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First Insights into Non-invasive Administration Routes for Non-viral Gene Therapy

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Abstract

Gene delivery has attracted increasing interest as a highly promising therapeutic method to treat various diseases, including both genetic and acquired disorders. However, its clinical application is still hampered by the lack of safe and effective gene delivery techniques, as well as by the need of non-invasive routes of administration in gene delivery platforms. Among the different approaches used to transport nucleic acids into target cells, non-viral vectors represent promising and safer alternatives to viruses. Non-invasive administration routes are currently being studied, such as intranasal administration to target the brain, topical retinal administration for ocular diseases and aerosolized formulations for inhalation for the treatment of pulmonary diseases. Reasonable evidence suggests that future gene delivery systems might be based on effective non-viral vectors administered through non-invasive routes, which would constitute a safe, easy to produce, cheap and customizable alternative to the current viral gene delivery platforms. In this review, after briefly introducing the basis of gene therapy, we discuss the up-to-date and possible future strategies to improve DNA transfection efficiency using non-viral vectors and focusing on the non-invasive routes of administration.

Keywords: Gene therapy, non-viral vector, intra- and extracellular barriers, non-invasive routes of administration

1. Introduction

1.1. Concept and historical evolution of gene therapy

Gene therapy can be broadly defined as the introduction of genetic material into target cells in order to modify and control protein expression for therapeutic or experimental purposes [1]. Nowadays, the culmination of the Human Genome Project along with recent advances in molecular biology have provided a better understanding of cellular and pathogenic processes, and several genes have been identified as targets for therapeutic approaches. Additionally, the constant advance in the development of gene carriers for the delivery of nucleic acids into target cells has led to conceiving new therapeutic strategies for the treatment of pathologies by genetic and cell-based approaches, collectively known as gene therapy [1].

Researchers have been working for decades to bring gene therapy to the clinic, but very few patients have received an effective gene-therapy treatment. The potential of gene therapy in medical applications was recognized soon after the discovery of DNA as genetic material, and the concept of gene therapy arose during the 1960s and 1970s [2]. The first success of gene therapy on humans arrived in 1990, it was performed by researchers at the National Institute of Health, and the treated disease was a form of severe combined immune deficiency (SCID) due to defects in the gene encoding adenosine deaminase (ADA) [3]. However, a fatal event in 1991 raised serious concerns about gene therapy. An eighteen-year-old boy died as a result of his voluntary participation in a gene therapy trial, becoming the first known human victim of this technology [4]. The Food and Drug Administration (FDA) investigation concluded that the scientists involved in the trial did not foresee serious side effects or fatality and that they did not follow the federal rules to ensure the safety of the participants [4]. This tragic case caused a severe setback in the research field of gene therapy.

According to data updated to June 2014 and presented by *The Journal of Gene Medicine* [5], since the onset of the first gene therapy clinical trial in 1989, more than 2000 new clinical trials for gene therapy have been approved globally (Figure 1). As shown in Figure 2, these trials address the most challenging diseases of today, that is, cancer (64.1% of approved trials), monogenic diseases (9.1%) such as cystic fibrosis, infectious diseases (8.2%) and cardiovascular diseases (7.8%). Although in a lesser extent, neurological diseases (1.8%) and ocular diseases (1.6%) are also subject to clinical trials with gene therapy. However, despite the intensive study during the last few years, at present only 0.1% of all the gene therapy products approved for clinical trials have arrived to the phase IV (Figure 3). In 2012, the European Medicine Agency approved for the first time a gene therapy product, Glybera, an adeno-associated viral vector engineered to express lipoprotein lipase in the muscle for the treatment of lipoprotein lipase deficiency [5].

1.2. The need of carriers

One of the main reasons why gene therapy clinical trials are still few in number is the lack of suitable and safe approaches to deliver the genetic material to target cells. The success of gene

therapy critically depends on suitable transfection vectors, which should be able to: (i) protect nucleic acids against degradation by blood and interstitial nucleases, (ii) promote internalization of the genetic material into target cells and (iii) release the nucleic acids once inside the cell to the correct site [1]. Furthermore, an ideal gene delivery system should be effective, specific, long-lasting, safe, easy to use and as inexpensive as possible [6]. Broadly, gene delivery vectors are mainly classified into two categories: viral vectors and non-viral vectors. According to data updated to June 2014 and presented by *The Journal of Gene Medicine* [7], among the over 2,000 clinical trials for gene therapy approved globally nowadays, 70% correspond to trials using viral vectors. As shown in Figure 4, there is a 17.7% of the gene therapy clinical trials that use naked DNA, and 5.3% of trials use lipofection [7].
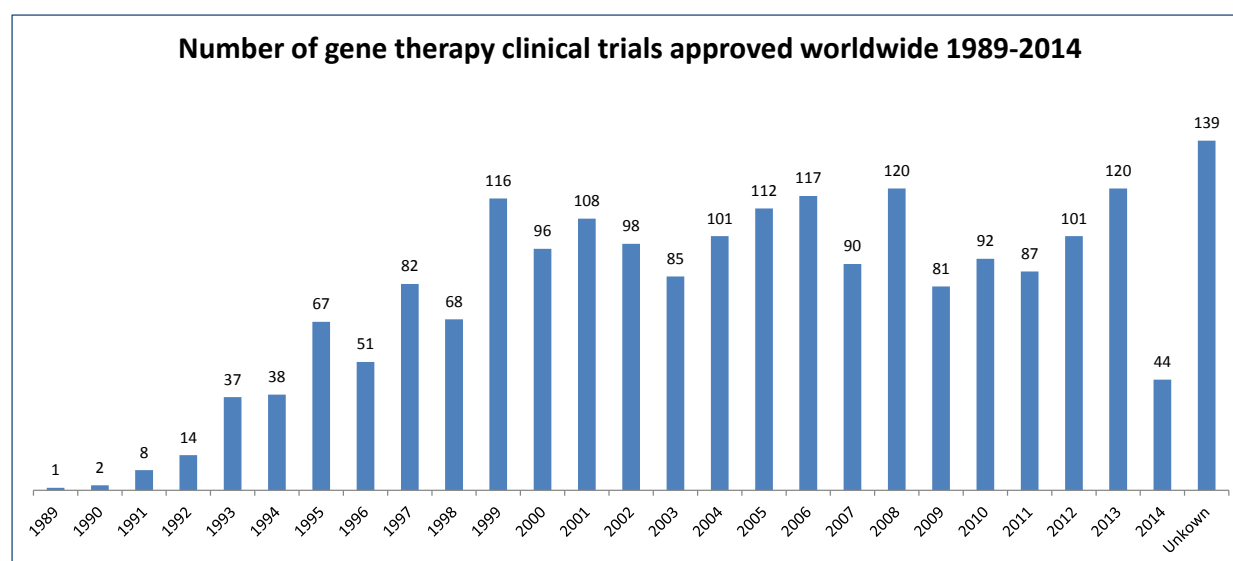

Figure 1. Number of gene therapy clinical trials approved worldwide 1989-2014 (adapted from http://www.wiley.co.uk/genmed/clinical).

Despite the still absolute predominance of viral-vector-based gene delivery platforms – which is due to their higher transfection efficiency – in clinical trials, non-viral vectors represent promising and safer alternatives to viruses. In addition, non-invasive routes of administration for gene delivery systems are currently being studied, such as intranasal administration to target the brain, topical administration on the surface of the eye to treat retinal inherited diseases and aerosolized formulations for inhalation for the treatment of pulmonary diseases. There is reasonable hope to suggest that future gene delivery systems might be based on effective non-viral vectors administered through non-invasive routes and that they would constitute a safe, easy to produce, cheap and customizable alternative to gene delivery platforms. Moreover, it is increasingly accepted that future gene delivery platforms may be based on multifunctional vectors specifically tailored for different applications [1].
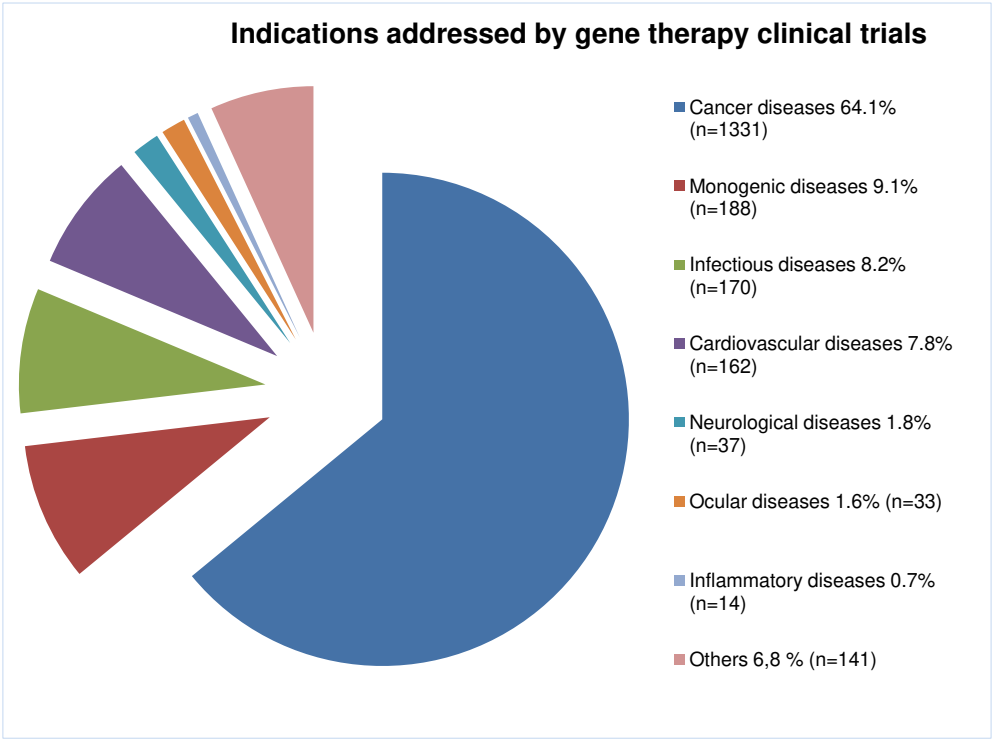
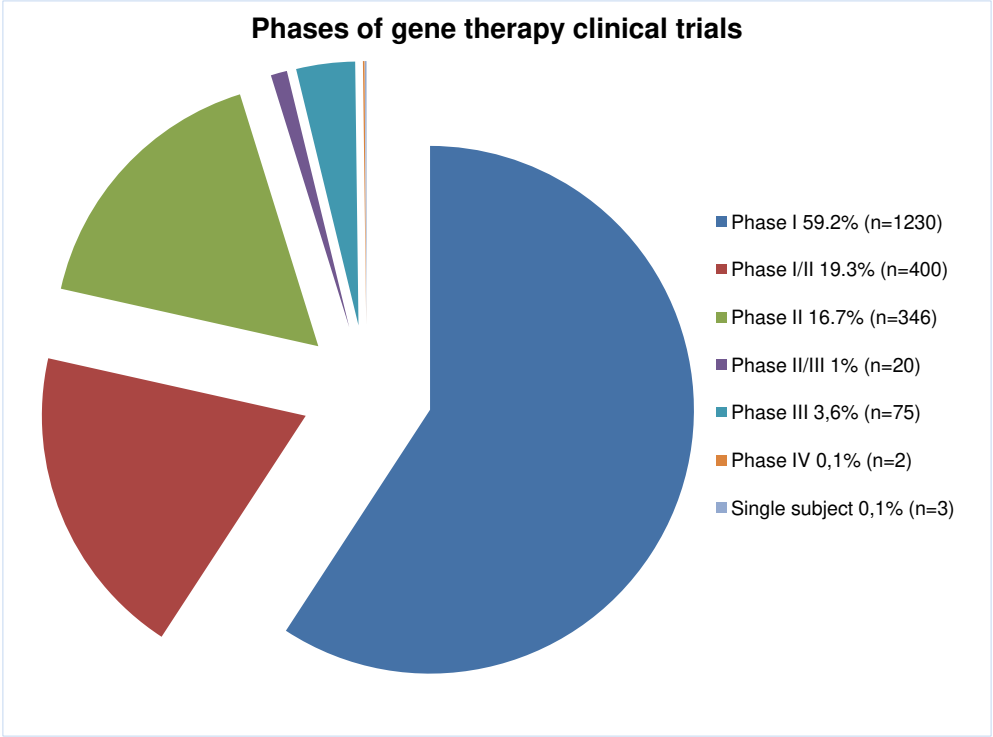**Figure 2.** Indications addressed by gene therapy clinical trials (adapted from http://www.wiley.co.uk/genmed/clinical).

