We are IntechOpen,
the world's leading publisher of
Open Access books
Built by scientists, for scientists

6,900
Open access books available

186,000
International authors and editors

200M
Downloads

Our authors are among the

154
Countries delivered to

TOP 1%
most cited scientists

12.2%
Contributors from top 500 universities

CLARIVATE ANALYTICS
BOOK CITATION INDEX
INDEXED

WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index
in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Non-Viral Delivery Systems in Gene Therapy

Alicia Rodríguez Gascón,
Ana del Pozo-Rodríguez and María Ángeles Solinís

Additional information is available at the end of the chapter

1. Introduction

Recent advances in molecular biology combined with the culmination of the Human Genome Project [1] have provided a genetic understanding of cellular processes and disease pathogenesis; numerous genes involved in disease and cellular processes have been identified as targets for therapeutic approaches. In addition, the development of high-throughput screening techniques (e.g., cDNA microarrays, differential display and database meaning) may drastically increase the rate at which these targets are identified [2,3]. Over the past years there has been a remarkable expansion of both the number of human genes directly associated with disease states and the number of vector systems available to express those genes for therapeutic purposes. However, the development of novel therapeutic strategies using these targets is dependent on the ability to manipulate the expression of these target genes in the desired cell population. In this chapter we explain the concept and aim of gene therapy, the different gene delivery systems and therapeutic strategies, how genes are delivered and how they reach the target.

2. Aim and concept of gene therapy with non-viral vectors

A gene therapy medicinal product is a biological product which has the following characteristics: (a) it contains an active substance which contains or consists of a recombinant nucleic acid used in administered to human beings with a view to regulating, repairing, replacing, adding or deleting a genetic sequence; (b) its therapeutic, prophylactic or diagnostic effect relates directly to the recombinant nucleic acid sequence it contains, or to the product of genetic expression of this sequence [4].

The most important, and most difficult, challenge in gene therapy is the issue of delivery. The tools used to achieve gene modification are called gene therapy vectors and they are the "key" for an efficient and safe strategy. Therefore, there is a need for a delivery system, which must first overcome the extracellular barriers (such as avoiding particle clearance mechanisms, targeting specific cells or tissues and protecting the nucleic acid from degradation) and, subsequently, the cellular barriers (cellular uptake, endosomal escape, nuclear entry and nucleic release) [5]. An ideal gene delivery vector should be effective, specific, long lasting and safe.

Gene therapy has long been regarded a promising treatment for many diseases, including inherited through a genetic disorder (such as hemophilia, human severe combined immunodeficiency, cystic fibrosis, etc) or acquired (such as AIDS or cancer). Figures 1 and 2 show the indications addressed and the gene types transferred in gene therapy clinical trials, respectively [6].
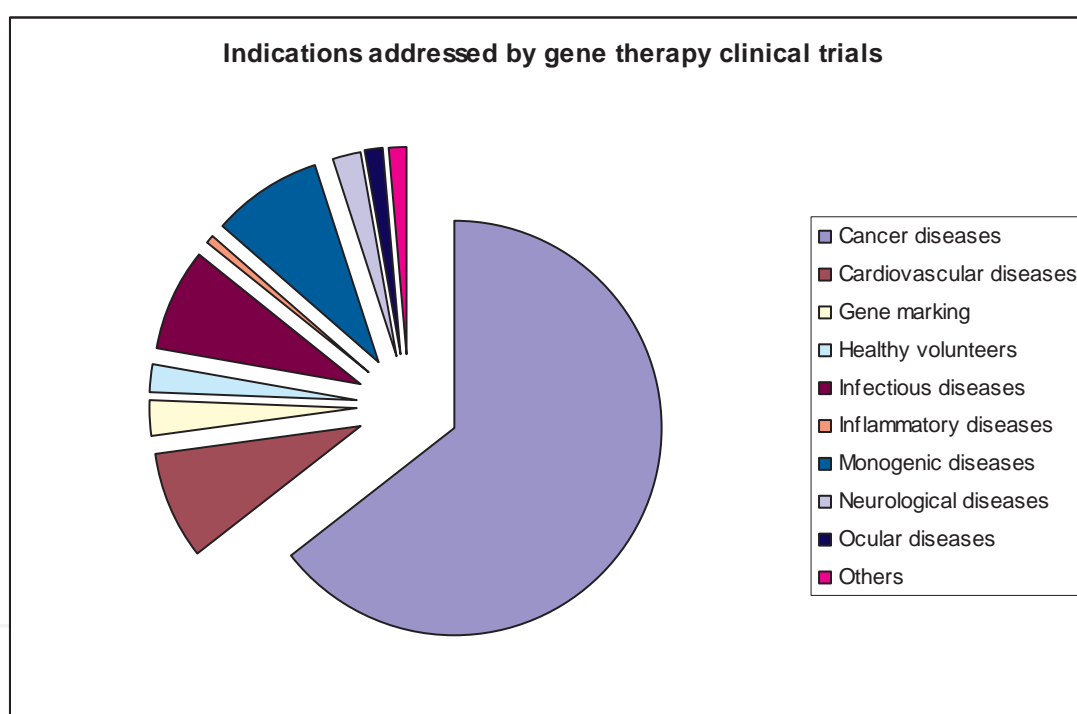

Figure 1. Indications addressed by gene therapy clinical trials (adapted from http://www.wiley.co.k/genmed/clinical).

Gene delivery systems include viral vectors and non-viral vectors. Viral vectors are the most effective, but their application is limited by their immunogenicity, oncogenicity and the small size of the DNA they can transport. Non-viral vectors are safer, of low cost, more reproducible and do not present DNA size limit. The main limitation of non-viral systems is their low transfection efficiency, although it has been improved by different strategies and the efforts are still ongoing [6]; actually, advances of non-viral delivery have lead to an increased number of products entering into clinical trials. However, viral vector has dominated the clinical trials in gene therapy for its relatively high delivery efficiency. Figure 3 shows the proportion of vector systems currently in human trials [7].
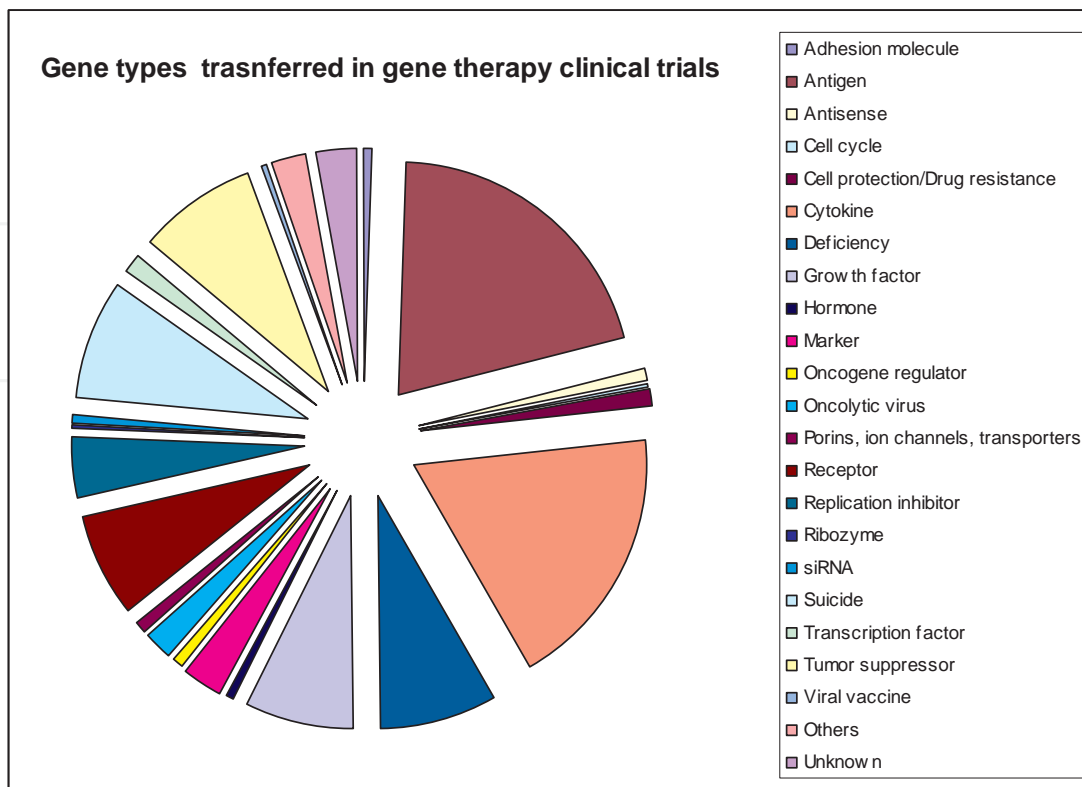
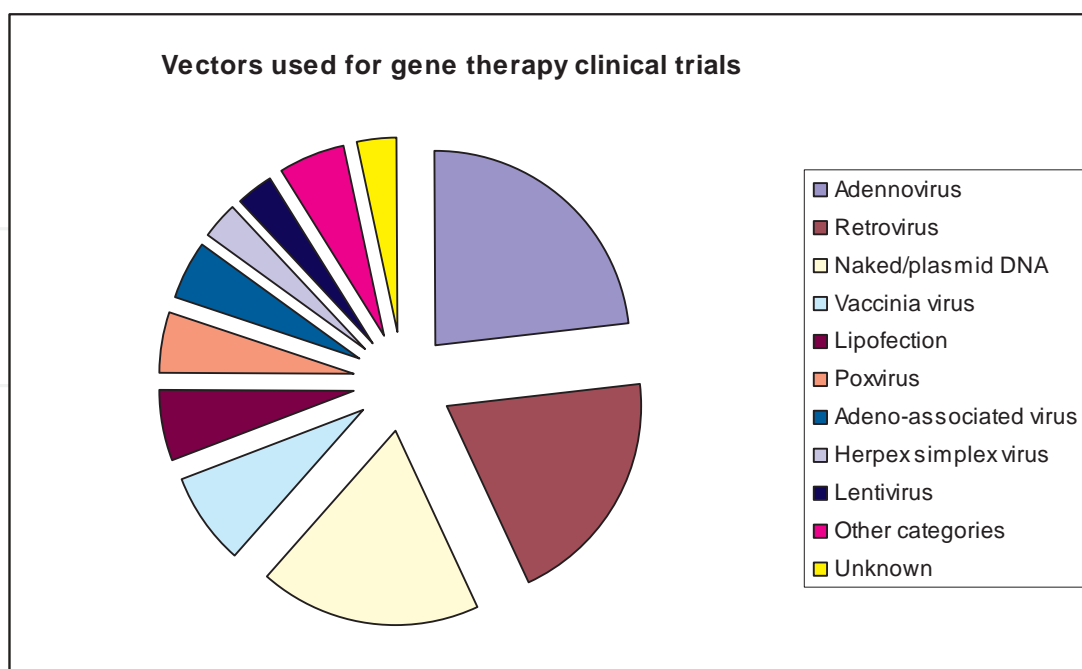**Figure 2.** Gene types transferred in gene therapy clinical trials (adapted from http://www.wiley.co.k/genmed/clinical).

