delivery that appears to be frequently overlooked is the release of DNA from the nanocomplexes. It is believed that the pDNA can be liberated either in the nucleus or in the cytoplasm. In case of decompaction of the nanocarrier in the cytoplasm, the pDNA has yet to be transported to the nucleus through an unknown mechanism. This pathway presents two barriers that must be overcome: degradation by cytoplasmatic enzymes and nuclear entry. Alternatively, the plasmid can enter the cell nucleus in the form of nanoparticles and avoid cytoplasmic degradation. Although essential for activity of the therapeutic agent, this is also a poorly characterized event *in vitro*. A potential approach to provide more insights is intranuclear injections, where a smaller degree of DNA decompaction correlates to lower gene expression.

An alternative to pDNA, to treat loss of function mutations, has been suggested recently – mRNA [128,129]. By carrying mRNA as the therapeutic agent the nanovector decreases the complexity of the delivery process by avoiding the barrier of nuclear entry. Moreover, similarities in size and composition might allow the knowledge regarding the delivery of siRNA and antisense oligonucleotides to be applied in the mRNA technology.

As illustrated in this review, although some aspects of *in vivo* delivery remain to be addressed, several of the existing non-viral nanovector strategies already comprise several features that are essential for successful therapeutics.

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