Figure 3. Phases of gene therapy clinical trials (adapted from http://www.wiley.co.uk/genmed/clinical).

Vectors used in gene therapy in clinical trials

- ■ Adenovirus 22.8% (n=488)
- ■ Retrovirus 19.1% (n=409)
- ■ Naked/Plasmid DNA 17.7% (n=379)
- ■ Vaccinia virus 7.6% (n=162)
- ■ Adeno-associated virus 5.5% (n=117)
- ■ Lipofection 5.3% (n=113)
- ■ Poxvirus 4.7% (n=100)
- ■ Lentivirus 4.2% (n=89)
- ■ Herpes simplex virus 2.9% (n=63)
- ■ Other vectors 10.3% (n=221)
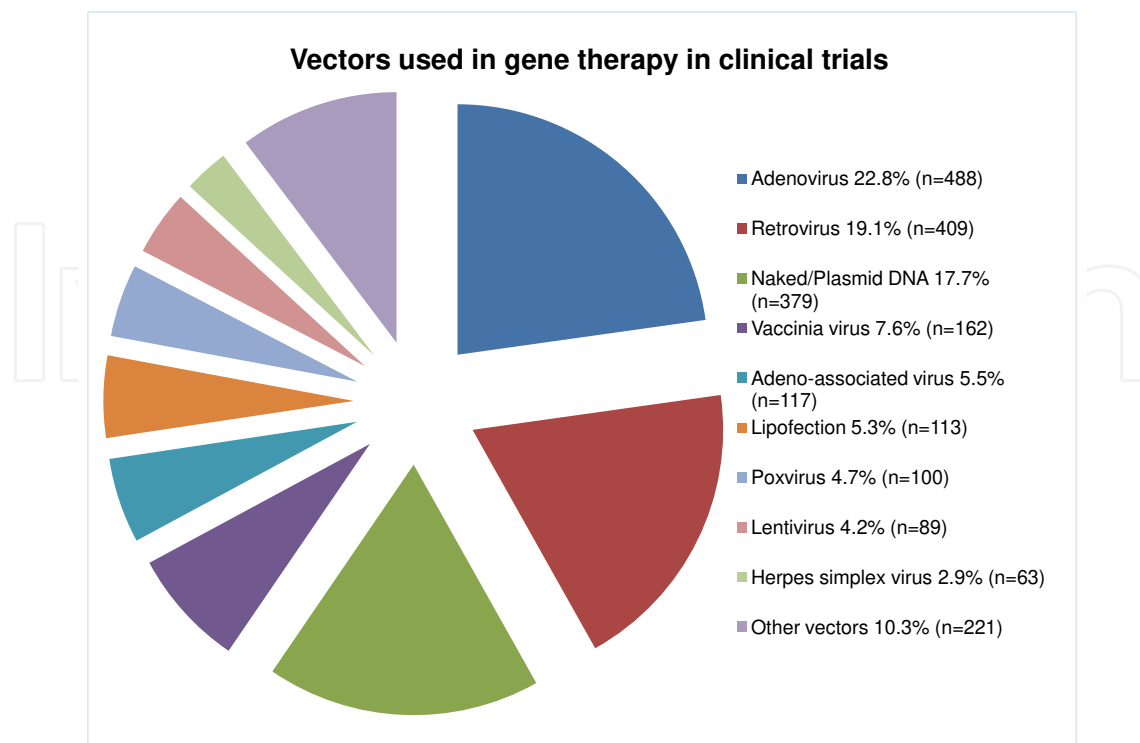
Figure 4. Vectors used in gene therapy in clinical trials (adapted from http://www.wiley.co.uk/genmed/clinical).

1.2.1. Viral vectors

In this review we will focus on non-viral-vector-based strategies for gene delivery platforms, but we will briefly discuss the most relevant aspects of viral gene therapy. Viruses are highly evolved biological machines that efficiently gain access into host cells, deliver their genetic material to cells and exploit the cellular machinery to facilitate their replication [8]. Therefore, viruses represent an excellent platform for the development of recombinant vectors containing foreign genes for gene delivery purposes [1]. However, viral vectors also present many impediments such as the low carrying capacity, the expensive and complex production and, most importantly, safety issues, since they can induce oncogenesis when randomly integrated in the host genome. In addition, the human immune system recognizes and combats viruses, shortening their effectiveness [1]. Table 1 summarizes the principal viral vectors used in gene therapy, as well as their main utilities and impediments. Viral vectors are, therefore, powerful tools but present important drawbacks for clinical use in humans.

1.2.2. Non-viral vectors

Non-viral vectors have emerged as a safer, cheaper and easier-to-produce alternative to viral vectors. In fact, non-viral vectors can be produced on a large scale with high reproducibility and acceptable costs, they are relatively stable to storage, they can be administered repeatedly with no or little immune response and the dimension of the genetic material they can carry is practically unlimited [1,9]. Nevertheless, the employment of non-viral gene delivery vectors is still strongly limited by their lower transfection efficiency as compared to viral vectors [1].

Viral vector	Integrative/ Episomal	Utility	Impediments
Adenovirus	Episomal	- Very efficient transfection in most tissues	- Induces inflammatory response
Adeno-associated virus (AAV)	Episomal (>90%)	- Not inducing inflammatory response	- Limited nucleic acid carrying capacity (<5 kb)
Retrovirus	Integrative	- Persistent gene expression	- Only transfects dividing cells - Risk of insertional mutagenesis
Lentivirus	Integrative	- Broad tropism - Persistent gene expression	- Risk of insertional mutagenesis
Herpes Simplex Virus-1 (HSV-1)	Episomal	- Large nucleic acid carrying capacity (up to 150 kb)	- Induces inflammatory response

Table 1. Principal viral vectors used in gene therapy, advantages and drawbacks. (Based on [8])

Non-viral vectors can be classified into two main categories depending on whether they are based on physical methods or on chemical methods. We will briefly review the most commonly employed non-viral DNA delivery systems for each category.

1.2.2.1. Physical methods

Physical methods for gene delivery purposes usually employ physical force to create transient membrane holes to cross the cell membrane and enhance gene transfer [6,10]. No particulate system is used to introduce the genetic material into the target cells [6]. Needle injection, ballistic DNA injection, electroporation, sonoporation, photoporation, magnetofection and hydroporation are the most utilized physical methods at present [6,11].

1.2.2.2. Chemical methods

Depending on the chemical feature, those methods can be classified into three groups: cationic lipids, cationic polymers and inorganic nanoparticles. Chemical vectors based on cationic lipids and cationic polymers form condensed complexes with negatively charged DNA through electrostatic interactions [10]. The complexes protect DNA and facilitate cell uptake intracellular delivery [10]. The principal characteristics of the non-viral chemical vectors are the following:

• *Vectors based on cationic lipids*

As shown in Figure 4, cationic lipid-mediated gene transfer or lipofection represents the most commonly used non-viral gene delivery system. Cationic lipids share four common functional

domains: (i) a hydrophilic head-group, which is responsible for the interaction with the DNA; (ii) a hydrophobic domain, which is usually derived from aliphatic hydrocarbon chains; (iii) a linker structure, which influences the flexibility, stability and biodegradability of the cationic lipid; and (iv) a backbone domain, which separates the polar head-group from the hydrophobic domain and it is usually a serinol or a glycerol group [12]. Changes in those domains can vary the transfection efficiency of different vectors elaborated with cationic lipids. The most employed cationic lipid formulations for gene delivery platforms are: (i) **liposomes** – vesicles made up of phospholipids; (ii) **niosomes** –non-ionic surfactant vesicles, with greater physic-chemical and storage stability than liposomes; and (iii) **solid lipid nanoparticles (SLN)** – particles with a solid lipid core, stabilized with surfactants [13].