Figure 3. Vector systems used in gene therapy clinical trials (adapted from http://www.wiley.co.k/genmed/clinical).

3. Non-viral methods for transfection

Currently, three categories of non-viral systems are available:

- Inorganic particles

- Synthetic or natural biodegradable particles

- Physical methods

Table 1 summarizes the most utilized non-viral vectors.

Category	System for gene delivery
Inorganic particles	Calcium phosphate
	Silica
	Gold
	Magnetic
Synthetic or natural biodegradable particles	1. Polymeric-based non-viral vectors:
	Poly(lactic-co-glycolic acid) (PLGA)
	Poly lactic acid (PLA)
	Poly(ethylene imine) (PEI)
	Chitosan
	Dendrimers
	Polymethacrylates
	2. Cationic lipid-based non-viral vectors:
	Cationic liposomes
	Cationic emulsions
	Solid lipid nanoparticles
	3. Peptide-based non-viral vectors:
	Poly-L-lysine
	Other peptides to functionalize other delivery systems: SAP, protamine
Physical methods	Needle injection
	Balistic DNA injection
	Electroporation
	Sonoporation
	Photoporation
	Magnetofection
	Hydroporation

Table 1. Delivery systems for gene therapy.

3.1. Inorganic particles

Inorganic nanoparticles are nanostructures varying in size, shape and porosity, which can be engineered to evade the reticuloendothelial system or to protect an entrapped molecular payload from degradation or denaturation [8]. Calcium phosphate, silica, gold, and several magnetic compounds are the most studied [9-11]. Silica-coated nanoparticles are biocompatible structures that have been used for various biological applications including gene therapy due to its biocompatibility [8]. Mesoporous silica nanoparticles have shown gene transfection efficiency "in vitro" in glial cells [12]. Magnetic inorganic nanoparticles (such as Fe_3O_4, MnO_2) have been applied for cancer-targeted delivery of nucleic acids and simultaneous diagnosis via magnetic resonance imaging [13,14]. Silica nanotubes have been also studied as an efficient gene delivery and imaging agent [13].

Inorganic particles can be easily prepared and surface-functionalized. They exhibit good storage stability and are not subject to microbial attack [13]. Bhattarai et al. [15] modified mesoporous silica nanoparticles with poly(ethylene glycol) and methacrylate derivatives and used them to deliver DNA or small interfering RNA (siRNA) "in vitro".

Gold nanoparticles have been lately investigated for gene therapy. They can be easily prepared, display low toxicity and the surface can be modified using various chemical techniques [16]. For instance, gold nanorods have been proposed to deliver nucleic acids to tumors [13]. They have strong absorption bands in the near-infrared region, and the absorbed light energy is then converted into heat by gold nanorods (photohermal effect). The near-infrared light can penetrate deeply into tissues; therefore, the surface of the gold could be modified with double-stranded DNA for controlled release [17]. After irradiation with near-infrared light, single stranded DNA is released due to thermal denaturation induced by the photothermal effect.

3.2. Synthetic or natural biodegradable particles

Synthetic or natural biocompatible particles may be composed by cationic polymers, cationic lipids or cationic peptides, and also the combination of these components [18-21]. The potential advantages of biodegradable carriers are their reduced toxicity (degradation leads to non-toxic products) and avoidance of accumulation of the polymer in the cells.

3.2.1. Polymer-based non-viral vectors

Cationic polymers condense DNA into small particles (polyplexes) and prevent DNA from degradation. Polymeric nanoparticles are the most commonly used type of nano-scale delivery systems. They are mostly spherical particles, in the size range of 1-1000 nm, carrying the nucleic acids of interest. DNA can be entrapped into the polymeric matrix or can be adsorbed or conjugated on the surface of the nanoparticles. Moreover, the degradation of the polymer can be used as a tool to release the plasmid DNA into the cytosol [22]. Table 1 shows several commonly used polymers used for gene delivery [16].

3.2.1.1. Poly(lactic-co-glycolic acid) (PLGA) and poly lactic acid (PLA)

Biodegradable polyesters, PLGA and PLA, are the most commonly used polymers for delivering drugs and biomolecules, including nucleic acids. They consist of units of lactic acid and glycolic acid connected through ester linkage. These biodegradable polymers undergo bulk hydrolysis thereby providing sustained delivery of the therapeutic agent. The degradation products, lactic acid and glycolic acid, are removed from the body through citric acid cycle. The release of therapeutic agent from these polymers occurs by diffusion and polymer degradation [16].

PLGA has a demonstrated FDA approved track record as a vehicle for drug and protein delivery [23,24]. Biodegradable PLA and PLGA particles are biocompatible and have the capacity to protect pDNA from nuclease degradation and increase pDNA stability [25,26].

PLGA particles typically less than 10 μm in size are efficiently phagocytosed by professional antigen presenting cells; therefore, they have significant potential for immunization applications [27,28]. For example, intramuscular immunization of p55 Gag plasmid adsorbed on PLGA/cetyl trimethyl ammonium bromide (CTAB) particles induced potent antibody and cytotoxic T lymphocyte responses. These particles showed a 250-fold increase in antibody response at higher DNA doses and more rapid and complete seroconversion, at the lower doses, compared to other adjuvants, including cationic liposomes [29].

The encapsulation efficiency of DNA in PLGA nanoparticles is not very high, and it depends on the molecular weight of the PLGA and on the hydrophobicity of the polymer, being the hydrophilic polymers those that provide higher loading efficiency [30]. To enhance the DNA loading, several strategies have been proposed. Kusonowiriyawong et al. [31] prepared cationic PLGA microparticles by dissolving cationic surfactants (like water insoluble stearylamine) in the organic solvent in which the PLGA was dissolved to prepare the microparticles. Another strategy was to reduce the negative charge of plasmid DNA by condensing it with poly(aminoacids) (like poly-L-lysine) before encapsulation in PLGA microparticles [32,33].

Normally, after an initial burst release, plasmid DNA release from PLGA particles occurs slowly during several days/weeks [22]. The degradation of the PLGA nanoparticles, through a bulk homogeneous hydrolytic process, determines the release of plasmid DNA. Consequently, it can be expected that the use of more hydrophilic PLGA not only improves the encapsulation efficiency of DNA, but also results in a faster release of plasmid DNA. Delivery of the plasmid DNA depends on the copolymer composition of the PLGA (lactic acid versus glycolic acid), molecular weight, particle size and morphology [22]. DNA release kinetics depends also on the plasmid incorporation technique; Pérea et al. [34] reported that nanoparticles prepared by the water-oil emulsion/diffusion technique released their content rapidly, whereas those obtained by the water-oil-emulsion method showed an initial burst followed by a slow release during at least 28 days.

PLGA and PLA based nanoparticles have also been used for "in vitro" RNAi delivery [35]. For instance, Hong et al. [36] have shown the effects of glucocorticoid receptor siRNA deliv-

ered using PLGA microparticles, on proliferation and differentiation capabilities of human mesenchymal stromal cells.

3.2.1.2. Chitosan

Chitosan [b(1-4)2-amino-2-deoxy-D-glucose] is a biodegradable polysaccharide copolymer of N-acetyl-D-glucosamine and D-glucosamine obtained by the alkaline deacetylation of chitin, which is a polysaccharide found in the exoskeleton of crustaceans of marine arthropods and insects [37]. Chitosans differ in the degree of N-acetylation (40 to 98%) and molecular weight (50 to 2000 kDa) [38]. As the only natural polysaccharide with a positive charge, chitosan has the following unique properties as carrier for gene therapy:

- it is potentially safe and non-toxic, both in experimental animals [39] and humans [40]

- it can be degraded into H_2O and CO_2 in the body, which ensures its biosafety

- it has biocompatibility to the human body and does not elicit stimulation of the mucosa and the derma

- its cationic polyelectrolyte nature provides a strong electrostatic interaction with negatively charged DNA [41], and protects the DNA from nuclease degradation [42]

- the mucoadhesive property of chitosan potentially leads to a sustained interaction between the macromolecule being "delivered" and the membrane epithelia, promoting more efficient uptake [43]

- it has the ability to open intercellular tight junctions, facilitating its transport into the cells [44]

Currently, there is a commercial transfection reagent based on chitosan (Novafect, NovaMatrix, FMC, US), and many other prototypes are under development. Most of the chitosanbased nanocarriers for gene delivery have been based on direct complexation of chitosan and the nucleic acid [45], whereas in some instances additional polyelectrolytes, polymers and lipids have been used in order to form composite nanoparticles [46-49] or chitosan-coated hydrophobic nanocarriers.

Many studies using cell cultures have shown that pDNA-loaded chitosan nanocarriers are able to achieve high transfection levels in most cell lines [50]. Chitosan nanocarriers loaded with siRNA have provided gene suppression values similar to the commercial reagent lipofectamine [51,52,18,53].

Chitosan of low molecular weight is more efficient for transfection than chitosan with high molecular weight. This enhancement in transfection efficacy observed with low molecular weight chitosan can be attributed to the easier release of pDNA from the nanocarrier upon cell internalization. Moreover, the presence of free low molecular chitosan has been deemed to be very important for the endosomal escape of the nanocarriers [50]. Concerning deacetylation degree, its influence on transfection is not still clear. "In vitro" studies have shown that the best transfection is achieved with highly deacetylated chitosan [54,55]. However, "in

vivo", higher transfection was achieved after intramuscular administration of chitosan complexes with a low deacetylation degree [55].

3.2.1.3. Poly(ethylene imine) (PEI)

PEI is one of the most potent polymers for gene delivery. PEI is produced by the polymerization of aziridine and has been used to deliver genetic material into various cell types both "in vitro" and "in vivo" [56,57]. There are two forms of this polymer: the linear form and the branched form, being the branched structure more efficient in condensing nucleic acids than the linear PEI [58].

PEI has a high density of protonable amino groups, every third atom being amino nitrogen, which imparts a high buffering ability at practically any pH [16]. Hence, once inside the endosome, PEI disrupts the vacuole releasing the genetic material in the cytoplasm. This ability to escape from the endosome, as well as the ability to form stable complexes with nucleic acids, make this polymer very useful as a gene delivery vector [56].

Depending on the type of polymer (e.g. linear or branched PEI), as well as the molecular weight, the particle sizes of the polyplexes formed are more or less uniformly distributed [59]. Transfection efficiency of PEI has been found to be dependent on a multitude of factors such as molecular weight, degree of branching, N/P ratio, complex size, etc [60].

The use of PEI for gene delivery is limited due to the relatively low transfection efficiency, short duration of gene expression, and elevated toxicity [61,62]. Conjugation of poly(ethylene glycol) to PEI to form diblock or triblock copolymers has been used by some authors to reduce the toxicity of PEI [63,64,65]. Poly(ethylene glycol) also shields the positive charge of the polyplexes, thereby providing steric stability to the complex. Such stabilization prevents non-specific interaction with blood components during systemic delivery [66].

