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Review

Strategies for cancer gene-delivery improvement by non-viral vectors

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ABSTRACT

Lack of selectivity together with severe side effects in conventional cancer treatment have afforded the development of new strategies based on gene therapy. Nowadays, gene therapy is employed through both viral and non-viral vectors. In spite of the high transfection activity of viral vectors, some drawbacks have pointed out to non-viral vectors as a safer alternative. To overcome low efficiency as well as other issues associated with the use of non-viral vectors, complexes formed by lipids and polymers with DNA, named lipoplexes and polyplexes respectively, have been modified in order to improve its features. Suitability of cancer gene therapy also requires the capacity to distinguish between normal and tumoral cells. This requirement has been solved by the addition of specific ligands that enable receptor binding and subsequent endocytosis. In this article we review the most recent approaches in structure modification of non-viral vectors through different methods comprising conjugation, addition of helper lipids or changes in design and synthesis as well as the strategy based on exploiting receptors that are usually overexpressed in malignancies. Such improvements confer specificity, efficient gene delivery, condensation, protection of DNA and low levels of toxicity avoiding off-target effects which turn into a potential tool to treat cancer.

1. Introduction

Most types of cancers are treated by a wide range of treatments such as radiotherapy, chemotherapy or immunotherapy. Although current therapy provides tumor regression and good results, some drawbacks such as high toxicity, drug resistance or off-target effects in organs and tissues have aroused interest in novel therapeutic approaches such as gene therapy (Schirrmacher, 2019).

Gene therapy is considered as a tool to transfer nucleic acids such as pDNA, siRNA or mRNA in order to treat or alleviate a wide range of disorders from infectious and inherited diseases to cancer. Regarding cancer approaches, gene therapy is focused on the therapeutic delivery into cells in order to overexpress or knock-down the gene of interest with the aim to eliminate or reduce tumor size by different mechanisms. Although the simplest way of gene therapy consists of the use of naked DNA, large size of DNA and its vulnerability to nuclease degradation attack in serum are problems that can be solved through the use of carriers, also known as vectors (Al-Dosari and Gao, 2009; Amer, 2014; Weichselbaum and Kufe, 1997). It is well known that vectors in gene therapy are divided in two principal types: viral and non-viral vectors.

Viral vectors (adenovirus, retrovirus, adeno-associated virus and herpes simplex virus) are characterized by presenting high transfection efficiency, however, toxicity and insufficient pharmaceutical quantities limit their use. On the other hand, although non-viral vectors are known to exhibit lower efficiency than viral vectors, present low toxicity, non-immunogenicity and the feasibility to be produced on a large scale. These properties together with the interest of gene therapy in focusing on receptor-mediated pathways by active targeting, make non-viral vectors the best option to develop in cancer approach (Al-Dosari and Gao, 2009; Kaneda and Tabata, 2006).

In this review, we will focus on recent advances in non-viral vectors emphasizing active targeting by endocytosis-mediated receptors in order to target specific cancer cells as well as improving transfection efficiency to make suitable vectors for cancer gene therapy.

2. Main vectors used in gene delivery

2.1. Viral vectors

Viral vectors are based on mediated-gene delivery by recombinant viruses that are able to transfer DNA or RNA into the host cell. The most common viruses used in gene therapy are: adenovirus, adeno-associated

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virus, retrovirus and herpes simplex virus. Although the use of viral vectors is extensively known due to high efficiency levels of transfection, disadvantages depending on the virus used, limit their use in order to avoid side effects or immune response (Sung and Kim, 2019; Weichselbaum and Kufe, 1997).

2.2. Non-viral vectors

In contrast to viral vectors, non-viral vectors are synthetic vectors based mainly on liposomes or polymers. The positive charge provided by cationic liposomes or polymers are able to interact and bind to DNA due to the presence of negative charges associated with the phosphate group, forming complexes. This electrostatically interaction between cationic non-viral vectors and DNA results in the compaction of DNA achieving small size (Al-Dosari and Gao, 2009; Kaneda and Tabata, 2006; Sung and Kim, 2019).