• *Vectors based on cationic polymers*

Vectors based on cationic polymers are mostly spherical particles ranging in the size 1-1000 nm and they condense DNA into polyplexes preventing DNA from degradation [6]. The DNA can be entrapped in the polymeric matrix or can be adsorbed or conjugated on the surface of the nanoparticles [6]. The most popular cationic polymers employed for DNA delivery purposes are: (i) poly(ethylene imine) – PEI, which has an excellent buffering capacity; (ii) chitosan – a linear polysaccharide derived from the deacetylation of the natural chitin; (iii) cyclodextrins – a series of natural cyclic oligosaccharids; (iv) dendrimers – tree-shaped synthetic molecules up to a few nanometers in diameter that are formed with a regular branching structure; and (vi) Poly(L-lysine) – PLL, which can form nanometer-size complexes with polynucleotides thanks to the presence of protonable amine groups on the lysine moiety [6,13,14].

• *Vectors based on inorganic nanoparticles*

Inorganic nanoparticles are nanostructures varying in size, shape and porosity, and calcium phosphate, silica, gold, and several magnetic compounds are the most studied [6,15]. Inorganic particles can be easily prepared and surface-functionalized. They exhibit good storage stability and are not subject to microbial attack [6,16].

In summary, non-viral vectors for gene delivery represent a safer alternative to conventional viral vectors. However, although tremendous progress has been made in this field in recent years, the clinical application of non-viral-vector-based gene therapy is still hampered by the lack of effective gene delivery techniques. In the present review, we will discuss the up-to-date and possible future strategies to improve DNA transfer efficacy using non-viral vectors and focusing on non-invasive routes of administration. First, the intracellular barriers that non-viral vectors have to overcome and the strategies to improve the transfection efficiency in this regard will be described. Second, we will review the extracellular barriers that hamper an efficient gene delivery, as well as the invasive and the alternative non-invasive routes of administration that elude those barriers. Finally, challenges for non-viral vectors to reach clinical trials will be discussed, focusing on the transfection efficiency, the targeting and the duration of the transfected gene expression.

2. Intracellular barriers and strategies to improve transfection efficiency

A key factor conditioning transfection efficiency is the ability of the gene delivery system to overcome the intracellular and extracellular barriers. In this section, we will describe the main intracellular barriers that gene delivery systems must overcome to reach an efficient transfection and the different strategies used for this purpose. Intracellular barriers involve all the obstacles that a gene delivery system must overcome from cell surface association to nuclear entry in target cells. The knowledge of the molecular features that command all these processes for the non-viral vectors and the overcoming of these hurdles are mandatory issues that need to be deeply considered in order to design efficient gene delivery methods. In this section, we will review the cellular uptake pathways and intracellular trafficking of non-viral vectors and we will discuss the existing methods to enhance the endosomal escape and the nuclear entry, which are the principal strategies to achieve an efficient transfection.

2.1. Cellular uptake pathways

Cell surface association is the first intracellular barrier that non-viral gene delivery platforms need to overcome and it can directly influence the next intracellular fates of the non-viral complexes [17]. Cell-binding interactions of non-viral vectors can be receptor-independent or receptor-mediated. Receptor-independent cell surface association occurs by electrophilic attraction between the positively charged non-viral complexes (i.e. cationic lipoplexes and cationic polyplexes) and the negatively charged cell surface proteoglycans [18]. This binding method can efficiently transfect many cell types *in vitro*, but therapeutic potential *in vivo* requires additional refinement. In fact, in order to specifically deliver a gene into a target tissue *in vivo*, non-specific cell binding would require very high and potentially toxic doses of the non-viral vector. The addition of cell-specific ligands or antibodies to the vectors reduces this problem, allowing the use of lower and safer vector doses and promoting tissue targeting [18]. For instance, transferrin (Tf), which is an iron-transporting protein, has been used to achieve brain delivery in view that the Tf receptor is expressed in neurons and in the capillary endothelial cells of the brain-blood-barrier (BBB) [19]. Ligand choice not only depends on the cell type being targeted, but it is also important to consider the type of cell entry pathway that will be induced after ligation. As discussed in the following section, the endocytic pathway used by the vector can depend on the targeting ligand.

Once bound to the cell surface, non-viral vectors need to cross the plasma membrane to enter the cell and initiate the intracellular trafficking to enter the nucleus. The cellular uptake of macromolecules and solutes into membrane-bound vesicles derived by the invagination and pinching off of pieces of the plasma membrane is known as endocytosis [20]. There are four principal endocytic pathways: clathrin-mediated endocytosis (CME), caveolae-mediated endocytosis (CvME), phagocytosis and macropinocytosis [17,21]. These endocytic pathways are described below.

2.1.1. Clathrin-mediated endocytosis

Clathrin-mediated endocytosis (CME) is a highly regulated and energy-dependent process, and it constitutes the major and best characterized endocytic pathway [20]. The first step in

CME is the strong binding of a ligand to a specific cell surface receptor. This triggers the localized accumulation of clathrin structures on the cytoplasmic surface of the plasma membrane, which helps to deform the membrane into a coated pit with a size about 100-150 nm [22]. As the clathrin lattice formation continues, the coated pits become deeply invaginated and they finally pinch off from the plasma membrane to form intracellular clathrin-coated vesicles (CCVs) [20]. The clathrin coats then depolymerize, resulting in early endosomes. A kind of GTPase named dynamin is necessary for the vesicle fission from the plasma membrane [23]. Cholesterol seems to be also important for CCV formation because its depletion impedes the coated pits to pinch off from the plasma membrane [24].

In the next step of the CME pathway, the endocyted vesicles internalized from the plasma membrane are integrated into late endosomes and those then deliver their cargos to lysosomes [25]. During maturation from early to late endosomes, proton pumps located on the endosome membrane produce the acidification of the compartment, and there is a further reduction to pH 5 in the progression from late endosomes to lysosomes [20]. The acid pH in endosomes seems to cause the dissociation of the ligands from their receptors. Most authors state that, in the absence of an endosomal escape mechanism, non-viral vector/DNA complexes are retained and degraded in the lysosomes due to the acid environment and the enzymatic activity in these compartments. The final result is that DNA molecules have little or almost no access to the nucleus [20].

Some authors suggest that in some cases, depending on the formulation of the non-viral vector, the CME pathway might be the most suitable to achieve a high transfection efficiency because the lysosomal activity facilitates the cytosolic release of nanoparticles and enhances the nuclear entry of DNA [26]. Depending on the composition of the vector, the most appropriate internalization mechanism may be modulated [26]. Therefore, it is crucial to have a comprehensive understanding of the cellular internalization pathways of non-viral gene delivery systems.

2.1.2. Caveolae-mediated endocytosis

Caveolae-mediated endocytosis (CvME) begins in membrane microdomains called caveolae, which are small, hydrophobic, and cholesterol- and sphingolipid-rich smooth invaginations [17,20]. As well as CME, CvME is a type of receptor-mediated and dynamin-dependent pathway in which cholesterol also plays an important role [27]. The main difference between the clathrin- and the caveolae-mediated pathways is that in CvME there are no endosomes. Instead, internalized molecules go into intracellular vesicles called caveosomes, which do not fuse with lysosomes and, therefore, the potential degradation process of the DNA is avoided in this pathway [17]. In fact, CvME is generally considered a non-acidic and non-digestive internalization pathway, meaning that the internalized molecules can be directly transported into their intracellular target sites without being degraded in lysosomes [17,28]. Nevertheless, this issue is still under debate because some authors have recently reported that sometimes caveosomes join the classical endocytic pathway, in which they eventually fuse with lysosomes [29]. Therefore, in this regard further evidence is needed in order to understand the relationship between caveosomes and lysosomes.

2.1.3. Phagocytosis

Phagocytosis is a special type of endocytic pathway that is primarily used by professional phagocytes such as macrophages, monocytes, neutrophils and dendritic cells, although other cells might use it too [17]. Cup-like membrane extensions larger than 1 μm mediate the phagocytic pathway, and it is usually employed by cells to internalize large particles such as bacteria or dead cells, although large lipoplexes and polyplexes can also be internalized through this pathway [17].

Phagocytosis usually involves three steps that are common to all molecules internalized through this pathway, including non-viral vectors. First, non-viral vector/DNA complexes are recognized by opsonins and, therefore, opsonized, in the bloodstream. Second, the opsonized complexes bind to the surface of macrophages through the interaction between macrophage receptors and the constant fragment of particle-adsorbed immunoglobulins [17]. Antibodies lacking the constant fragment can be employed in non-viral gene delivery systems to prevent their recognition and clearance by macrophages *in vivo* [30].

Finally, the union of the molecule or non-viral/DNA complex to the macrophage receptors activates Rho-family GTPases, which trigger actin assembly and cell surface extension formation [17]. The complexes are ingested by the macrophages when the surface extension zippers up around them [20]. The vesicles internalized in the cells though the phagocytic pathway are called phagosomes and they usually have a diameter of 0.5–1.0 μm [20]. The phagosomes carrying the internalized complexes form mature phagolysosomes when they fuse with lysosomes, where the complexes undergo an acidification process [20]. In view of the fact that the intracellular fate of the phagocytic pathway is the fusion with lysosomes, the nucleic acids carried in the non-viral vector complexes will probably be degraded in this internalization pathway [31].