3.2.1.4. Dendrimes

Dendrimers are polymer-based molecules with a symmetrical structure in precise size and shapes, as well as terminal group functionality [8]. Dendrimers contain three regions: i) a central core (a single atom or a group of atoms having two or more identical chemical funcionalities); ii) branches emanating from core, which are composed of repeating units with at least one branching junction, whose repetition is organized in a geometric progression that results in a series of radially concentric layers; and iii) terminal function groups. Dendrimers bind to genetic material when peripheral groups, that are positively-charged at physiological pH, interact with the negatively-charged phosphate groups of the nucleic acid [67,68]. Due to their nanometric size, dendrimers can interact effectively and specifically with cell components such as membranes, organelles, and proteins [69].

For instance, Qi et al. [70] showed the ability of generations 5 and 6 (G5 and G6) of poly(amidoamine) (PAMAM) dendrimers, conjugated with poly(ethylene glycol) to efficiently transfect both "in vitro" and "in vivo" after intramuscular administration to neonatal mice. PAMAM has also the ability to deliver siRNAs, especially "in vitro" in cell culture sys-

tems [71-73]. Recent studies showed that the dendrimer-mediated siRNA delivery and gene silencing depends on the stoichiometry, concentration of siRNA and the dendrimer generation [71]. In a recent study, a PAMAM dendrimer-delivered short hairpin RNA (shRNA) showed the ability to deplect a human telomerase reverse transcriptase, the catalytic subunit of telomerase complex, resulting in partial cellular apoptosis, and inhibition of tumor outgrowth in xenotransplanted mice [74].

The toxicity profile of dendrimers is good, although it depends on the number of terminal amino groups and positive charge density. Moreover, toxicity is concentration and generation dependent with higher generations being more toxic as the number of surface groups doubles with increasing generation number [75,76].

3.2.1.5. Polymethacrylates

Polymethacrylates are cationic vinyl-based polymers that possess the ability to condense polynucleotides into nanometer size particles. They efficiently condense DNA by forming inter-polyelectrolyte complexes. A range of polymethacrylates, differing in molecular weights and structures, have been evaluated for their potential as gene delivery vector, such us poly[2-dimethylamino) ethyl methacrylate] (DMAEMA) and its co-polymers [16]. The use of polymethactrtylates for DNA transfection is, however, limited due to their low ability to interact with membranes.

In order to optimise the use of these compounds for gene transfer, Christiaens et al. [77] combined polymethacrylates with penetratin, a 16-residue water-soluble peptide that internalises into cells through membrane translocation. Penetratin mainly enhanced the endolysosomal escape of the polymethacrylate–DNA complexes and increased their cellular uptake using COS-1 (kidney cells of the African green monkey). Nanoparticles with a methacrylate core and PEI shell prepared via graft copolymerization have also been employed lately for gene delivery [78,79]. This conjugation resulted in nanoparticles with a higher transfection efficiency and lower toxicity as compared with PEI.

3.2.2. Cationic-lipid based non-viral vectors

Cationic lipids have been among the more efficient synthetic gene delivery reagents "in vitro" since the landmark publications in the late 1980s [80]. Cationic lipids can condense nucleic acids into cationic particles when the components are mixed together. This cationic lipid/nucleic acid complex (lipoplex) can protect nucleic acids from enzymatic degradation and deliver the nucleic acids into cells by interacting with the negatively charged cell membrane [81]. Lipoplexes are not an ordered DNA phase surrounded by a lipid bilayer; rather, they are a partially condensed DNA complex with an ordered substructure and an irregular morphology [82,83]. Since the initial studies, hundreds of cationic lipids have been synthesized as candidates for non-viral gene delivery [84] and a few made it to clinical trials [85,86].

Cationic lipids can be used to form lipoplexes by directly mixing the positively charged lipids at the physiological pH with the negatively charged DNA. However, cationic lipids are

more frequently used to prepare lipoplex structures such as liposomes, nanoemulsions or solid lipid nanoparticles [81].

3.2.2.1. Cationic liposomes

Liposomes are spherical vesicles made of phospholipids used to deliver drugs or genes. They can range in size from 20 nm to a few microns. Cationic liposomes and DNA interact spontaneously to form complexes with 100% loading efficiency; in other words, all of the DNA molecules are complexed with the liposomes, if enough cationic liposomes are available. It is believed that the negative charges of the DNA interact with the positively charged groups of the liposomes [87]. The lipid to DNA ratio, and overall lipid concentration used in forming these complexes, are very important for efficient gene delivery and vary with applications [88].

Liposomes offer several advantages for gene delivery [87]:

- they are relatively cheap to produce and do not cause diseases

- protection of the DNA from degradation, mainly due to nucleases

- they can transport large pieces of DNA

- they can be targeted to specific cells or tissues

Successful delivery of DNA and RNA to a variety of cell types has been reported, including tumour, airway epithelial cells, endothelial cells, hepatocytes, muscle cells and others, by intratissue or intravenous injection into animals [89,90].

Several liposome-based vectors have been assayed in a number of clinical trials for cancer treatment. For instance, Allovectin-7® (Vical, San Diego, CA, USA), a plasmid DNA carrying HLAB and ß2-microglobulin genes complexed with DMRIE/DOPE liposomes have been assessed for safety and efficacy in phase I and II clinical trials [91,92].

3.2.2.2. Lipid nanoemulsions

An emulsion is a dispersion of one immiscible liquid in another stabilized by a third component, the emulsifying agent [93]. The nanoemulsion consists of oil, water and surfactants, and presents a droplet size distribution of around 200 nm. Lipid-based carrier systems represent drug vehicles composed of physiological lipids, such as cholesterol, cholesterol esters, phospholipids and tryglicerides, and offer a number of advantages, making them an ideal drug delivery carrier [94]. Adding cationic lipids as surfactants to these dispersed systems makes them suitable for gene delivery. The presence of cationic surfactants, like DOTAP, DOTMA or DC-Chol, causes the formation of positively charged droplets that promote strong electrostatic interactions between emulsion and the anionic nucleic acid phosphate groups [95,96]. For instance, Bruxel et al. [97] prepared a cationic nanoemulsion with DOTAP as a delivery system for oligonucleotides targeting malarial topoisomerase II.

Lipid emulsions are considered to be superior to liposomes mainly in a scaling-up point of view. On the one hand, emulsions can be produced on an industrial scale; on the other

hand, emulsions are stable during storage and are highly biocompatible [94]. In addition, the physical characteristics and serum-resistant properties of the DNA/nanoemulsion complexes suggest that cationic nanoemulsions could be a more efficient carrier system for gene and/or immunogene delivery than liposomes. One of the reasons for the serum-resistant properties of the cationic lipid nanoemulsions may be the stability of the nanoemulsion/DNA complex [98]. However, in spite of extensive research on emulsions, very few reports using cationic amino-based nanoemulsions in gene delivery have been published. After "in vivo" administration, cationic nanoemulsions have shown higher transfection and lower toxicity than liposomes [99].

The incorporation of noninonic surfactant with a branched poly(ethylene glycol), such as Tween 80®, increments the stability of the nanoemulsion and prevent the formation of large nanoemulsion/DNA complexes, probably because of their stearic hindrance and the generation of a hydrophilic surface that may enhance the stability by preventing physical aggregation [94]. In addition, this strategy may prevent from enzymatic degradation in blood, and due to the hydrophilic surface, they are taken up slowly by phagocytic cells, resulting in prolonged circulation in blood [100,101].

3.2.2.3. Solid lipid nanoparticles (SLN)

Solid lipid nanoparticles are particles made from a lipid being solid at room temperature and also at body temperature. They combine advantages of different colloidal systems. Like emulsions or liposomes, they are physiologically compatible and, like polymeric nanoparticles, it is possible to modulate drug release from the lipid matrix. In addition, SLN possess certain advantages. They can be produced without use of organic solvents, using high pressure homogenization (HPH) method that is already successfully implemented in pharmaceutical industry [102]. From the point of view of application, SLN have very good stability [103] and are subject to be lyophilized [104], which facilitates the industrial production.

Cationic SLN, for instance, SLN containing at least one cationic lipid, have been proposed as non-viral vectors for gene delivery [105,20]. It has been shown that cationic SLN can effectively bind nucleic acids, protect them from DNase I degradation and deliver them into living cells. Cationic lipids are used in the preparation of SLN applied in gene therapy not only due to their positive surface charge, but also due to their surfactant activity, necessary to produce an initial emulsion, which is a common step in most preparation techniques. By means of electrostatic interactions, cationic SLN condense nucleic acids on their surface, leading generally to an excess of positive charges in the final complexes. This is beneficial for transfection because condensation facilitates the mobility of nucleic acids, protects them from environmental enzymes and the cationic character of the vectors allows the interaction with negatively charged cell surface. The characteristics of the resulting complexes depend on the ratio between particle and nucleic acid; there must be an equilibrium between the binding forces of the nucleic acids to SLN to achieve protection without hampering the posterior release in the site of action [106]. Release of DNA from the complexes may be one of the most crucial steps determining the optimal ratio for cationic lipid system-mediated transfection [107].

Our research group showed for the first time the expression of a foreign protein with SLNs in an "in vivo" study [108]. After intravenous administration of SLN containing the EGFP plasmid to BALB/c mice, protein expression was detected in the liver and spleen from the third day after administration, and it was maintained for at least 1 week. In a later study [109], we incorporated dextran and protamine in the SLN and the transfection was improved, being detected also in lung. The improvement in the transfection was related to a longer circulation in the bloodstream due to the presence of dextran on the nanoparticle surface. The surface features of this new vector may also induce a lower opsonization and a slower uptake by the RES. Moreover, the high DNA condensation of protamine that contributes to the nuclease resistance may result in an extended stay of plasmid in the organism. The presence of nuclear localization signals in protamine, which improves the nuclear envelope translocation, and its capacity to facilitate transcription [110] may also explain the improvement of the transfection efficacy "in vivo".

SLN have also been applied for the treatment of ocular diseases by gene therapy. After ocular injection of a SLN based vector to rat eyes, the expression of EGFP was detected in various types of cells depending on the administration route: intravitreal or subretinal. In addition, this vector was also able to transfect corneal cells after topical application [111].

SLN may also be used as delivery systems for siRNA or oligonucleotides. Apolipoprotein-free low-density lipoprotein (LDL) mimicking SLN [112] formed stable complexes with siRNA and exhibited comparable gene silencing efficiency to siRNA complexed with the polymer PEI, and lower citotoxicity. Afterwards, Tao et al. [113] showed that lipid nanoparticles caused 90% reduction of luciferase expression for at least 10 days, after a single systemic administration of 3 mg/kg luciferase siRNA to a liver-luciferase mouse model. CTAB stabilized SLN bearing an antisense oligonucleotide against glucosylceramide synthase (asGCS) reduced the viability of the drug resistant NCI/ADR-RES human ovary cancer cells in the presence of the chemotherapeutic doxorubicin [114].