2.2.1. Lipoplexes

Lipoplexes are structures formed after interaction between negative charges associated with DNA and the positively-charged liposome. The fact that positive charges surrounded DNA, provide protection against nuclease attack, as well as, the linkage between lipoplexes and negatively-charged molecules on the cell membrane, improving cellular uptake. Lipids are divided into three main types according to this nature: cationic, anionic and neutral. Several lipids have been used in gene delivery such as 1-palmitoyl-2-oleoyl- *sn*-glycero-3-ethylphosphocholine (EPOPC), poly (lactic-co-glycolic) acid (PLGA), Dioleoylphosphatidylcholine (DOPC), 2,3-dioleyloxy-N-[2-(sperminecarboxamido)ethyl]-N,N-dimethyl −1-propanaminium trifluoroacetate (DOSPA), N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), although the most common are 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), 1,2-dioleoyl-sn-glycero-3-phosphatidyl ethanol amine (DOPE) and cholesterol (Al-Dosari and Gao, 2009; Felgner et al., 1987; Kaneda and Tabata, 2006).

Although most common cationic lipids have been used to form lipoplexes, some drawbacks such as short half-life in circulation need to be attended in order to improve transfection efficiency (Al-Dosari and Gao, 2009). It is well known that PEGylation mechanism appears to be effective in reducing particle aggregation as well as increasing half-time in lipoplexes and polyplexes (Harvie et al., 2000). However, one of the major disadvantages is its low biological activity. In order to improve biological activity, Xu et al. (2011) incorporated a cholesterol domain in PEGylated lipoplexes achieving highest levels of transfection when 0.4% PEG-cholesterol was incorporated to DOTAP/Chol/DNA lipoplexes with 69% (wt%) cholesterol in KB cells compared to other cholesterol percentages. Moreover, maximal transfection in the presence of 50% of serum was achieved at PEG-cholesterol above 1 mol% in KB cells (Xu et al., 2011).

Another way to improve transfection efficiency is by using helper lipids such as DOPE which is able to facilitate endosomal escape (Xu and Szoka, 1996). Changes in design and synthesis of cationic lipids such as Gemini Cationic lipids (GCL) can lead to a non-viral vector with better properties such as stabilization, compaction and protection of gene material in order to improve *in vivo* biological activity. Martínez-Negro et al. (2018a) demonstrated the non-viral vector suitability of lipoplexes comprised by a gemini-bolaamphiphilic lipid ($C_6C_{22}C_6$), DOPE and pDNA through lipid mixture presenting protection against DNase I, compaction and good transfection efficiency (TE). $C_6C_{22}C_6$-DOPE-pDNA lipoplexes formed a nanosized multillamellar Lα structure and exhibited higher expressions levels of luciferase in COS-7 cell line at both molar fractions (α), α = 0.2, α = 0.5 at effective charge ratio (ρ_{eff}), ρ_{eff} = 4 comparable to control (Lipofectamine 2000) together with 60–80% of viability, except for α = 0.2 ρ_{eff} = 10 (Martínez-Negro et al., 2018a).

A different approach to enhance transfection efficiency was also described by Martínez-Negro et al. (2018b) through replacing 2 imidazole groups by 2 histidine residues in head group of a GCL obtaining $C_3(C_{16}His)_2$/DOPE-pDNA lipoplexes. This change in GCL structure

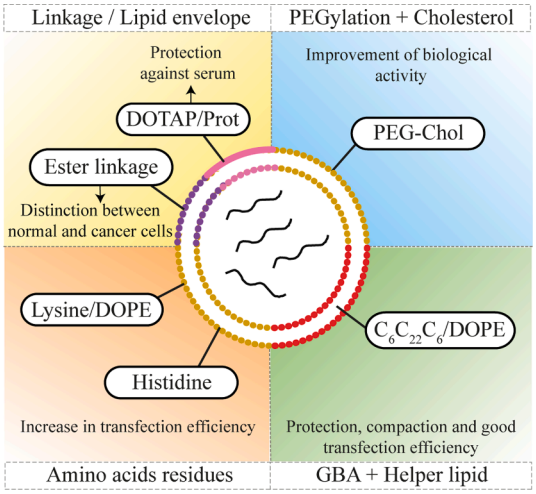

Fig. 1. Schematic representation of recent strategies to improve lipoplexes. Strategies are based on: incorporation of a cholesterol domain in PEGylated lipoplexes (Blue); using a gemini-bolaamphiphilic lipid (GBA) with a helper lipid (DOPE) (Green); addition of amino acid residues such as histidine and lysine/DOPE (Orange); modifying the linkage of the hydrophobic-alkyl chain or encapsulating DNA by a lipid envelope made of DOTAP/Protamine (Yellow).