2.1.4. Macropinocytosis

Macropinocytosis is an internalization pathway based on fluid-phase endocytosis, since it non-specifically takes up a large amount of fluid-phase contents [17]. Similarly to the phagocytic pathway, macropinocytosis also happens through the formation of actin-directed membrane protuberances. Nevertheless, here the protrusions do not zipper up the ligand-coated particle. Alternatively, as shown in Figure 5, in the macropinocytic pathway the protuberances fuse with the plasma membrane. Macropinosomes are also different from clathrin-coated vesicles (CCVs) and caveosomes, since macropinosomes have no coat structures and, even if they are heterogeneous in size, they use to be larger than 0.2 μm in diameter [32]. As well as the other endocytic pathways, macropinocytosis also depends on small GTPase proteins since they are necessary for the vesicle fission from the plasma membrane [17].

The connection between macropinosomes and lysosomes remains still unknown. In some studies, early macropinosomes have been reported to show the same markers as early endosomes, and late macropinosomes have been reported to present lysosome markers [17]. However, macropinosomes have been shown to present different intracellular fates depending on the cell type, even if the explanation of this event remains unclear, and they do not always fuse with lysosomes [17].

2.2. Endosomal escape mechanisms

As mentioned before, most non-viral vectors are internalized in the cells mainly through the clathrin-mediated endocytic pathway. The major problem here is the intracellular fate of the endosomes that fuse with lysosomes and this can potentially lead to the degradation of the nucleic acids. In order to avoid this effect, while taking advantage of the CME pathway for cellular uptake, several attempts have been made to promote the early endosomal escape of non-viral gene delivery systems.

Many pathogens, mainly viruses and bacteria, have evolved different mechanisms to promote endosomal escape when internalized in cells. Several endosomal escape agents derive from virus (i.e. haemagglutinin protein of influenza virus) and bacteria (i.e. diphtheria toxin), and some derive from plants (i.e, ricin), human (i.e. fibroblast growth factors) or animals (i.e. melittin form bee venom) too [33]. The understanding of the mechanism used by pathogens allows to design and to ameliorate endosomal escape strategies applicable to non-viral gene delivery systems. Nowadays, several synthetic peptides with specific sequences and length are designed (i.e. the amphiphatic Sweet Arrow Peptide), as well as specific chemical agents (i.e. the polymer polyethylenimine PEI) for endosomal escape induction [33]. In the following paragraphs, the principal endosomal escape mechanisms are described.

2.2.1. Pore formation in the endosomal membrane

Pore formation is based on the interplay between a membrane tension that enlarges the pore and a line tension that closes the pore. Some peptides have a high affinity for the edge of the pore, and binding of those peptides to the edge of the pore produces a reduction of the line tension [33].

Some studies have reported that the union of cationic amphiphilic peptides to the lipid bilayer produces a strong internal membrane tension able to create pores in the lipid membrane [33].

2.2.2. pH-buffering effect (the proton sponge effect)

In this endosomal escape mechanism, the low pH of the endosomal environment leads to the protonation of the entrapped agents with a high buffering capacity. Protonation causes an influx of ions (H^+ and Cl^-) and water into endosomes, resulting in osmotic swelling and endosome rupture [33].

The proton-sponge effect has been observed in certain cationic polymers with a high H^+ buffering capacity over a wide pH range [34]. These polymers usually contain protonable secondary or tertiary amine groups with pKa close to endosomal/lysosomal pH. As explained before, during the maturation of endosomes, the membrane-bound ATPase proton pumps actively translocate protons from the cytosol into endosomes, causing the acidification of the endosomal compartments. At this point, cationic polymers with high buffering capacity become protonated and resist the acidification of endosomes, which results in more protons pumped into the endosome in an attempt to decrease the pH [34]. The proton pumping action is followed by passive chloride ions entry, increasing ionic concentration and, consequently,

water influx [34]. The high osmotic pressure produces the swelling and the rupture of endosomes, releasing their contents to the cytosol [34]. Histidine-rich molecules show a buffering effect upon protonation [33] and histidine can be included in non-viral vectors to enhance transfection efficiency by facilitating endosomal escape.

2.2.3. The flip-flop mechanism

This endosomal escape mechanism can be useful for endocytozed lipoplexes. Lipoplexes are endocytozed and become entrapped inside the early endosomes. There is an electrostatic interaction between the cationic lipoplexes and the anionic lipids of the endosomal membrane [34]. The anionic lipids of the endosomal membrane laterally diffuse into the lipoplexes and form charge-neutralized ion pair with cationic lipids of the lipoplexes, resulting in the nucleic acids being displaced from the lipoplexes and released in the cytoplasm [34].

2.2.4. Fusion in the endosomal membrane

This mechanism of endosomal escape is based on the destabilization of the endosomal membrane by water soluble and partly hydrophobic, and/or polybasic peptides known as cell-penetration peptides or CPPs. CPPs were originally derived from viruses, and they constitute short sequences of amino acids (10-30 residues) that use to be cationic and/or amphiphatic [34]. The main features of CPPs are their abilities to penetrate the cell membrane at low molecular concentrations without causing significant membrane damage and to internalize electrostatically or covalently bound biologically active cargoes (including proteins, peptides and nucleic acids) with high efficiency and low toxicity [35]. CPPs either form complexes with nucleic acids, through electrostatic interaction, or can be incorporated into polymeric and lipidic delivery systems [34]. To date, the internalization mechanism of CPPs still remains controversial, since there is evidence for both energy-independent and endocytic processes for cellular uptake of CPPs. Nowadays, it is generally accepted that endocytosis is the major internalization mechanism for most CPPs. However, it seems plausible that several CPPs utilize two or more cellular uptake pathways depending on the experimental conditions [35]. Further research would be needed in order to elucidate the exact uptake mechanisms and to identify the precise factors influencing these processes.

There are different criteria to categorize CPPs into different families. In general, CPPs can be classified into two categories [36]: (i) Cationic peptides that usually contain arginine and lysine residues; and (ii) amphiphatic peptides that consist of both hydrophobic and hydrophilic segments. Two examples of CPPs currently used to improve transfection efficiency of non-viral gene delivery platforms are the transcriptional activator protein or TAT (which belongs to the first category and was the first CPP identified, derived from the transcription activating factor of human immunodeficiency virus 1 (HIV-1)) [33,37] and the Sweet Arrow Peptide or SAP (which belongs to the second category and is a proline-rich amphipathic peptide of synthetic origin) [38].

2.2.5. Photochemical disruption of the endosomal membrane

Photochemical internalization (PCI) is a light-directed delivery technology that utilizes photosensitizers to facilitate the transport of membrane-impermeable macromolecules from endocytic vesicles into the cytoplasm [34]. Photosensitizers are usually amphiphilic compounds that can bind and localize in the plasma membrane. In this mechanism, photosensitizers bind to and localize in the plasma membrane, and they can be taken up by endocytosis together with the non-viral gene delivery systems. Photosensitizers are confined to the endosomal membrane and remain inactive until they are triggered by light with specific wavelengths matching their absorption spectra [39]. Once activated, they induce the formation of highly reactive oxygen species, causing the rupture of endosomes' and lysosomes' membrane. As a result, macromolecules that are trapped inside the endosomes/lysosomes can be liberated into the cytosol [34].

In general, the enhancement of endosomal escape is believed to be a crucial factor in non-viral-vector-based DNA delivery platforms. Different strategies for endosomal escape have different characteristics. A safe endosomal escape agent applicable in the clinic should have low immunogenicity and toxicity, high efficiency, ease of use and production, modular attachment of targeting ligands and the potential for cost-effective large-scale manufacture [33].

2.3. Nuclear import

In the previous section, we have seen several strategies suitable for non-viral gene delivery to avoid endosomal degradation of the DNA and to enhance its release to the cytoplasm. However, in order to achieve an effective transfection, the DNA molecules have to enter the nucleus. Here, we will discuss the principal strategies to transport DNA to the nucleus once released in the cytoplasm.

To enter the nucleus, molecules must pass through nuclear pore complexes (NPCs), which are multimeric structures with a central channel of 9 nm that prevents molecules with a molecular weight higher than 45 kDa from passively diffusing into the nucleus [40]. In the case of naked DNA, molecules smaller than 300 bp can passively diffuse into the nucleus, but larger DNA molecules, even when condensed by a non-viral vector, are excluded from the nucleus except when cells are undergoing mitosis [40-42]. During cell division, the integrity of the nuclear membrane is lost, which allows the nuclear entry of DNA-vector complexes within the daughter cells [21,40]. This is the case in the *in vitro* transfection with dividing cells, but *in vivo* transfection often targets slow dividing or terminally differentiated cells [21,40]. Therefore, the nuclear envelope cannot be neglected in *in vivo* situations, and there is considerable interest in improving the nuclear import efficiency of non-viral vectors [21,40].