3.2.3. Peptide-based gene non-viral vectors

Many types of peptides, which are generally cationic in nature and able to interact with plasmid DNA through electrostatic interaction, are under intense investigation as a safe alternative for gene therapy [115]. There are mainly four barriers that must be overcome by non-viral vectors to achieve successful gene delivery. The vector must be able to tightly compact and protect DNA, target specific cell-surface receptors, disrupt the endosomal membrane, and deliver the DNA cargo to the nucleus [115]. Peptide-based vectors are advantageous over other non-viral systems because they are able to achieve all of these goals [116]. Cationic peptides rich in basic residues such as lysine and/or arginine are able to efficiently condense DNA into small, compact particles that can be stabilized in serum [117,118]. Attachment of a peptide ligand to a polyplex or lipoplex allows targeting to specific receptors and/or specific cell types. Peptide sequence derived from protein transduction domains are able to selectively lyse the endosomal membrane in its acidic environment leading to cytoplasmic release of the particle [119,120]. Finally, short peptide sequences taken

from longer viral proteins can provide nuclear localization signals that help the transport of the nucleic acids to the nucleus [121,122].

3.2.3.1. Poly-L-lysine

Poly-L-lysine is a biodegradable peptide synthesized by polimerization on N-carboxy-anhydride of lysine [123]. It is able to form nanometer size complexes with polynucleotides owing to the presence of protonable amine groups on the lysine moiety [16]. The most commonly used poly-L-lysine has a polymerization degree of 90 to 450 [124]. This characteristic makes this peptide suitable for "in vivo" use because it is readily biodegradable [116]. However, as the length of the poly-L-lysine increases, so does the cytotoxicity. Moreover, poly-L-lysine exhibits modest transfection when used alone and requires the addition of an edosomolytic agent such as chloroquine or a fusogenic peptide to allow for release into the cytoplasm. An strategy to prevent plasma protein binding and increase circulation half-life is the attachment of poly(ethylene glycol) to the poly-L-lysine [125,126].

3.2.3.2. Peptides in multifunctional delivery systems

Due to the advantages of peptides for gene delivery, they are frequently used to funtionalize cationic lipoplexes or polyplexes. Since these vectors undergo endocytosis, decorating them with endosomolytic peptides for enhanced cytosolic release may be helpful. Moreover, combination with peptides endowed with the ability to target a specific tissue of interest is highly beneficial, since this allows for reduced dose and, therefore, reduced side effects following systemic administration [127]. In a study carried out by our group [19], we improved cell transfection of ARPE-19 cells by using a cell penetration peptide (SAP) with solid lipid nanoparticles. Kwon et al. [128] covalently attached a truncated endosomolytic peptide derived from the carboxy-terminus of the HIV cell entry protein gp41 to a PEI scaffold, obtaining improved gene transfection results compared with unmodified PEI. In other study [20], protamine induced a 6-fold increase in the transfection capacity of SLN in retinal cells due to a shift in the internalization mechanism from caveolae/raft-mediated to clathrin-mediated endocytosis, which promotes the release of the protamine-DNA complexes from the solid lipid nanoparticles; afterwards the transport of the complexes into the nucleus is favoured by the nuclear localization signals of the protamine.

3.3. Physical methods for gene delivery

Gene delivery using physical principles has attracted increasing attention. These methods usually employ a physical force to overcome the membrane barrier of the cells and facilitate intracellular gene transfer. The simplicity is one of the characteristics of these methods. The genetic material is introduced into cells without formulating in any particulate or viral system. In a recent publication, Kamimura et al. [87] revised the different physical methods for gene delivery. These methods include the following:

3.3.1. Needle injection

The DNA is directly injected through a needle-carrying syringe into tissues. Several tissues have been transfected by this method [87]: muscle, skin, liver, cardiac muscle, and solid tumors. DNA vaccination is the major application of this gene delivery system [129]. The efficiency of needle injection of DNA is low; moreover, transfection is limited to the needle surroundings.

3.3.2. Ballistic DNA injection

This method is also called particle bombardment, microprojectile gene transfer or gene gun. DNA-coated gold particles are propelled against cells, forcing intracellular DNA transfer. The accelerating force for DNA-containing particles can be high-voltage electronic discharge, spark discharge or helium pressure discharge. One advantage of this method is that it allows delivering precise DNA doses. However, genes express transiently, and considerable cell damage occurs at the centre of the discharge site. This method has been used in vaccination against the influenza virus [130] and in gene therapy for treatment of ovarian cancer [131].

3.3.3. Electroporation

Gene delivery is achieved by generating pores on a cell membrane through electric pulses. The efficiency is determined by the intensity of the pulses, frequency and duration [132]. Electroporation creates transient permeability of the cell membrane and induces a low level of inflammation at the injection site, facilitating DNA uptake by parenchyma cells and antigen-presenting cells [133]. As drawbacks, the number of cells transfected is low, and surgery is required to reach internal organs. This method has been clinically tested for DNA-based vaccination [134] and for cancer treatment [135].

3.3.4. Sonoporation

Sonoporation utilizes ultrasound to temporally permeabilize the cell membrane to allow cellular uptake of DNA. It is non-invasive and site-specific and could make it possible to destroy tumor cells after systemic delivery, while leave non-targeted organs unaffected [13]. Gene delivery efficiency seems to be dependent on the intensisty of the pulses, frequency and duration [87]. This method has been applied in the brain, cornea, kidney, peritoneal cavity, muscle, and heart, among others. Low-intensity ultrasonund in combination with microbubbles has recently acquired much attention as a safe method of gene delivery [13]. The use of microbubbles as gene vectors is based on the hypothesis that destruction of DNA-loaded microbubbles by a focused ultrasound beam during their microvascular transit through the target area will result in localized transduction upon disruption of the microbubble shell while sparing non-targeted areas. The therapeutic effect of ultrasound-targeted microbubble destruction is relative to the size, stability, and targeting function of microbubbles.

3.3.5. Photoporation

The photoporation method utilizes a single laser pulse as the physical force to generate transient pores on a cell membrane to allow DNA to enter [87]. Efficiency seems to be controlled by the size of the focal point and pulse frequency of the laser. The level of transgene expression reported is similar to that of electroporation. Further studies are needed before this highly sophisticted procedure becomes a practical technique for gene delivery.

3.3.6. Magnetofection

This method employs a magnetic field to promote transfection. DNA is complexed with magnetic nanoparticles made of iron oxide and coated with cationic lipids or polymers through electrostatic interaction. The magnetic particles are then concentrated on the target cells by the influence of an external magnetic field. Similar to the mechanism of non-viral vector-based gene delivery, the cellular uptake of DNA is due to endocytosis and pinocytosis [136]. This method has been successfully applied to a wide range of primary cells, and cells that are difficult to transfect by other non-viral vectors [137].

3.3.7. Hydroporation

Hydroporation, also called hydrodynamic gene delivery method, is the most commonly method used for gene delivery to hepatocytes in rodents. Intrahepatic gene delivery is achieved by a rapid injection of a large volume of DNA solution via the tail vein in rodents, that results in a transient enlargement of fenestrae, generation of a transient membrane defect on the plasma membrane and gene transfer to hepatocytes [87]. This method has been frequently employed in gene therapy research. In order to apply this simple method of gene administration to the clinic, efforts have been made to reduce the injection volume and avoid tissue damage.

4. Strategies to improve transfection mediated by non-viral vectors

The successful delivery of therapeutic genes to the desired target cells and their availability at the intracellular site of action are crucial requirements for efficient gene therapy. The design of safe and efficient non-viral vectors depends mainly on our understanding of the mechanisms involved in the cellular uptake and intracellular disposition of the therapeutic genes as well as their carriers. Moreover, they have to overcome the difficulties after "in vivo" administration.

4.1. Target cell uptake and intracellular trafficking

Nucleic acid must be internalized to interact with the intracellular machinery to execute their effect. The positive surface charge of unshielded complexes facilitates cellular internalization. The non-viral vector can be functionalized with compounds that are recognized by the desire specific target cell type. Peptides, proteins, carbohydrates and small molecules

have been used to induce target cell-specific internalization [138]. For instance, SLN have been combined with peptides that show penetrating properties, such as the dimeric HIV-1 TAT (Trans-Activator of Transcription) peptide [139] or the synthetic SAP (Sweet Arrow Peptide) [19].

Endocytosis has been postulated as the main entry mechanism for non-viral systems. Various endocytosis mechanisms have been described to date: phagocytosis, pinocytosis, clathrin-mediated endocytosis, caveolae/raft-mediated endocytosis and chathrin and caveolae independent endocytosis. Clathrin-mediated endocytosis leads to an intracellular pathway in which endosomes fuse with lysosomes, which degrade their content, whereas caveolae/raft-mediated endocytosis avoids the lysosomal pathway and its consequent vector degradation [20]. Cytosolic delivery from either endosomes or lisosomes has been reported a major limitation in transfection [140]. In consequence, some research groups have used substances that facilitate endosomal escape before lysosomal degradation. For clathrin-mediated endocytosis, the drop in pH is a useful strategy for endosomal scape via proton destabilization conferred by the cationic carrier, or by pH-dependent activation of membrane disruptive helper molecules, such as DOPE or fusogenic peptides [141-143]. More recently, Leung et al. [144] have patented lipids with 4-amino-butiric acid (FAB) as headgroup to form lipid nanoparticles able to introduce nucleic acids, specifically siRNA, into mammalian cells. FAB lipids also demonstrated membrane destabilizing properties.

Once genes are delivered in the cytoplasm they have to diffuse toward the nuclear region. DNA plasmids have difficulties to diffuse in the cytoplasm because they are large in size. Therefore, packaging and complexing them into small particles facilitates its displacement intracellularly. Diffusion is a function of diameter; hence, smaller particles move faster than larger ones. Thus, another way to optimize gene delivery to the nucleus would be to decrease the size of the particles to increase the velocity of passive diffusion through the cytoplasm [145].

The pass through the nuclear membrane is the next step, and it is in general, quite difficult. There are two mechanisms large molecules can use to overcome that barrier: disruption of the nuclear membrane during mitosis, which is conditioned by the division rate of targeted cells, or import through the nuclear pore complex (NPC). This latter mechanism requires nuclear localization signals, which can be used to improve transfection by non-viral vectors [146]. In this regard, protamine is a peptide that condenses DNA and presents sequences of 6 consecutive arginine residues [147], which make this peptide able to translocate molecules such as DNA from the cytoplasm to the nucleus of living cells. Although protamine/DNA polyplexes are not effective gene vectors [148], the combination of protamine with SLN produced good results in both COS-1 and Na 1330 (murine neuroblastoma) culture cells [149,150]. Precondensation of plasmids with this peptide, to form protamine-DNA complexes that are later bound to cationic SLN, is another alternative that has shown higher transfection capacity in retinal cells compared to SLN prepared without protamine [20].

Once inside the nucleus, level of transgene expression depends on the copy number of DNA and its accessibility for the transcription machinery. Studies have shown that the minimum number of plasmids delivered to the nucleus required for measurable transgene expression

depends on the type of vectors [145]. Comparisons between different delivery vehicles showed that higher copy numbers of DNA molecules in the nucleus do not necessarily correlate with higher transfection efficiency. At similar plasmid/nucleus copies, lipofectamine mediated 10-fold higher transfection efficiency than PEI. This suggests that the DNA delivered by PEI is biologically less active than the DNA delivered by lipofectamine. It also emphasizes that a deeper understanding of the nuclear events in gene delivery is required for future progress.