provoked an increase in transfection efficiency of pCMV-IL12 together with good cell viability (80%) in COS-7 cell lines at two molar fractions α = 0.5 (ρ_{eff} = 4 and 10) and α = 0.2 (ρ_{eff} = 4) when compared to commercial Lipofectamine 2000 (Martínez-Negro et al., 2018b). Following the strategy of adding amino acid components in order to potentiate transfection levels, a lysine-residue linked to a C12 chain (LYC1, α = 0.2) in combination with DOPE (80%) was also studied. This combination formed well organized stabilized lipoplexes (LYC1/DOPE-pDNA) in a lamellar lyotropic liquid crystal phase capable of showing higher ng of luciferase per mg of protein (α = 0.2 and ρ_{eff} = 10) compared to Lipofectamine 2000 in COS-7 cell line (Martínez-Negro et al., 2019).

Changes in design and synthesis also influence size, zeta potential and transfection efficiency. Huang et al. (2011) synthetized different cationic lipids consisting of the same protonated cyclen and imidazolium salt as a headgroup and differing in the linkage of the hydrophobic alkyl chain, via methylene (L1) or via ester with different ester orientation (L2 and L3). Although L1 and L3-lipoplexes showed better hydrodynamic diameter (100–300 nm) with positive zeta potential compared to L2-lipoplexes, it was demonstrated that higher levels of transfection were achieved by L3-lipoplexes (lipid/DOPE; 1:2) (3.8-fold than L1) even surpassing Lipofectamine 2000 with low levels of cytotoxicity. Moreover, transfection efficiency studies revealed that L3 was able to transfect HepG2 and H460 cells but no luciferase levels were found in HEK293 cells, noticing that L3-lipoplexes are able to distinguish between normal and cancer cells (Huang et al., 2011).

Another hurdle to overcome is the interaction between DNA and serum proteins that lead to inactivation (Escriou et al., 1998; Yang and Huang, 1997). In this respect, Caracciolo et al. (2011) encapsulated protamine/pDNA by a DOTAP lipid envelope (DOTA/P-DNA) achieving higher transfection efficiency in different cell lines when compared to a complex system without envelope (DOTAP/DNA). Encapsulation of protamine/DNA was possible at lower amounts of cationic lipid (DOTAP) which makes it less toxic to cells. DNA protection showed no differences in the absence or presence of serum. Moreover, physical–chemical properties played an important role in DNA release and interaction with cellular membranes. The more fusogenic capacity of lipid/protamine/DNA (LPD) promotes DNA releasing from endosomes while lipoplexes accumulate at the nuclear membrane (Caracciolo et al., 2011). Fig. 1 summarizes all the aforementioned strategies to improve

the features of lipoplexes.

2.2.2. Polyplexes

When positive charges associated with cationic polymers are complexed with negative charges from DNA, they form complexes called polyplexes. Besides being more stable than lipoplexes, polyplexes are able to achieve a better compaction of genetic material. The main polymers used for gene delivery are the cationic and the amphiphilic polymers (Al-Dosari and Gao, 2009; Kaneda and Tabata, 2006; Sung and Kim, 2019).