Classically, proteins that are destined for the nucleus contain a nuclear localization signal (NLS), which is abundant in basic amino acids and it can be recognized by cytoplasmic proteins known as importins [17] that mediate energy-dependent transport through the NPC [40,43]. The same approach can be used to enhance non-viral gene delivery to the nucleus [21]. An NLS-containing vector can be added to the DNA-vector formulation or an NLS sequence can be directly bound to the DNA in order to promote its transport to the nucleus by the importins

[40]. In addition, highly basic polymers such as polylysine and protamine, the highly basic sequence of which resembles typical NLS sequences, have been used as potential agents to enhance nuclear targeting when complexed with DNA [40].
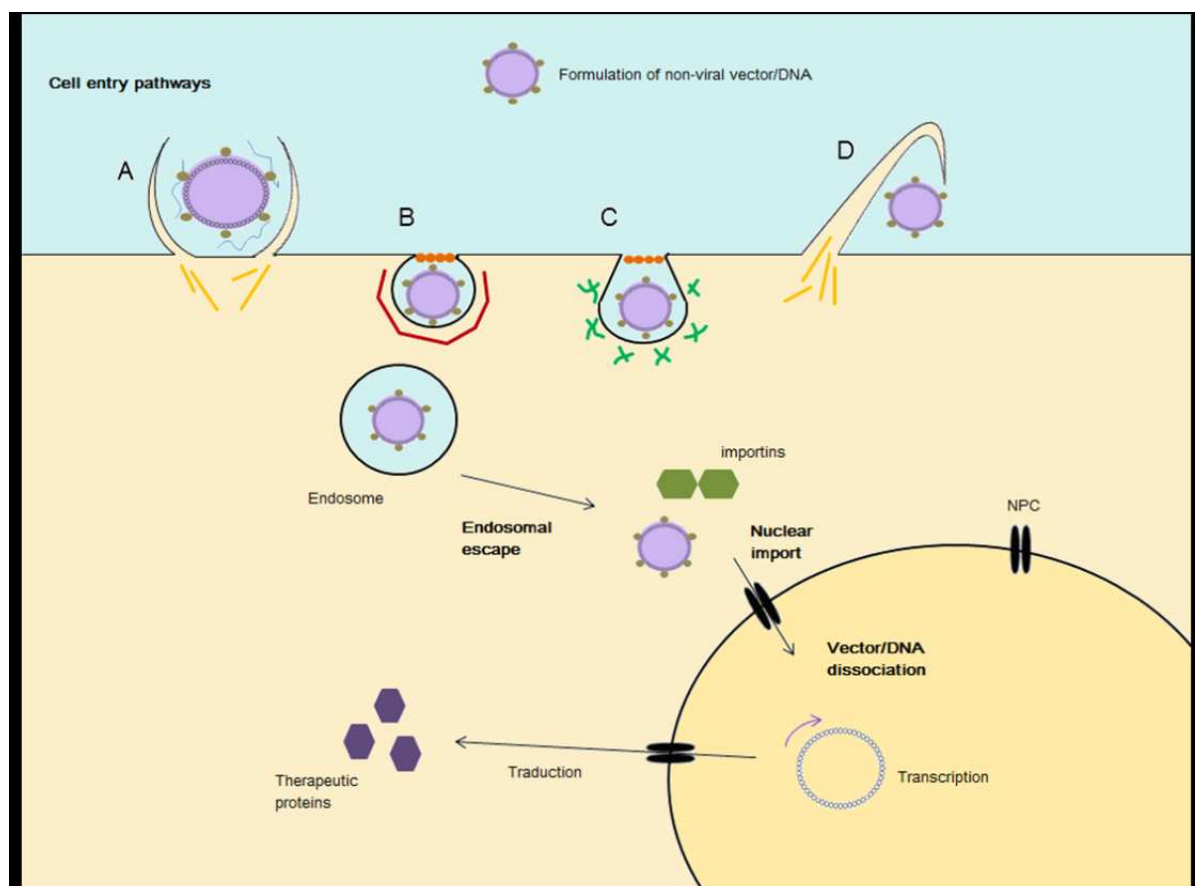

Figure 5. Cell entry pathways and intracellular trafficking of non-viral gene delivery systems. A) Phagocytosis. B) Clathrin-mediated endocytosis (CME). C) Caveolae-mediated endocytosis (CvME). D) Macropinocytosis. Internalized vector/DNA complexes following the CME pathway escape from endosomes and can be transported into the nucleus by the importins if they contain an NLS. When vector and DNA dissociate from each other, the transgene is expressed in the target cells. Yellow filaments represent actin; red chain represents clathrin coat; green filaments represent caveolin dimers; orange little circles represent the small GTPase dynein; blue filaments represent opsonins. (Adapted from [23; 24])

Finally, it should be considered that, once inside the nucleus, the non-viral vector itself may constitute a barrier to transgene expression. In fact, the agent used to condense the DNA could potentially interfere with the access of the cellular transcription machinery to the transgene promoter, thereby reducing or preventing its expression [40]. Still, premature release of DNA from the vector may expose the DNA to enzymatic degradation before expression can occur [40,44]. For liposomal-based vectors, DNA displacement from the vector seems to be connected to endosomal escape, driven by the anionic lipids of the endosomal membrane that neutralize the charge of the cationic lipids in the liposomal formulation [40,45,46]. In contrast, polycation/DNA complexes appear to release from each other in the nucleus through exchange of the polycations in the complexes with the protein components of the surrounding proteins

[40,47,48]. However, it seems plausible that additional mechanisms other than competitive charge interactions may be involved in the dissociation of DNA from polycations, and a deeper understanding of chromatin remodelling mechanisms may shed further light on this issue.

In summary, non-viral vectors for DNA delivery systems must overcome several intracellular barriers from cell-surface association to nuclear entry and DNA release. Depending on the cell type and on the cellular internalization pathway, some intracellular barriers may differ. Most non-viral vectors are taken up in cells through the CME pathway, which presents some problems such as the acid environment of endosomal and lysosomal compartments and the risk of DNA degradation. Therefore, several endosomal escape agents and mechanisms are currently being studied to avoid DNA degradation and to enhance its cytosolic release. In addition, DNA molecules have to enter the nucleus for the transgene expression. The most employed strategy for this purpose is the incorporation of an NLS in the vector-DNA complex. Finally, once inside the nucleus or earlier during the endocytic pathway, the vector-DNA complex needs to dissociate in order to allow the transcriptional machinery of the cell to access the transgene promoter. Figure 5 summarizes the most relevant aspects described in this section.

3. Extracellular barriers to overcome. Non-invasive routes of administration

Depending on the administration route and the target organ, gene delivery systems must overcome several extracellular barriers *in vivo* before reaching the target cells. As mentioned earlier, cancer diseases represent 60% of all clinical trials in gene therapy; yet other pathologies such as infectious, neurodegenerative, ocular and pulmonary diseases merit special attention, which, in sum, represent 10% of clinical trials in gene therapy. The principal and most studied route of administration of non-viral gene delivery systems for those diseases is the intravenous administration. Here, vectors need to be properly designed in order to overcome all the hurdles this route presents. Moreover, when specific tissues need to be targeted, such as the brain, the eye or the lungs, additional extracellular barriers appear and vectors have to be able to surpass them too. In this section, we will review the principal systemic barriers following the intravenous administration of non-viral gene delivery systems, as well as the additional tissue-specific barriers DNA/vector complexes have to overcome. We will also describe several attempts that have been made in order to overcome those barriers using invasive and alternative non-invasive routes of administration. Many efforts are being conducted to achieve effective strategies for safe non-viral gene delivery platforms based on non-invasive administration routes.

3.1. Intravenous administration

For many cancer forms, and specially disseminated cancer diseases, treatment needs to be administered systemically. Thus, intravenously administered current gene delivery systems to treat cancer should be able to transport and deliver the genetic cargo into cancerous cells.

The principal challenge of systemically administered DNA is to resist the extracellular enzymatic degradation, since DNA is subject to enzymatic degradation from the point of entry. However, it is possible to considerably surmount this hurdle by condensing the negatively charged DNA with the positively charged non-viral vectors [13].

Secondly, the other major extracellular barrier in the systemic route is the non-specific binding of the non-viral vector/DNA complex, which has a net positive charge, with blood cells and serum proteins such as albumin, complements, immunoglobulins and fibronectin,which have a negative surface charge [49]. These interactions could potentially end in aggregation or dissociation of vector/DNA complexes, resulting in their rapid clearance and elimination by the reticuloendothelial systems [49].

A third obstacle in systemic delivery might be the colloidal instability of non-viral vector/DNA complex formulations in the extracellular environment, which can also result in the aggregation of the complexes [49]. Fourth, vascular system is an extracellular barrier to be considered since it limits the size of the nanoparticles that can pass through the endothelial cells, which are relatively small and have tight junctions [49].

Finally, the activation of the immune system by the foreign vector/DNA complexes is an extracellular issue to be taken into consideration as well. In fact, foreign synthetic vectors can also induce an inflammatory response and/or complement activation, and hydrophobic particles can be eliminated by mononuclear phagocytic system through opsonization [49].

In order to overcome all these systemic barriers, non-viral vectors should be structurally modified. Formulation of gene delivery vectors is a key factor in determining their bioavailability and transfection efficiency *in vivo* [49]. Some of the strategies that are being developed to improve the properties of nanoparticles in the extracellular environment are discussed below.

The most employed strategy to increase the stability of vector/DNA complexes is shielding the outer surface of complexes with poly(ethylene glycol) or PEG [50]. Because of its highly hydrophobic nature, PEG produces a steric barrier against nuclease degradation and aggregation of nanoparticles in blood circulation [49]. However, despite the promising results, some difficulties exist in conjugation of PEG to gene delivery systems. PEGylation could decrease binding ability of non-viral vectors to DNA causing instability of lipo- or polyplexes in blood circulation. It may also affect the binding of vector/DNA complexes to receptors on the cell membrane [51]. Moreover, PEGylation can induce accelerated blood clearance due to activation of splenic synthesis of anti-PEG IgM antibody after first injection, resulting in the opsonization of the subsequent doses [49]. The length and the degree of PEGylation can also affect the ability of DNA condensation and biodistribution of gene carriers *in vivo*, and the optimal PEG length and content depends on gene carrier systems [52].

Consequently, considerable research has been made with the aim of compensating the negative effect of PEGylation in non-viral gene delivery systems. As reported in a recent study, one possible solution is to replace PEG by some hydrophilic polymers such as poly(N-vinyl-2-pyrrolidone) (PVP), poly(4-acryloylmorpholine), or poly(N,N-dimethylacrylamide) [53]. Coating of nanoparticles with these polymers led to extended residence of the nanoparticles

in blood circulation in rats, although they had a shorter half-life than the PEG-coated nano-particles [53]. Other strategies include providing stability against serum compounds and enzymatic digestion using copolymers of poly(L-lysine) and poly(2-methyl-2-oxazoline) [54], the pH-sensitive shielding of DNA polyplexes or lipoplexes (e.g. with PEG-acetal-MAL or maleimide moiety) [55], the use of enzymatically cleavable PEG linkers (e.g. PEG-peptide-DOPE or PPD that is cleaved in a matrix metalloproteinase-rich environment) [56], or the production of reducible PEG nanoparticles (e.g. PEG and chitosan bound through disulphide bridges) [57].