4.2. "In vivo" optimization

Vectors mediating high transfection efficiency "in vitro" often fail to achieve similar results "in vivo". One possible reason is that lipidic and polymeric vectors are optimized "in vitro" using two-dimensional (2D) cultures that lack extracellular "in vivo" barriers and do not realistically reflect "in vivo" conditions. While cells "in vitro" grow in monolayers, cells "in vivo" grow in 3D tissue layers held together by the extracellular matrix [145]. This results in cells with reduced thicknesses but larger widths and lengths. Particles that are taken up directly above the nucleus (supranuclear region) have the shortest transport distance to the nucleus and hence a greater chance of delivery success. The spatiotemporal distribution of carriers, however, determines the optimal time for endosomal escape and the optimal intracellular pathway [151]. This highlights the need to develop adequate "in vitro" models that mimics as much as possible the "in vivo" conditions to optimize carriers for gene therapy.

After intravenous administration, plasma nuclease degradation of the nucleic acid is the first barrier that needs to be overcome for therapeutic nucleic acid action. Nucleic acids can be degraded by hydrolytic endo- and exo-nucleases. Both types of nucleases are present in blood. Therefore, increasing nuclease resistance is crucial for achieving therapeutic effects. Naked nucleic acids are not only rapidly degraded upon intravenous injection, they are also cleared from the circulation rapidly, further limiting target tissue localization [138]. To improve nuclease resistance and colloidal stability, complexation strength is an important factor. Shielding the non-viral vectors with poly-L-lysine or poly(ethylene glycol), as mentioned previously, prolongs the circulation time in blood of the vectors.

Vectors delivered "in vivo" by systemic administration not only have to withstand the bloodstream, but also have to overcome the cellular matrix to reach all cell layers of the tissue. While large particles seem to have an advantage "in vitro" due to a sedimentation effect on cells, efficient delivery of particles deep into organs requires particles <100 nm. Small particles (40 nm) diffuse faster and more effectively in the extracellular matrix and inner layers of tissues, whereas larger particles (>100 nm) are restricted by steric hindrance [152].

The net cationic charge of the synthetic vector is a determinant of circulation time, tissue distribution and cellular uptake of synthetic vectors by inducing interactions with negatively blood constituents, such as erythrocytes and proteins. The opsonisation of foreign particles by plasma proteins actually represents one of the steps in the natural process of removal of foreign particles by the innate immune system [153]. This may result in obstruction of small capillaries, possibly leading to serious complication, such as pulmonary embolism [154]. Part of the complexes end up in the reticuoloendothelial system (RES), where they are re-

moved rapidly by phagocytosis or by trapping in fine capillary beds [155]. The nanocarriers, when circulating in blood, can activate the complement system and it seems that the complement activation is higher as the surface charge increases [156,157].

The interaction with blood components is related to the intrinsic properties of the cationic compound (side chain end groups, its spatial conformation and molecular weight), as well as the applied Nitrogen:Phosphate (N:P) ratio [138]. Shielding of the positive surface charge of complexes is currently an important strategy to circumvent the aforementioned problems. The most popular strategy is based on the attachment of water-soluble, neutral, flexible polymers, as poly(ethylene glycol), poly(vinylpyrrolidone) and poly(hydroxyethyl-L-asparagine). The efficacy of the shielding effect of these polymers is determined by the molecular weight and grafting density of the shielding polymer [158]. Longer chains are usually more effective in protecting the particle (surface) from aggregation and opsonisation.

The nanocarriers must arrive to the target tissue to exert their action. Although most commonly used targeting strategies consist of proteins and peptides, carbohydrates have also been utilized [159]. The access of non-viral vector to tumors has been investigated extensively. The discontinuous endothelial cell layer has gaps that give the nanocarriers the opportunity to escape the vascular bed and migrate into the tumoral mass. The most common entities used for tumor targeting include transferrin, epidermal growth factor, and the integrin-binding tripeptide arginine-glycine-aspartic acid (RGD) [159]. Brain targeting has also a great interest; most gene vector do not cross the blood-brain barrier (BBB) after intravenous administration and must be administered through intracerebral injection, which is highly invasive and does not allow for delivery of the gene to other areas of the brain. Injection in the cerebrospinal fluid is also another strategy. Commonly used ligands for mediated uptake are insuline-like growth factors, transferrin or low-density lipid protein [159]. Targeting to the liver has been also investigated in a great extension by many researchers. Carbohydrate-related molecules, such as galactose, asialofetuin, N-acetylgalactosamine and folic acid are the most commonly molecules used for liver targeting [159]. Targeting to endothelial cells provides avenues for improvement of specificity and effectiveness of treatment of many diseases, such as cardiovascular or metabolic diseases [160]. Among other endothelial cell surface determinants, intercellular adhesion molecule-1 (CD54 or ICA-1, a 110-KDa Ig-like transmembrane constitutive endothelial adhesion molecule) is a good candidate target for this goal. ICAM-1 targeting can be achieved by coupling Anti-ICAM-1 antibodies to carriers [161].

5. Conclusion

The success of gene therapy is highly dependent on the delivery vector. Viral vectors have dominated the clinical trials in gene therapy for its relatively high delivery efficiency. However, the improvement of efficacy of non-viral vectors has lead to an increased number of products entering into clinical trials. A better understanding of the mechanisms governing the efficiency of transfection, from the formation of the complexes to their intracellular delivery, will lead to the design of better adapted non-viral vectors for gene therapy applica-

tions. A number of potentially rate-limiting steps in the processes of non-viral-mediated gene delivery have been identified, which include the efficiency of cell surface association, internalization, release of gene from intracellular compartments such as endosomes, transfer via the cytosol and translocation into the nucleus and transcription efficacy. Insight into molecular features of each of these steps is essential in order to determine their effectiveness as a barrier and to identify means of overcoming these hurdles. Although non-viral vectors may work reasonably well "in vitro", clinical success is still far from ideal. Considering the number of research groups that focus their investigations on the development of new vectors for gene therapy, together with the advances in the development of new technologies to better understand their "in vitro" and "in vivo" behavior, the present limitations of non-viral vectors will be resolved rationally.

Author details

Alicia Rodríguez Gascón, Ana del Pozo-Rodríguez and María Ángeles Solinís

*Address all correspondence to: alicia.rodriguez@ehu.es

Pharmacokinetics, Nanotechnology and Gene Therapy Group, Faculty of Phamacy, University of the Basque Country UPV/EHU, Spain

References

[1] Venter JC, Adams MD, Myers EW, et al. The Sequence of the Human Genome. Science 2001;291(5507) 1304-51.

[2] Kassner PD. Discovery of Novel Targets with High Throughput RNA Interference Screening. Combinatorial Chemistry & High Throughput Screen 2008;11(3) 175-184.

[3] Wiltgen M, Tilz GP. DNA Microarray Analysis: Principles and Clinical Impact. Hematology 2007;12(4) 27-87.

[4] Directive 2009/120/EC of the European Parliament. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2009:242:0003:0012:EN:PDF (accessed 07 August 2012).

[5] Zhang Y, Satterlee A, Huang L. In Vivo Gene Delivery by Nonviral Vectors: Overcoming Hurdles?. Molecular Therapy 2012; 20(7) 1298-1304.

[6] Gene Therapy Clinical Trials Worldwide.Provided by the Journal of Gene Medicine. Jon Wiley and Sons Ltd, 2012; http://www.wiley.co.k/genmed/clinical (accessed 01 August 2012).

[7] Li SD, Huang L. Non-viral is Superior to Viral Gene Delivery. Journal of Controlled Release 2007;123(3) 181-183.

[8]   Pérez-Martinez FC, Carrión B, Ceña V. The Use of Nanoparticles for Gene Therapy in the Nervous System. Journal of Alzheimer's Disease 2012;31(4) 697-710.

[9]   Roy I, Mitra S, Maitra A, Mozumdar S. Calcium Phosphate Nanoparticles as Novel Non-viral Vectors for Targeted Gene Delivery. International Journal of Pharmaceutics 2003;250(1) 25-33.

[10] Armatas GS, Kanatzidis MG. Mesostructured Germanium with Cubic Pore Symmetry. Nature 2006;441(7097) 1122-1125.

[11] Zou X, Conradsson T, Klingstedt M, Dadachov MS, O'Keeffe M. A Mesoporous Germanium Oxide with Crystalline Pore Walls and its Chiral Derivative. Nature 2005;437(7059) 716-719.

[12] Slowing II, Vivero-Escoto JL, Wu CW, Lin VS. Mesoporous Silica Nanoparticles as Controlled Release Drug Delivery and Gene Transfection Carriers. Advanced Drug Delivery Reviews 2008;60(11) 1278-1288.

[13] Guo X, Huang L. Recent Advances in Nonviral Vectors for Gene Delivery. Accounts of Chemical Research 2012;45(7) 971-979.

[14] Skolova V, Epple M. Inorganic Nanoparticles as Carriers of Nucleic Acids into Cells. Angewandte Chemie International Edition 2008;47(8) 1382-1395.

[15] Bhattarai SR, Muthuswamy E, Wani A, et al. Enhanced Gene and siRNA Delivery by Polycation-Modified Mesoporous Silica Nanoparticles Loaded with Chloroquine. Pharmaceutical Research 2010;27(12) 2556-2568.

[16] Basarkar A, SinghJ. Nanoparticulate Systems for Polynucleotide Delivery. International Journal of Nanomedicine 2007;2(3) 353-360.

[17] Yamashita S, Fukushima H, Akiyama Y, et al. Controlled-Release System of Single Stranded DNA Triggered by the Photothermal Effect of Gold Nanorods and its In Vivo Application. Bioorganic & Medicinal Chemistry 2011;19(7) 2130-2135.

[18] Raviña M, Cubillo E, Olmeda D, et al. Hyaluronic Acid/Chitosan-g-poly(ethylene glycol) Nanoparticles for Gene Therapy: an Application for pDNA and siRNA Delivery. Pharmaceutical Research 2010;27(12) 2544-2555.

[19] del Pozo-Rodríguez A, Pujals S, Delgado D, et al. A Proline-Rich Peptide Improves Cell Transfection of Solid Lipid Nanoparticle-Based Non-Viral Vectors. Journal of Controlled Release 2009;133(1) 52-59.

[20] Delgado D, del Pozo-Rodríguez A, Solinís MA, Rodríguez-Gascón A. Understanding the Mechanism of Protamine in Solid Lipid Nanoparticle-Based Lipofection: the Importance of the Entry Pathway. European Journal of Pharmaceutics and Biopharmaceutics 2011;79(3) 495-502.

[21] Nie H, Khew ST, Lee LY, Poh KL, Tong YW, Wang C-H. Lysine-Based Peptide-Functionalized PLGA Foams for Controlled DNA Delivery. Journal of Controlled Release 2009;138(1) 64-70.