Besides PEGylation, other chemical and structural modifications can be applied to gene delivery agents in order to overcome the systemic barriers. In the case of cationic lipid-based non-viral vectors, incorporation of cholesterol can stabilize lipoplexes against binding to red blood cells [49]. In the case of cationic polymer-based non-viral vectors, conjugation of lactose to chitosan polyplexes has shown excellent DNA binding ability, good protection of DNA from nuclease, and the suppression of self-aggregation and serum-induced aggregation [58]. Therefore, current research has focused on multifunctional and diverse non-viral gene carriers that can be adjusted for each particular condition.

3.2. Targeting specific tissues: additional extracellular barriers

When targeting specific tissues, vectors have to be able to surpass additional barriers as well. Here, we will focus on specific extracellular barriers present in gene delivery to the central nervous system (CNS), to the eye and to the lungs. The invasive and alternative non-invasive routes of administration that avoid those barriers will also be discussed.

3.2.1. Gene delivery to CNS

The CNS possesses particular anatomical and physiological properties that make gene delivery to CNS specially challenging. The CNS is protected by the blood-brain-barrier (BBB), which consists of tightly joined capillary endothelial cells [19], and it is considered to be impermeable for almost 100% of the macromolecular drugs and over 98% of small molecule drugs [59]. The spinal cord is part of the CNS and it is protected by the blood-cerebrospinal fluid barrier (BCSFB), which is constituted of choroid plexus epithelial cells and restricts the free diffusion of molecules into the cerebrospinal fluid (CSF) [19]. Transport into the CNS of essential nutrients, such as glucose and amino acids, occurs through specific receptors present in the BBB and the BSCFB [19]. Within the CNS, distinct cell types exist including neurons and different types of glial cells; neurons are particularly challenging to transfect and it is thought this is attributable to their post-mitotic nature, their complex structure and the complexity of neuronal networking [19].

Most of the strategies to cross the BBB upon systemic administration of non-viral vector/DNA complexes exploit receptor-mediated uptake of molecules such as transferrin (Tf), lactoferrin and insulin, since receptors of those molecules are expressed on many cell types, including neurons and the capillary endothelial cells of the BBB [19]. By attaching a ligand for those receptors to the non-viral delivery system, one can enhance the transport of the vector/DNA

complex towards the CNS. Another strategy known as 'Molecular Trojan Horse' uses peptidomimetic monoclonal antibodies that are designed to target specific receptors on the BBB and induce receptor-mediated transcytosis of the non-viral delivery system into the CNS [60]. Other approaches investigated for CNS delivery of conventional pharmaceutics upon systemic administration include transient mechanical disruption of the BBB and RNAi-mediated knockdown of tight junction proteins [19].

The ultimate goal for CNS gene therapeutics is delivery by systemic route, which is the most acceptable for clinical use. Nevertheless, in view of the high amount of extracellular barriers that vectors must overcome, many studies have attempted different routes of administration. To date, several pre-clinical studies have essayed local administration to the brain, either by injection or by infusion. However, even if local administration to the brain eludes the extracellular barriers to access the CNS, the need for brain surgery to infuse a gene therapy vector clearly limits the clinical applicability for this approach.

Intranasal delivery offers a novel and non-invasive means by which non-viral gene delivery systems can gain access to the brain. The mechanisms by which intranasally delivered substances enter the CNS have not been fully elucidated, but an accumulating amount of evidence suggests that substances can reach the brain through a combination of perineuronal, perivascular and lymphatic transport pathways. In addition, the prevailing nose-to-brain pathway will largely depend on the region where the delivered agent is placed within the nasal cavity and the physicochemical properties of the therapeutic being administered [61]. It is currently accepted that the intranasally administered substance can reach the CNS by three main pathways: (i) direct paracellular or transcellular transport via the olfactory neurons or olfactory epithelial cells ('olfactory neural pathway'), (ii) transport via the trigeminal nerves ('trigeminal pathway') or (iii) indirectly, *via* blood vasculature and/or lymphatic system ('systemic pathway') [62]. The nasal mucosa is highly vascularized, and the blood vessels allow passage of drugs following nasal administration in nano-drug delivery systems; however, the substance that has been absorbed into the systemic circulation has to cross the BBB in order to reach the CNS [61]. Following olfactory and trigeminal nerve pathways, drug is delivered to the olfactory bulbs and to more caudal brain areas, respectively [63]. Within the brain, pulsatile flow in perivascular spaces has been postulated to allow for widespread transport of molecules within interstitial fluid to sites deep in parenchyma [63,64].

Advantages of intranasal administration include ease of administration (non-invasive), rapid dose absorption via highly vascularized mucosa, large nasal mucosa surface area for dose absorption, avoidance of the gastrointestinal tract, first-pass metabolism and fewer side effects, among others [65]. Moreover, intranasal administration confers improved convenience and compliance compared to other more invasive routes and it allows self-administration [65]. Disadvantages of this route include that nasal congestion could interfere with dose absorption, that the amount of dose that reaches the CNS varies with each agent and that the frequent use of this route leads to mucosal damage [65]. In addition, the administered formulation can undergo rapid clearance from nasal cavity by the mucociliary system [59,66]. This latter drawback can be overcome by adding a mucoadhesive substance to the formulations. For non-viral-vector-based gene delivery systems, chitosan is an attractive excipient that can confer

both bio-adhesion and absorption properties, and it is the most widely investigated absorption enhancer material both in terms of efficiency and safety [63]. Chitosan is able to interact through its positively charged amino groups with the anionic counterpart present in the mucus layers, mainly sialic acid, and to affect permeability of the epithelial membrane by the transient opening of the tight junctions in the epithelial cells [67]. In a recent study, another substance, the non-ionic surfactant laureate sucrose ester has been also reported to be an effective intranasal absorption enhancer [68].

Recently, the first report that intranasal delivery of DNA nanoparticles can bypass the BBB and transfect and express the encoded protein in rat brain has been published, thereby affording a non-invasive approach for gene therapy in CNS disorders [63]. Authors demonstrated that intranasal delivery of unimolecularly compacted DNA nanoparticles, which consist of single molecules of plasmid DNA encoding enhanced green fluorescent protein (eGFP) compacted with 10 kDa commercial peptide (PEG-substituted lysine 30 mers or CK30PEG10k), successfully transfects cells and leads to the expression of the eGFP in the rat brain [63]. The results further suggest that the cells transfected within the brain are likely to be pericytes, and that the distribution of nasally administered substances occurs via perivascular transport [63]. Additionally, another recent study has reported brain (cortex and hippocampus) transfection upon intranasal administration of chitosan and polyethyleneimine (PEI)-coated magnetic micelles [69]. Even if those nanoparticles were able to reach the brain presumably because of a transient disruption of the BBB following mild traumatic brain injury, the results show that the intranasal route might be useful for targeting the brain. Although further optimization of the dose, dosing regimen and dose interval is needed to achieve appropriate levels of transgene expression [63], the promising results will certainly encourage the research in the field of intranasal administration of non-viral-vector-based gene delivery systems, which has clinical importance due to its non-invasive nature.

3.2.2. Gene delivery to the eye

The eye is an attractive target organ for gene therapy because of its unique characteristics. The tissue volume to be treated is small, the therapeutic concentration to be administered is relatively low and the diffusion of active products from the eye to the circulation is minimal [70]. In addition, the eye benefits from a relative immune privilege, minimizing the potential immune and inflammatory reactions that may follow the intraocular injections of foreign agents [70].

In general, gene delivery systems for eye diseases range from simple eye drops and ointments to more advanced bio- and nanotechnology-based systems such as muco-adhesive systems, polymers, liposomes and ocular inserts. Most of these technologies were developed for front-of-the-eye ophthalmic therapies and are not applicable as back-of-the-eye delivery systems [71].

When the systemic administration is used to target the eye, non-viral vector/DNA complexes must cross the blood-ocular-barrier (BOB) to reach the ocular tissue. This constitutes a real challenge, since the BOB is composed of tight epithelial junctions. Two principal strategies to overcome this barrier are the use of vectors smaller than 100 nm to allow intracellular passage

across the BOB [72] and the use of ligand-equipped vectors that recognize specific receptors in the BOB [73]. Therefore, even if the intravenous route permits the delivery of larger volumes of the formulations as well as repeated administrations, the therapeutic effect achieved by this method is often limited by the factors restricting the access to the eye.

Invasive methods such as intravitreal injection, subconjuntival injection and subretinal injection can bypass some of those barriers, and intravitreal and subretinal injections are currently considered as the most effective and common methods of gene delivery to retinal ganglion cells and to inner layers of the retina, respectively. However, these methods are very invasive and repeated gene delivery to the eye using such methods can cause further damage of the eye like retinal detachment, haemorrhages, and sub- or pre-retinal fibrosis [71]. Therefore, non-invasive and effective methods for ocular gene delivery are needed. In this regard, topical administration in the form of eye drops is a non-invasive delivery method that can be performed repeatedly with minimal side effects [71].

However, the non-invasive route of administration is perhaps the most ambitious goal because the barriers associated with topical gene delivery to the posterior ocular tissue are the most challenging. First, vector/DNA complexes have to surpass the tear film, which is an aqueous layer covered by lipids and underlined by mucin that covers the corneal and conjuctival layers [71]. This tear film restricts the bioavailability of applied formulations because of the tear turnover rate and the lacrimal and nasolacrimal drainage [85]. Strategies to overcome this barrier include addition of viscosity enhancers such as cellulose derivatives or thermoreversible poloxamer gels [71] and, most importantly, the incorporation of muco-adhesive polymers such as chitosan and hyaluronic acid derivatives in gene delivery systems [74]. Second, ocular tissue barriers such as the cornea, conjunctiva, sclera and choroid, contain epithelial tight junctions, proteoglycan matrices and fibril collagen networks within their structures, which contribute to restrict the passage of vector/DNA complexes to the neuroretina [71]. Finally, the vitreous is an aqueous biogel composed of collagen, hyaluronan and proteoglycans that hinders transfection of the retinal cells [71]. Strategies to overcome those barriers include all the above-mentioned methods from the use of vectors of suitable dimensions to the use of specific ligands and muco-adhesive polymers [71].

The majority of success in ocular gene therapy research thus far was accomplished for applications involving the anterior part of the eye, using mainly viral-based delivery systems and invasive delivery methods. Interestingly, effective gene delivery to the retina and retinal pigment epithelium using non-viral vectors has been recently reported; however, in this study magnetic nanoparticles were administered invasively, through intravitreal and subretinal injections [75]. Rather than in gene delivery, significant advances have been made in drug delivery systems to target the posterior part of the eye using non-invasive administration routes. For instance, in a recent study, it was reported that surface-modified submicron-sized lipid emulsions could be promising vehicles of hydrophobic drug delivery to the ocular posterior segment [76]. In that study, researchers performed surface modification of the lipid emulsions using a positive charge inducer and the functional polymers chitosan and poloxamer 407. Authors suggested that poloxamer 407 increased the lipid emulsion retention time on the eye surface by its adhesive properties, therefore enhancing gene delivery to the ocular

posterior segment. Additionally, another study has reported successful drug delivery to the posterior segment of the eye of rats and rabbits using annexin A5-associated liposomes [77]. Here, authors suggested that annexin A5 mediated endocytosis can enhance the delivery of associated lipidic drug delivery vehicles across biological barriers. Moreover, a novel study has reported the topical drug delivery to retinal pigment epithelium with microfluidizer produced small liposomes, which might be an attractive option for drug delivery to the posterior segment tissues of the eye [78]. It may be reasonable that some of the advances in drug delivery will be applicable for gene delivery systems as well, and they will probably inspire further strategies for non-invasive, non-viral gene therapy platforms aimed at targeting the posterior segment of the eye.

3.2.3. Gene delivery to the lungs

Pulmonary gene therapy is considered for the treatment of a variety of lung diseases like cystic fibrosis, asthma, emphysema and lung cancer [79]. Depending on the respiratory disease to be treated, the target cells in the lung can vary from epithelial cells, alveolar cells, macrophages, respiratory stem cells or endothelial cells [79]. Besides, the nucleic acid cargo needs to be delivered to cells in the target region of the lung. Nevertheless, this is severely limited by the pulmonary architecture, the presence of mucus, the clearance mechanisms and the activation of the immune system [79]. Inhalation, intranasal instillation, intratracheal instillation, and intratracheal intubation are techniques that can be used to administer materials of interest to the lungs. Considering the interest of non-invasive administration routes for clinical applications, aerosolized non-viral vector/DNA complexes for inhalation would be the ideal choice for lung gene therapy. There are many advantages to administering medications to the lungs as an aerosol, such as the high local concentration by delivery directly to the airways, and the pain- and needle-free delivery.

Respiratory secretions, which include mucus and alveolar fluid, are the most important extracellular barriers for lung gene delivery. Respiratory mucus is one of the most important defence mechanisms and it is mainly composed by a three-dimensional network of cross-linked mucin chains, which gives the mucus viscoelastic properties [79]. The major proteins in respiratory mucus are albumin, proteases, anti-proteases, immunoglobulins, lysozyme and lactoferrin, and the respiratory secretions of patients with cystic fibrosis or respiratory infections also contain huge amounts of DNA and actin [79]. The alveolar fluid is a thin continuous layer of pulmonary surfactant that covers the alveolar epithelium and it comprises phospholipids and specific surfactant-associated proteins [79].

Respiratory mucus can act as a barrier towards pulmonary gene delivery in several ways. The biopolymer network of mucus limits the diffusion of complexes by sterical obstruction or by binding the complexes [79]. Also, negatively charged and non-cross-linked macromolecules of mucus, as well as other components present in the mucus such as antibodies, can bind to the surface of vector/DNA complexes. These interactions may cause: (i) entrapment of the vector/DNA complexes in the mucus, (ii) aggregation of the complexes due to neutralization of their surface charges, (iii) release of the DNA cargo from the vector, and (iv) an inefficient cell binding of the complex due to shielding of their positive charges or their receptor binding

ligands [79]. Finally, the mucus blanket is continuously removed via mucociliary transport or coughing. Therefore, the vector/DNA complex should be able to cross the mucus before they are cleared from the respiratory tract. The diffusion coefficient of the complexes in the mucus, the thickness of the mucus layer and the rate of mucus clearance will determine whether the vector/DNA complexes will reach the epithelial cells [79].

Regarding the alveolar fluid as a barrier towards pulmonary gene therapy, the presence of this surfactant layer can inhibit transfection of cationic lipid-based vector/DNA complexes [79]. It has been suggested that this inhibitory effect results from disintegration of the lipoplexes by the negatively charged lipids present in the surfactant layer, leading to accessibility of nucleases to the DNA cargo and thus its degradation and loss of function [80]. On the other hand, non-viral vectors based on cationic polymers such as PEI might be more resistant to detrimental effects by pulmonary surfactant [79].

Several strategies have been developed in order to overcome the extracellular barriers of lung gene delivery. Size and surface properties of non-viral vector/DNA complexes have a pivotal role in determining their behaviour in respiratory secretions. Most efforts have been conducted to increase the mobility of vector/DNA complexes in respiratory mucus and to avoid interactions of complexes with respiratory secretions.

There are different methods to increase non-viral vector/DNA complex mobility through the respiratory mucus. One straightforward mechanism consists of adding mucolytic agents that hydrolyze mucins present in the mucus [79]. Also, it has been demonstrated that N-acetylcysteine and its derivatives lower the viscosity and elasticity of mucus by reducing the disulphide bridges between the subunits of mucins [81]. As future directions, research is focusing on functionalized nanoparticles with mucolytic agents able to cut a way through the mucus, enhancing their transport across the extracellular matrix [79].

On the other hand, the principal strategy to avoid interactions between vector/DNA complexes and components of biological fluids is the shielding of the complexes by modification with biocompatible hydrophilic but biologically inert polymers [79]. Shielding of vector/DNA complexes may not only be important to reduce interaction with mucus and alveolar fluid components but also diminish clearance by alveolar macrophages. For instance, shielding the positive surface charges of vector/DNA complexes with neutral hydrophilic polymers such as polyethylene glycol (PEG) favours their physicochemical stability and their gene transfer capacity [79].

A number of challenges must be overcome before pulmonary gene therapy becomes a reality, such as the development of gene vectors that can more efficiently penetrate the mucus barrier [82]. However, real advances have been made in recent years, novel aerosol therapeutic modalities are currently being investigated for lung cancer, and inhaled gene therapy has already presented safety and effectiveness in cystic fibrosis [83].

As summarized in Figure 6, non-viral vector/DNA complexes have to overcome several extracellular barriers before binding the target cells and initiating the intracellular trafficking towards the nucleus, where transgene expression will occur. Intravenous injection is the most widely used administration route but it presents many hurdles that hamper effective gene

delivery. Local administration routes are being explored in order to directly target the tissue of interest and avoid systemic barriers, but they often implicate invasive procedures (e.g. brain surgery). Many efforts are currently focused on the study of non-invasive routes of administration that can equally avoid the systemic barriers (e.g. intranasal administration). Besides, local administration into specific tissues such as the eye or the lungs, involve additional barriers that gene delivery systems have to elude. Surface modifications of the vector/DNA formulations are the most employed strategies to overcome both systemic and tissue-specific barriers. It is expected that future non-viral gene delivery systems will be based on multifunctional vectors and that they will allow a non-invasive administration of therapeutic genes into target tissues.
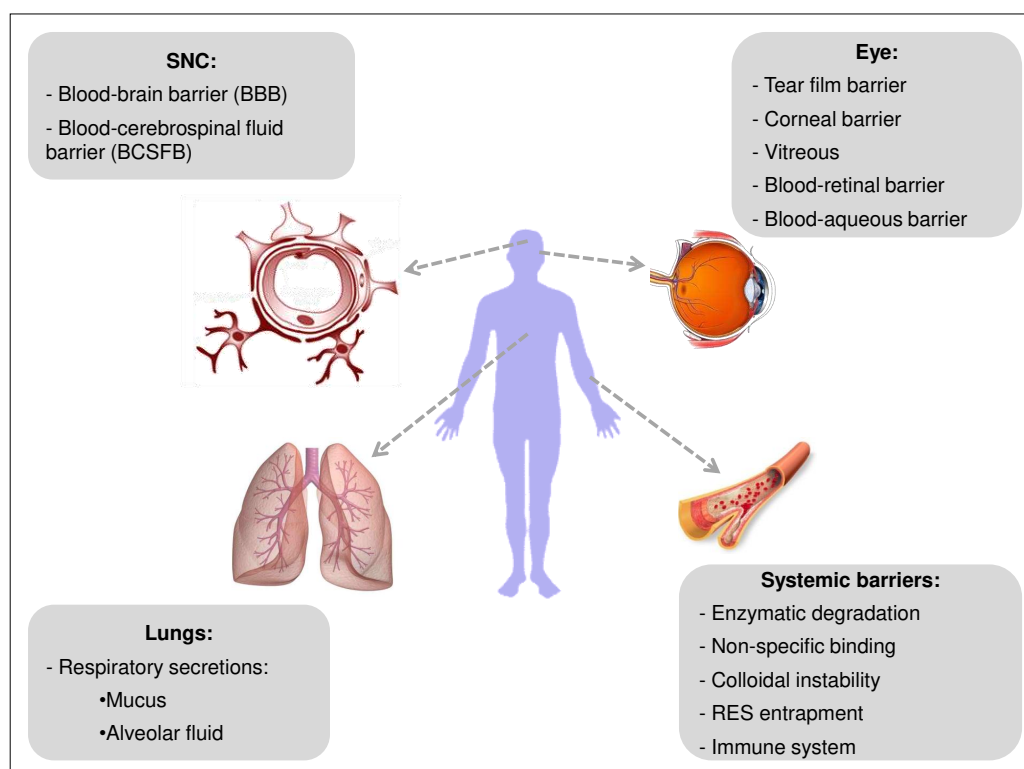

Figure 6. Overview of the systemic and tissue-specific (SNC, eye, lungs) extracellular barriers in non-viral gene therapy.