[22] Luten J, van Nostrum CF, De Smedt SC, Hennink WE. Biodegradable Polymers as Non-Viral Carriers for Plasmid DNA Delivery. Journal of Controlled Release 2008;126(2) 97-110.

[23] Bala I, Hariharan S, Kumar M. PLGA Nanoparticles in Drug Delivery: The State of the Art. Critical Reviews in Therapeutics Drug Carrier Systems 2004;21(5) 387–422.

[24] Panyam J, Labhasetwar V. Biodegradable Nanoparticles for Drug and Gene Delivery to Cells and Tissue. Advanced Drug Delivery Reviews 2003;55(3) 329–347.

[25] Shive MS, Anderson JM. Biodegradation and Biocompatibility of PLA and PLGA Microspheres. Advanced Drug Delivery Reviews 1997;28(1) 5–24.

[26] Wang D, Robinson DR, Kwon GS, Samuel J. Encapsulation of Plasmid DNA in Biodegradable poly(D,L-lactic-co-glycolic acid) Microspheres as a Novel Approach for Immunogene Delivery. Journal of Controlled Release 1999;57(1) 9–18.

[27] Banchereau J, Steinman RM. Dendritic Cells and the Control of Immunity. Nature 1998;392(6673) 245–252.

[28] Walter E, Dreher D, Kok M, et al. Hydrophilic poly(DL lactide- co-glycolide) Microspheres for the Delivery of DNA to Human-Derived Macrophages and Dendritic cells. Journal of Controlled Release 2001;76(1-2)149–168.

[29] Singh M, Ugozzoli M, Briones M, Kazzaz J, Soenawan E, O'Hagan DT. The Effect of CTAB Concentration in Cationic PLG Microparticles on DNA Adsorption and In Vivo Performance. Pharmaceutical Research 2003;20(2) 247–251.

[30] Walker E, Moelling K, Pavlovic J, Merkle HP. Microencapsulation of DNA Using poly(DL-lactide-co-glycolide): Stability Issues and Release Characteristics. Journal of Controlled Release 1999;61(3) 361-374.

[31] Kusonowiriyawong C, Atuah K, Alpar OH, Merkle HP, Walter E. Cationic Stearylamine-Containing Biodegradable Microparticles for DNA Delivery. Journal of Microencapsulation 2004;21(1) 25-36.

[32] Benoit MA, Ribet C, Distexhe J, et al. Studies on the Potential of Microparticles Entrapping pDNA-poly(aminoacids) Complexes as Vaccine Delivery Systems. Journal of Drug Targeting 2001;9(4) 253-266.

[33] Nie Y, Zhang ZA, Duan YR. Combined Use of Polycationic Peptide and Biodegradable Macromolecular Polymer as a Novel Gene Delivery System: a Preliminary Study. Drug Delivery 2006;13(6) 441-446.

[34] Pérea C, Sánchez A, Putnam D, Ting D, Langer R, Alonso MJ. Poly(lactic acid)-poly(ethylene glycol) Nanoparticles as New Carriers for the Delivery of Plasmid DNA. Journal of Controlled Release 2001;75(1) 211-224.

[35] Shinde RR, Bachmann MH, Wang Q, Kasper R, Contag CH. PEG-PLA/PLGA Nanoparticles for In-Vivo RNAi Delivery. Nanotech: conference technical proceedings, May 20-23, 2007, Santa Clara Convention Centar, Santa Clara, California, USA.

[36] Hong L, Wei N, Joshi V, et al. Effects of Glucocorticoid Receptor Small Interfering RNA Delivered Using Poly Lactic-co-Glycolic Acid Microparticles on Proliferation and Differentiation Capabilities of Human Mesenchymal Stromal Cells. Tissue Engineering Part A 2012;18 (7-8) 775-784.

[37] Zheng F, Shi XW, Yang GF, et al. Chitosan Nanoparticle as Gene Therapy Vector Via Gastrointestinal Mucosa Administration: Results of an In Vitro and In Vivo Study. Life Sciences 2007;80(4) 388–396.

[38] Hejazi R, Amiji M. Chitosan-based Gastrointestinal Delivery Systems. Journal of Controlled Release 2003;89(2) 151–165.

[39] Rao SB, Sharma CP. Use of Chitosan as a Biomaterial: Studies on its Safety and Hemostatic Potential. Journal of Biomedical Materials Research 1997;34(1) 21–28.

[40] Aspden TJ, Mason JD, Jones NS, Lowe J, Skaugrud O, Illum L. Chitosan as a Nasal Delivery System: the Effect of Chitosan Solution on In Vitro and In Vivo Mucociliary Transport Rates in Human Turbinates and Volunteers. Journal of Pharmaceutical Sciences 1997;86(4) 509–513.

[41] Fang N, Chan V, Mao HQ, Leong KW. Interactions of Phospholipid Bilayer with Chitosan: Effect of Molecular Weight and pH. Biomacromolecules 2001;2(4) 1161–1168.

[42] Cui Z, Mumper RJ. Chitosan-based Nanoparticles for Topical Genetic Immunization. Journal of Controlled Release 2001;75(3) 409–419.

[43] Takeuchi H, Yamamoto H, Niwa T, Hino T, Kawashima Y. Enteral Absorption of Insulin in Rats from Mucoadhesive Chitosan-coated Liposomes. Pharmaceutical Research 1996;13(6) 896–901.

[44] Illum L. Chitosan and its Use as a Pharmaceutical Excipient. Pharmaceutical Research 1998;15(9) 1326–1331.

[45] Leong KW, Mao HQ, Truong-Le VL, Roy K, Walsh SM, August JT. DNA-Polycation Nanospheres as Non-Viral Gene Delivery Vehicles. Journal of Controlled Release 1998;53(1) 183–193.

[46] Csaba N, Köping-Höggård M, Fernandez-Megia E, Novoa-Carballal R, Riguera R, Alonso MJ. Ionically Crosslinked Chitosan Nanoparticles as Gene Delivery Systems: Effect of PEGylation Degree on In Vitro and In Vivo Gene Transfer. Journal of Biomedical Nanotechnology 2009;5(2) 162–171.

[47] de la Fuente M, Seijo B, Alonso MJ. Bioadhesive Hyaluronan-Chitosan Nanoparticles can Transport Genes Across the Ocular Mucosa and Transfect Ocular Tissue. Gene Therapy 2008;15(9) 668–676.

[48] de la Fuente M, Seijo B, Alonso MJ. Novel Hyaluronic Acid-Chitosan Nanoparticles for Ocular Gene Therapy. Investigative Ophthalmology & Visual Science 2008;49(5) 2016–2024.

[49] Katas H, Alpar HO. Development and Characterisation of Chitosan Nanoparticles for siRNA Delivery. Journal of Controlled Release 2006;115(2) 216–225.

[50] Garcia-Fuentes M, Alonso MJ. Chitosan-based Drug Nanocarriers: Where do we Stand?.Journal of Controlled Release 2012;161(2) 496-504

[51] Rojanarata T, Opanasopit P, Techaarpornkul S, Ngawhirunpat T, Ruktanonchai U. Chitosan-Thiamine Pyrophosphate as a Novel Carrier for siRNA Delivery. Pharmaceutical Research 2008;25(12) 2807–2814.

[52] Ji AM, Su D, Che O, et al. Functional Gene Silencing Mediated by Chitosan/siRNA Nanocomplexes. Nanotechnology 2009;20(40) 405103.

[53] Yuan Q, Shah J, Hein S, Misra RD. Controlled and Extended Drug Release Behaviour of Chitosan-based Nanoparticle Carrier. Acta Biomaterialia 2010;6(3) 1140–1148.

[54] Huang M, Fong CW, Khor E, Lim LY. Transfection Efficiency of Chitosan Vectors: Effect of Polymer Molecular Weight and Degree of Deacetylation. Journal of Controlled Release 2005;106(3) 391–406.

[55] Kiang T, Wen J, Lim HW, Leong KW. The Effect of the Degree of Chitosan Deacetylation on the Efficiency of Gene Transfection. Biomaterials 2004;25(22) 5293–5301.

[56] Boussif O, Lezoualc'h F, Zanta MA et al. A Versatile Vector for Gene and Oligonucleotide Transfer into Cells in Culture and In Vivo: Polyethylenimine. Proceedings of the National Academy of Sciences of the United States of America 1995;92(16) 7297-7301.

[57] Abdallah B, Hassan A, Benoist C, Goula D, Behr JP, Demeneix BA. A Powerful Nonviral Vector for In Vivo Gene Transfer into the Adult Mammalian Brain: Polyethylenimine. Human Gene Therapy 1996;7(16) 1947-1954.

[58] Dunlap DD, Maggi A, Soria MR, Monaco L. Nanoscopic Structure of DNA Condensed for Gene Delivery. Nucleic Acids Research 1997;25(15) 3095-3101.

[59] Debus H, Beck-Broichsitter M, Kissel T. Optimized Preparation of pDNA/poly(ethyleneimine) Polyplexes Uusing a Microfluidic System. Lab on a Chip 2012;12(14) 2498-2506.

[60] Thomas M, Ge Q, Lu JJ, et al. Cross-Linked Small Polyethylenimines: While Still Nontoxic, Deliver DNA Efficiently to Mammalian Cells In Vitro and In Vivo. Pharmaceutical Research 2005;22(3) 373-380.

[61] Chollet P, Favrot MC, Hurbin A, Coll JL. Side-Effects of a Systemic Injection of Linear Polyethylenimine-DNA Compleses. The Journal of Gene Medicine 2002;4(1): 84-91.

[62] Moghimi SM, Symonds P, Murray JC, Hunter AC, Debska G, Szewczyk A. A Two-stage Poly(ethylenimine)-Mediated Cytotoxicity: Implications for Gene Transfer/ Therapy. Molecular Therapy 2005;11(6) 990-995.

[63] Choi HS, Ooya T, Yui N. One-Pot Synthesis of a Polyrotaxane Via Selective Threading of a PEI-b-PEG-b-PEI Copolymer. Macromolecular Biosciences 2006;6(6) 420-424.

[64] Park MR, Han KO, Han IK, et al. Degradable Polyethyleneimine-alt-poly(ethylene glycol) Copolymers as Novel Gene Carriers. Journal of Controlled Release 2005;105(3) 367-380.

[65] Zhong Z, Feijen J, Lok MC, et al. Low Molecular Weight Linear Polyethyleneimine-bpoly(ethylene glycol)-b-polyethylenimine Triblock Copolymers: Synthesis, Characterization, and In Vitro Gene Transfer Properties. Biomacromolecules 2005;6(6) 33440-33448.

[66] Kursa M, Walker GF, Roessler V et al. Novel Shielded Transferring-Polyethylene Glycol-Polyethylenimine/DNA Complexes for Systemic Tumor-Targeted Gene Transfer. Bioconjugate Chemistry 2003;14(1) 222-231.

[67] Nandy B, Santosh M, Maiti PK. Interaction of Nucleic Acids with Carbon Nanotubes and Dendrimers Journal of Biosciences 2012;37(3) 457–474.

[68] Lee H, Larson RG. Lipid Bilayer Curvature and Pore Formation Induced by Charged Linear Polymers and Dendrimers: the Effect of Molecular Shape. The Journal of Physical Chemistry B 2008;112(39) 12279–12285.

[69] Dutta T, Jain NK, McMillan NA, Parekh HS. Dendrimer Nanocarriers as Versatile Vectors in Gene Delivery. Nanomedicine 2010;6(1) 25–34.

[70] Qi R, Gao Y, Tang Y et al. PEG-conjugated PAMAM Dendrimers Mediate Efficient Intramuscular Gene Expression. The AAPS Journal 2009;11(3): 395–405.

[71] Liu X, Huang H, Wang J et al. Dendrimers-Delivered Short Hairpin RNA Targeting hTERT Inhibits Oral Cancer Cell Growth In Vitro and In Vivo. Biochemical Pharmacology 2011;82(1) 17-23.

[72] Khurana B, Goyal AK, Budhiraja A, Arora D, Vyas SP. siRNA Delivery Using Nanocarriers - an Efficient Tool for Gene Silencing. Current Gene Therapy 2010;10(2) 139-155.

[73] Pavan GM, Albertazzi L, Danani A. Ability to Adapt: Different Generations of PA-MAM Dendrimers show Different Behaviors in Binding siRNA. The Journal of Physical Chemistry B 2010;114 (8) 2667–2675.

[74] Liu X, Huang H, Wang J et al. Dendrimers-Delivered Short Hairpin RNA Targeting hTERT Inhibits Oral Cancer Cell Growth In Vitro and In Vivo. Biochemical Pharmacology 2011;82(1) 17-23.

[75] Choi YJ, Kang SJ, Kim YJ, Lim YB, Chung HW. Comparative Studies on the Genotoxicity and Cytotoxicity of Polymeric Gene Carriers Polyethylenimine (PEI) and Polyamidoamine (PAMAM) Dendrimer in Jurkat T-cells. Drug and Chemical Toxicology 2010;33(4) 357–366.

[76] Duncan R, Izzo L. Dendrimer Biocompatibility and Toxicity. Advanced Drug Delivery Reviews 2005;57 (15) 2215–2237

[77] Christiaens B, Dubruel P, Grooten J, et al. Enhancement of Polymethacrylate-Mediated Gene Delivery by Penetratin. European Journal of Pharmaceutical Sciences 2005;24(5) 525-537.

[78] Li P, Zhu JM, Sunintaboom P, Harris FW. New Route to Amphiphilic Core-Shell Polymer Nanospheres: Graft Copolymerization of Methyl Methacrylate from Water-Soluble Polymer Chains Containing Amino Groups. Langmuir 2002;18(22) 8641-8646.

[79] Feng M, Lee D, Li P. Intracellular Uptake and Release of Poly(ethyleneimine)-co-poly(methyl methacrylate) Nanoparticle/pDNA Complexes for Gene Delivery. International Journal of Pharmaceutics 2006;311(1-2) 209-214.

[80] Felgner PL, Gadek TR, Holm M, et al. Lipofection: a Highly Efficient, Lipid-Mediated DNA-Transfection Procedure. Proceedings of the National Academy of Sciences of the United States of America 1987;84(21) 7413-7417.

[81] Gascon AR, Pedraz JL. Cationic Lipids as Gene Transfer Agents: a Patent Review. Expert Opinion on Therapeutic Patents 2008;18(5) 515-524.

[82] Xu Y, Hui SW, Frederik P, Szoka FC Jr. Physicochemical Characterization and Purification of Cationic Lipoplexes. Biophysical Journal 1999;77(1) 341-353.

[83] Radler JO, Koltover I, Salditt T, Safinya CR. Structure of DNA-Cationic Liposome Complexes: DNA Intercalation in Multilamellar Membranes in Distinct Interhelical Packing Regimes. Science 1997;275(5301) 810-814.

[84] Mahato RI., ed. Kim SW. Pharmaceutical Perspectives of Nucleic Acid-Based Therapeutics. London: Taylor & Francis; 2002.

[85] Edelstein ML, Abedi MR, Wixon J, Edelstein RM. Gene Therapy Clinical Trials Worldwide 1989-2004 - an Overview. The Journal of Gene Medicine 2004;6(6) 597-602.

[86] Edelstein ML, Abedi MR, Wixon J. Gene Therapy Clinical Trials Worldwide to 2007 – an Update. The Journal of Gene Medicine 2007;9(10) 833-842.

[87] Kamimura K, Suda T, Zhang G, Liu D. Advances in gene delivery systems. Pharmaceutical Medicine 2011;25(5) 293-306.

[88] Zhou X, Huang L. DNA Transfection Mediated by Cationic Liposomes Containing Lipopolylysine: Characterization and Mechanism of Action. Biochimica et Biophysica Acta 1994;1189(2) 195-203.

[89] Xu L, Huang CC, Huang W, et al. Systemic Tumor-Targeted Gene Delivery by Anti-transferrin Receptor scFv-Immunoliposomes. Molecular Cancer Therapeutics 2002;1(5) 337-346.

[90] Pardridge WM. Re-engineering Biopharmaceuticals for Delivery to Brain with Molecular Trojan Horses.Bioconjugate Chemistry 2008;19(7) 1327-1338.

[91] Stopeck AT, Hersh EM, Akporiaye ET, et al. Phase I Study of Direct Gene Transfer of an Allogeneic Histocompatibility Antigen, HLA-B7, in Patients with Metastatic Melanoma. Journal of Clinical Oncology 1997;15(1) 341-349.

[92] Stopeck AT, Jones A, Hersh EM, et al. Phase II Study of Direct Intralesional Gene Transfer of Allovectin-7, an HLA-B7/beta2-microglobulin DNA-Liposome Complex, in Patients with Metastatic Melanoma. Clinical Cancer Research 2001;7(8): 2285–2291.

[93] Becher P., ed. Emulsions, Theory and Practice. New York: Reinhold; 1965.

[94] Verissimo LM, Lima LF, Egito LC, de Oliveira AG, do Egito ES. Pharmaceutical Emulsions: a New Approach for Gene Therapy. Journal of Drug Targeting 2010;18(5) 333-342.

[95] Martini E, Fattal E, de Oliveira MC, Teixeira H. Effect of Cationic Lipid Composition on Properties of Oligonucleotide/emulsion Complexes: Physico-chemical and Release Studies. International Journal of Pharmaceutics 2008;352(1–2) 280–286.

[96] Marty R, N'soukpoé-Kossi CN, Charbonneau D, Weinert CM, Kreplak L, Tajmir-Riahi HA. Structural Analysis of DNA Complexation with Cationic Lipids. Nucleic Acids Research 2009;37(3) 849–857.

[97] Bruxel F, Cojean S, Bochot A, et al. Cationic Nanoemulsion as a Delivery System for Oligonucleotides Targeting Malarial Topoisomerase II. International Journal of Pharmaceutics 2011;416(2) 402-409.

[98] Yi SW, Yune TY, Kim TW, et al. A Cationic Lipid Emulsion/DNA Complex as a Physically Stable and Serum-Resistant Gene Delivery System. Pharmaceutical Research 2000;17(3) 314-320.

[99] Kwon SM, Nam HY, Nam T, et al. In Vivo Time-Dependent Gene Expression of Cationic Lipid-Based Emulsion as a Stable and Biocompatible Non-Viral Gene Carrier. Journal of Controlled Release 2008;128(1) 89-97.

[100] Buyens K, Demeester J, De Smedt SS, Sanders NN. Elucidating the Encapsulation of Short Interfering RNA in PEGylated Cationic Liposomes. Langmuir 2009;25(9) 4886-4891.

[101] Kim TW, Kim YJ, Chung H, Kwon IC, Sung HC, Jeong SY. The Role of Non-Ionic Surfactants on Cationic Lipid Mediated Gene Transfer. Journal of Controlled Release 2002;82(2-3) 455-465.

[102] Teeranachaideekul V, Müller RH, Junyaprasert VB. Encapsulation of Ascorbylpalmitate in Nanostructured Lipid Xarriers (NLC)--Effects of Formulation Parameters on Physicochemical Stability. International Journal of Pharmaceutics 2007;340(1-2) 198-206.

[103] Müller R, Mäder K, Gohla S. Solid Lipid Nanoparticles (SLN) for Controlled Drug Delivery - a Review of the State of the Art. European Journal of Pharmaceutics and Biopharmaceutics 2000; 50(1) 161-177.

[104] del Pozo-Rodríguez A, Solinís MA, Gascón AR, Pedraz JL. Short- and Long-Term Stability Study of Lyophilized Solid Lipid Nanoparticles for Gene Therapy. EuropeanJournal of Pharmaceutics and Biopharmaceutics 2009;71(2) 181-189.

[105] del Pozo-Rodríguez A, Delgado D, Solinís MA, Gascón AR, Pedraz JL. Solid Lipid Nanoparticles: Formulation Factors Affecting Cell Transfection Capacity. International Journal of Pharmaceutics 2007;339(1-2) 261-268.

[106] del Pozo-Rodríguez A, Delgado D, Solinís MA, Gascón AR. Lipid Nanoparticles as Vehicles for Macromolecules: Nucleic Acids and Peptides. Recent Patents on Drug Delivery & Formulation 2011;5(3) 214-226.

[107] Sakurai F, Inoue R, Nishino Y, Okuda A, Matsumoto O, Taga T. Effect of NA/Liposome Mixing Ratio on the Physicochemical Characteristics, Cellular Uptake and Intracellular Trafficking of Plasmid DNA/Cationic Liposome Complexes and Subsequent Gene Expression. Journal of Controlled Release 2000;66(2-3) 255-269.

[108] del Pozo-Rodríguez A, Delgado D, Solinís MA, et al. Solid Lipid Nanoparticles as Potential Tools for Gene Therapy: In Vivo Protein Expression After Intravenous Administration. International Journal of Pharmaceutics 2010;385(1-2) 157-162.

[109] Delgado D, Gascón AR, del Pozo-Rodríguez A, et al. Dextran-Protamine-Solid Lipid Nanoparticles as a Non-Viral Vector for Gene Therapy: In Vitro Characterization and In Vivo Transfection after Intravenous Administration to Mice. International Journal of Pharmaceutics 2012;425(1-2) 35-43.

[110] Masuda T, Akita H, Harashima H. Evaluation of Nuclear Transfer and Transcription of Plasmid DNA Condensed with Protamine by Microinjection: the Use of a Nuclear Transfer Score. FEBS Letters 2005;579(10) 2143-2148.

[111] Delgado D, del Pozo-Rodríguez A, Solinís MA, et al. Dextran and Protamine-based Solid Lipid Nanoparticles as Potential Vectors for the Treatment of X-linked Juvenile Retinoschisis. Human Gene Therapy 2012;23(4) 345-355.

[112] Kim HR, Kim IK, Bae KH, Lee SH, Lee Y, Park TG. Cationic Solid Lipid Nanoparticles Reconstituted from Low Density Lipoprotein Components for Delivery of siRNA. Molecular Pharmaceutics 2008;5(4) 622-631.