4. Challenges of non-viral gene therapy and future prospects

Non-viral gene therapy has emerged as a promising therapeutic approach for gene delivery. Even if this field is still far from clinical practice, much progress has been made in the last few years regarding both the optimization of the non-viral vector formulations and the exploration of alternative routes of administration. For transgene expression to occur, optimal non-viral vectors should not elicit an immune response and should be able, among other aspects, to protect the DNA cargo from degradation in circulation, to enable extravasation from the

bloodstream, to traverse cellular membranes, to enhance endosomal escape and to facilitate DNA transport to the nucleus [84]. The comprehensive understanding of the extracellular and intracellular barriers that vector/DNA complexes have to overcome in order to achieve an efficient transfection, has allowed the development of several strategies to surpass all those barriers, most of them based on formulation modifications of the complexes and on the use of local routes of administration. Regarding this latter aspect, considerable evidence suggests that the optimization of non-invasive routes of administration may provide safer and more effective gene delivery platforms in the future; therefore, it might be relevant to guide some efforts in this direction.

The major limitation of non-viral gene delivery, as mentioned repeatedly, is the low transfection efficiency. Several strategies discussed along the chapter increase transfection efficiency by providing the nanoparticles the ability to overcome extra- and intracellular barriers. However, there are two other aspects that are also essential for developing optimal gene delivery platforms: *targeting* and *long-term expression* of the transgene. Both are crucial to bring non-viral gene delivery systems into the clinic, since they provide specificity and sustained effect of the treatment, respectively. Many efforts have been made in this regard; however, further research is still required. In addition, other aspects such as the toxicity of the nanoparticles and the manufacturing and regulatory issues have to be carefully considered. Here, we will briefly discuss the current strategies for targeting the desired cells or tissues and for achieving a long-term expression of the transgene. We will also highlight the importance of considering the toxicity, manufacturing and regulatory issues of the nanoparticle formulations.

4.1. Targeting

Targeting to the desired cells or tissue can be achieved by modifying either the vehicle (the non-viral vector) or the cargo (the plasmid DNA). The most employed strategy is the attachment to the non-viral vector specific ligands (such as transferrin for targeting the SNC) that recognize particular receptors present in the target cells or tissues. As discussed earlier, this approach has proved effective in several studies. Also, in cancer gene therapy, some strategies are based on the exploitation of the tumour-specific physiological changes (the tumour microenvironment) to specifically conduct the nanoparticles to cancer cells [85].

On the other hand, another possibility is to introduce modifications in the DNA cargo (instead of the vector) to achieve targeted expression of the transgene, this approach is known as "transcriptional targeting." This strategy is based on the use of DNA expression cassettes that contain regulatory regions that are recognized by transcription factors specifically present or selectively expressed by the target cell population [85]. In this strategy the DNA would, in theory, be delivered to all tissues, but the expression of the transgene would only occur in the cell populations where the particular transcription factors are present, that is, in the target cell populations [84]. The success of this method of targeting needs prior knowledge of a difference in transcription factor expression between the target and normal tissue [84].

Targeting is an essential requirement in gene delivery systems. Beneficial aspects of targeted gene delivery include, among others, increased bioavailability of the therapeutic product in the diseased tissue; reduced accumulation in healthy tissues and, hence, reduced side effects;

reduction of drug dosage and reduced dosing frequency, which enhances patient compliance. All these aspects help to increase the therapeutic efficacy and permit to reduce treatment costs [86].

4.2. Duration of gene expression

Long-term or sustained expression of the transgene delivery constitutes a real challenge in non-viral gene therapy, and it is a considerable limiting factor, since transient expression requires repeated dosing and makes the therapeutic effect unsustained. Transgene expression can decrease in time due to several factors, including destruction by nucleases, loss by recombination, distribution to non-nuclear compartments and/or recognition and subsequent silencing of foreign DNA [85]. In addition, in dividing cells the percentage of transfected cells decreases at each division, because while cells replicate, plasmids do not.

Strategies to increase duration of transgene expression have focused on plasmid DNA modifications rather than on vector modifications. Some of those strategies are aimed at integrating the transgenes into the host genome using viral integrases, site-specific recombinases and transposases, which are enzymes with capacity of inserting foreign DNA into the host genome [85]. However, this approach cannot be clinically applicable in humans because of its associated risks, such as the induction of insertional mutagenesis in the host cells.

A different strategy to achieve sustained transgene expression is the use of autonomously replicating plasmids or episomes, which does not require integration in the host genome and, hence, avoids insertional mutagenesis risks [85]. In addition, episomally replicating plasmids usually yield high levels of transgene expression. These strategies incorporate genes that encode necessary cofactors for transcription of the plasmid to the therapeutic plasmid DNA, making the transgene expression less dependent on host factors. Incorporation of viral DNA that allows the plasmid to replicate extrachromosomally is an efficient approach, but it presents a major drawback, since those replication-inducing viral DNA elements are associated with induction of immune response and risk of transformation and oncogenicity [85]. Alternatively, mammalian scaffold/matrix attachment regions (S/MARs) have been identified that can be incorporated to plasmid DNA instead of the aforementioned viral sequences. These sequences can also enhance episomal replication of the plasmids, probably by bringing the plasmids into contact with the host replication machinery [73]. Episomally replicating plasmids are especially important in cancer gene therapy, where maintenance and vertical transfer of the therapeutic plasmid will be essential because of the presence of dividing tumour cells [85].

Some other strategies for achieving sustained expression focus on the prevention of transgene silencing, since cellular gene silencing mechanisms can impede transgene expression [85].

Further modifications can be also applied to the therapeutic plasmid DNA in order to increase the strength or the specificity of the therapeutic transgene expression. For instance, positive feedback loops can be incorporated. To do this, a promoter that drives the expression of both the transgene and of a strong artificial transcriptional activator is used. This transcriptional activator is capable of interacting with appropriate binding sites within the promoter and, that way, upregulating transgene expression, as well as its own expression [85]. Technologies

incorporating positive feedback loops are estimated to increase strength of weak but highly specific regulatory elements [85].

4.3. Toxicity, manufacturing and regulatory issues

Besides increasing transfection efficiency through targeting and other strategies, careful consideration of toxicity, manufacturing and regulatory issues of non-viral delivery systems is mandatory. Scalability and long-term storage requirements are essential factors to be taken into serious consideration when developing non-viral formulations for potential commercial application and introduction in clinical practice [19]. A generally accepted advantage of non-viral vectors is their ease of large-scale manufacture. However, this can become more complex as formulations increase in complexity, incorporating stabilizing components and bioactive targeting ligands.

Regarding toxicity of non-viral vectors, characteristics such as size, charge, surface function-alization, shape, and architecture may contribute to the toxicity profile of nanoparticles. Non-viral vectors are thought to cause toxicity through different mechanisms, including membrane destabilization and lysis, inducing oxidative stress, initiating inflammatory response, inducing global changes in gene expression profiles, among others [85]. Also, the properties of the biomaterials used can influence toxicity, depending on the rate of degradation and persistence in organs [19]. Persisting and accumulating biomaterials are more likely to induce an inflam-matory response; also, products of the degradation of nanoparticles could potentially cause toxicity. However, knowledge about how non-viral vectors are disassembled and metabol-ically processed is still very scarce and further research is necessary in this regard [19].

To conclude, the concept of a unique universal non-viral vector is nowadays abandoned, and it is increasingly accepted that future non-viral gene delivery platforms will be based on multifunctional vectors specifically tailored for different applications [1]. However, there are some generally assumed features that all non-viral vectors should accomplish for efficient gene delivery. In short, three main factors should be taken into consideration when developing non-viral gene delivery platforms: (i) formulation components (of both the vector and the DNA) – the nanoparticle should be able to protect DNA over extra- and intracellular barriers and deliver the cargo into the nucleus of target cells; once inside the nucleus, the plasmids can be addressed to the nuclear matrix for episomal replication and sustained expression; (ii) manufacturing issues – non-viral vector formulations should have potential for scale-up; and (iii) safety and regulatory issues – formulations should be non-toxic, non-immunogenic and should have suitable storage conditions. Although non-viral vectors are still far from clinical practice, they represent a safer alternative to conventional viral vectors. Several formulations and strategies are under investigation with the aim of overcoming extra- and intracellular barriers, enhancing targeted transfection and, in general, increasing transfection efficiency. In addition, those formulations would ideally be suitable for administration through non-invasive routes, such as the intranasal administration to target the brain, topical ocular administration for the retina and aerosols for pulmonary diseases. Finally, the inclusion of novel functional modules within both the carrier and the DNA molecule will produce a range of non-viral vectors tailored for specific applications, including the safe and long-term expression of therapeutic genes in humans [40]. Therefore, reasonable hope suggests that next-

generation gene delivery systems may be based on non-viral vector systems tailored for specific applications and suitable for non-invasive administration routes, representing an ideal platform to effectively shuttle the genetic material to target cells in a safe and controlled way.

Acknowledgements

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