[113] Tao W, Davide JP, Cai M, et al. Noninvasive Imaging of Lipid Nanoparticle-Mediated Systemic Delivery of Small-Interfering RNA to the Liver. Molecular Therapy 2010;18(9) 1657-1666.

[114] Siddiqui A, Patwardhan GA, Liu YY, Nazzal S. Mixed Backbone Antisense Glucosylceramide Synthase Oligonucleotide (MBO-asGCS) Loaded Solid Lipid Nanoparticles: In Vitro Characterization and Reversal of Multidrug Resistance in NCI/ADR-RES Cells. International Journal of Pharmaceutics 2010;400(1-2) 251-259.

[115] Mahato RI. Nonviral Peptide-based Approaches to Gene Delivery. Journal of Drug Targeting 1999;7(4) 249-268.

[116] Martin ME, Rice KG. Peptide-guided Gene Delivery. The AAPS Journal 2007;9(1) E18-E29.

[117] Adami RC, Rice KG. Metabolic Stability of Glutaraldehyde Cross-linked Peptide DNA Condensates. Journal of Pharmaceutical Sciences 1999;88(8) 739-746.

[118] McKenzie DL, Kwok KY, Rice KG. A Potent New Class of Reductively Activated Peptide Gene Delivery Agents. The Journal of Biological Chemistry 2000;275(14) 9970-9977.

[119] Gupta B, Levchenko TS, Torchilin VP. Intracellular Delivery of Large Molecules and Small Particles by Cell-Penetrating Proteins and Peptides. Advanced Drug Delivery Reviews 2005; 57(4) 637-651.

[120] Deshayes S, Morris MC, Divita G, Heitz F. Cell-Penetrating Peptides: Tools for Intra-cellular Delivery of Therapeutics. Cellular and Molecular Life Science 2005;62(16) 1839-1849.

[121] Goldfarb DS, Gariepy J, Schoolnik G, Kornberg RD. Synthetic Peptides as Nuclear Localization signals. Nature.1986;322(6080) 641-644.

[122] Nigg EA. Nucleocytoplasmic Transport: Signals, Mechanisms and Regulation. Nature. 1997;386(6627) 779-787.

[123] Zhang S, Xu Y, Wang B, Qiao W, Liu D, Li Z. Cationic Compounds Used in Lipoplex-es and Polyplexes for Gene Delivery. Journal of Controlled Release 2004;100(2) 165-180.

[124] Wadhwa MS, Collard WT, Adami RC, McKenzie DL, Rice KG. Peptide-Mediated Gene Delivery: Influence of Peptide Structure on Gene Expression. Bioconjugate Chemistry 1997;8(1) 81-88.

[125] El-Aneed A. An Overview of Current Delivery Systems in Cancer Gene Ttherapy. Journal of Controlled Release 2004;94(1) 1-14.

[126] Tiera MJ, Winnik FO, Fernandes JC. Synthetic and Natural Polycations for Gene Therapy: State of the Art and New Perspectives. Current Gene Therapy 2006;6(1) 59-71.

[127] Hoyer J, Neundorf I. Peptide Vectors for the Nonviral Delivery of Nucleic Acids. Accounts of Chemical Research 2012;45(7) 1048-1056.

[128] Kwon EJ, Liong S, Pun SH. A Truncated HGP Peptide Sequence that Retains Endoso-molytic Activity and Improves Gene Delivery Efficiencies. Molecular Pharmaceutics 2010;7(4) 1260-1265.

[129] Prausnitz MR, Mikszta JA, Cormier M, Andrianov AK. Microneedle-based Vaccines. Current Topics in Microbiology and Immunology 2009;333 369-393.

[130] Yager EJ, Dean HJ, Fuller DH. Prospects for Developing an Effective Particle-Mediated DNA Vaccine Against influenza. Expert Review of Vaccines. 2009;8(9) 1205-1220.

[131] Kaur T, Slavcev RA, Wettig SD. Addressing the Challenge: Current and Future Directions in Ovarian Cancer Therapy. Current Gene Therapy 2009;9(6) 434-458.

[132] Heller LC, Ugen K, Heller R. Electroporation for Targeted Gene Transfer. Expert Opinion on Drug Delivery 2005;2(2) 255-268.

[133] van DrunenLittel-van den Hurk S, Hannaman D. Electroporation for DNA Immunization: Clinical Application. Expert Review of Vaccines 2010;9(5) 503-517.

[134] Bodles-Brakhop AM, Heller R, Draghia-Akli R. Electroporation for the Delivery of DNA-based Vaccines and Immunotherapeutics: Current Clinical Developments. Molecular Therapy 2009;17(4) 585-592.

[135] Heller R, Jaroszeski MJ, Glass LF, et al. Phase I/II Trial for the Treatment of Cutaneous and Subcutaneous Tumors using Electrochemotherapy. Cancer 1996;77(5) 964-971.

[136] Plank C, Anton M, Rudolph C, Rosenecker J, Krötz F. Enhancing and Targeting Nucleic Acid Delivery by Magnetic Force. Expert Opinion on Biological Therapy 2003;3(5) 745-758.

[137] Mykhaylyk O, Antequera YS, Vlaskou D, Plank C. Generation of Magnetic Nonviral Gene Transfer Agents and Magnetofection In Vitro. Nature Protocols 2007;2(10) 391-2411.

[138] Schiffelers RM, de Wolf HK, van Rooy I, Storm G. Synthetic Delivery Systems for Intravenous Administration of Nucleic Acids. Nanomedicine 2007;2(2)169-181.

[139] Rudolph C, Schillinger U, Ortiz A, et al. Application of Novel Solid Lipid Nanoparticle (SLN)-Gene Vector Formulations Based on a Dimeric HIV-1 TAT-Peptide In Vitro and In Vivo. Pharmaceutical Research 2004;21(9) 1662-1669.

[140] El-Sayed A, Futaki S, Harashima H. Delivery of Macromolecules Using Arginine-Rich Cell-Penetrating Peptides: Ways to Overcomes Endosomal Entrapment. The AAPS Journal 2009;11(1) 13-22.

[141] Choi SH, Jin SE, Lee MK, et al. Novel Cationic Solid Lipid Nanoparticles Enhanced p53 Gene Transfer to Lung Cancer Cells. European Journal of Pharmaceutics and Biopharmaceutics 2008;68(3) 545-554.

[142] Dincer S, Turk M, Piskin E. Intelligent Polymers as Nonviral Vectors. Gene Therapy 2005;12(1) S139-S145.

[143] Yessine MA, Leroux JC. Membrane-Destabilizing Polyanions: Interaction with Lipid Bilayers and Endosomal Escape of Biomacromolecules. Advanced Drug Delivery Reviews 2004;56(7) 999-1021.

[144] Leung KK, Masuna S, Ciufolini M, Cullis PR. Reverse Head Group Lipids, Lipid Compositions Comprising Reverse Headgroup Lipids, and Methods for Delivery of Nucleic Acids. WO2011056682; 2011.

[145] Nguyen J, Szoka FC. Nucleic Acid Delivery: the Missing Pieces of the Puzzle? Accounts of Chemical Research 2012;45(7) 1153-1162.

[146] Boulanger C, Di Giorgio C, Vierling P. Synthesis of Acridine-Nuclear Localization Signal (NLS) Conjugates and Evaluation of their Impact on Lipoplex and Polyplex-based Ttransfection. European Journal of Medicinal Chemistry 2005;40(12) 1295-1306.

[147] Biegeleisen K.The Probable Structure of the Protamine-DNA Complex. Journal of Theoretical Biology 2006;241(3) 533-540.

[148] Xu Z, Gu W, Chen L, Gao Y, Zhang Z, Li Y. A Smart Nanoassembly Consisting of Acid-Labile Vinyl Ether PEG-DOPE and Protamine for Gene Delivery: Preparation and "In Vitro" Transfection. Biomacromolecules 2008;9(11) 3119-3126.

[149] Vighi E, Ruozi B, Montanari M, Battini R, Leo E. pDNA Condensation Capacity and In Vitro Gene Delivery Properties of Cationic Solid Lipid Nanoparticles. International Journal of Pharmaceutics 2010;389(1-2) 254-261.

[150] Vighi E, Montanari M, Ruozi B, Tosi G, Magli A, Leo E. Nuclear Localization of Cationic Solid Lipid Nnanoparticles Containing Protamine as Transfection Promoter. European Journal of Pharmaceutics and Biopharmaceutics 2010;76(3) 384-393.

[151] Dinh AT, Pangarkar C, Theofanous T, Mitragotri S. Understanding Intracellular Transport Processes Pertinent to Synthetic Gene Delivery Via Stochastic Simulations and Sensitivity Analyses. Biophysical Journal 2007;92(3) 831-846.

[152] Ng CP, Pun SH. A Perfusable 3D Cell-Matrix Tissue Culture Chamber for In Situ Evaluation of Nanoparticle Vehicle Penetration and Transport. Biotechnology and Bioengineering 2008;99(6) 1490-1501.

[153] Owens DE 3rd, Peppas NA. Opsonization, Biodistribution, and Pharmacokinetics of Polymeric Nanopartices. International Journal of Pharmaceutics 2006;307(1) 93-102.

[154] Ogris M, Brunner S, Schuller S, Kircheis R, Wagner E. PEGylated DNA Transferring-PEI Complexes: Reduced Interaction with Blood Components, Extended Circulation in Blood and Potential for Systemic Gene Delivery. Gene Therapy 1999;6(4) 595-605.

[155] Merdan T, Kopeced J, Kissel T. Prospects for Cationic Polymers in Gene and Oligonucleotide Therapy Against Cancer. Advanced Drug Delivery Reviews 2002;54(5) 715-758.

[156] Moghimi SM, Szebeni J. Stealth Liposomes and Long Circulating Nanoparticles: Critical Issues in Pharmacokinetics, Opsonisation and Protein-Binding Properties. Progress in Lipid Research 2003;42(6) 463-478.

[157] Krieg AM. CpG Motifs in Bacterial DNA and their Iimmune Effects. Annual Review of Immunology 2002;20 709-760.

[158] Kunath K, von Harpe A, Petersen H, et al. The Structure of PEG-Modified Poly(ethyl-
eneimines) Influences Biodistribution and Pharmacokinetics of their Complexes with
NF-kappaB Decoy in mice. Pharmaceutical Research 2002;19(6) 810-817.

[159] Viola JR, El-Andaloussi S, Oprea II, Smith CI. Non-viral Nanovectors for Gene Deliv-
ery: Factors that Govern Successful Therapeutics. Expert Opinion on Drug Delivery
2010;7(6) 721-735.

[160] Muro S, Dziubla T, Qiu W, et al. Endothelial Targeting of High-Affinity Multivalent
Polymer Nanocarriers Directed to Intercellular Adhesion Molecule 1. The Journal of
Pharmacology and Experimental Therapeutics 2006;317(3) 1161-1169.

[161] Koren E, Torchilin VP. Drug Carriers for Vascular Drug Delivery. IUBMB Life
2011;63(8) 586-595.