Although polyethylenimine (PEI) is one of the most used cationic polymers in gene delivery because of its excellent transfection efficiency, disadvantages such as non-biodegradability and high toxicity limit its use (Al-Dosari and Gao, 2009; Sung and Kim, 2019). Toxicity and non-degradable drawbacks were overcome by Ding et al. (2020) through incorporation of biodegradable acrylated (PLA) to branched PEI. Besides its biodegradability due to PLA addition, PLA-PEI copolymer showed an increase in cell viability compared to PEI cytotoxicity in HCT116, HepG2 and SKOV3 cell lines. Moreover, PEI-PLA@siRNA revealed high internalization efficiency equivalent to Lipofectamine 2000, making it a good vector candidate (Ding et al., 2020). Another strategy to reduce cytotoxicity and improve biodegradability and transfection levels was developed by Wu et al. (2020) through incorporation of histidine and lysine amino acids containing linkages to low molecular weight PEI (600 Da). Although PEI 600 Da exhibited low cytotoxicity due to its low molecular weight, showed poor transfection levels when compared to PEI 25 kDa. However, incorporation of histidine (HisP) and lysine (LysP) through Michael addition demonstrated high transfection efficiency levels in HeLa and B16 cell lines even in the presence of serum, thus improving gene delivery via the caveolae-mediated endocytosis pathway (Wu et al., 2020). Furthermore, Park et al. (2013) demonstrated high transfection efficiency as well as minimal toxicity when using O-carboxymethyl chitosan-grafted branched polyethylenimine (OCMPEI). High luciferase transfection levels were achieved with 10–15% pDNA/OCMPEI polyplexes at a weight ratio of 16 w/w in HEKD293, HCT119 and LoVo cell lines together with good cells viabilities (80% or higher) compared to branched polyethylenimine (bPEI). Furthermore, a silencing capacity was also demonstrated when HCT119 cells were pretreated with pEGFP-OCMPEI before transfection with GFP-siRNA, revealing an important decrease in GFP expression. This indicates the suitability of this vector to deliver pDNA as well as siRNA as a new approach in order to combine and potentiate therapeutic effects (Park et al., 2013).

Another way to improve gene delivery with no toxicity was developed by Navarro et al. (2011) through micelle-like-nanoparticles (MNPs) based on the combination of phospholipid-Polyethylenimine conjugates (PLPEI), with siRNA covered by 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), cholesterol and 1,2-disrearoyl-*sn*-glycero-3- phosphoethanolamine-N-[methoxy (polyethyleneglycol)-2000] (PEG-PE). MNPs presented a good hydrodynamic size (213 nm) and neutral surface charge at N/P ratio of 10 with good stability, protection and condensation of siRNA. Downregulation of GFP expression was demonstrated when MNPs with loaded with GFP-siRNA were assessed in GFP-expressing c166 cell lines, exhibiting a 20% of reduction of GFP fluorescence when compared to negative-siRNA loaded MNPs, as well as no toxicity in B16F10 and NIH/3T3 cell lines; presenting a cell viability of 80% when compared with positive control PEI 25 kDa (Navarro et al., 2011).

Other strategies are based on improving transfection efficiency with low levels of toxicity by synthesis of novel dendriplexes. Arnáiz et al. (2012) showed an increase in transfection efficiency by using amine-terminated carbosilane dendriplexes via Huisgen *cyclo*-addition with an ammonium group per branch in its structure, named as Family 1 that comprises one (F1G1), second (F1G2) and third generation (F1G3). Second generation (F1G2) dendriplexes at 5/1 charge ratio as well as third generation (F1G3) at 20/1 charge ratio exhibited higher levels of transfection in HepG2 and HeLa cell lines respectively compared to control fourth generation Poly(amidoamine) (4G PAMAM). Regarding *in vivo* studies, F1G3 was administered systemically in mice at a charge ratio of 20/1, obtaining higher levels of transfection in the lung and the liver when compared to control group (4G PAMAM at 5/1 charge ratio) (Arnáiz et al., 2012).

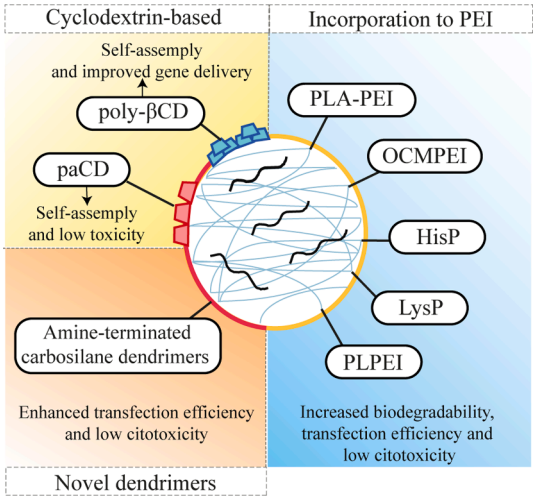

Fig. 2. Schematic representation of recent strategies to improve polyplexes. Strategies are based on: incorporation of biodegradable acrylated (PLA-PEI), O-Carboxymethyl chitosan (OCMPEI), histidine (HisP) or lysine (LysP) amino acids or conjugate phospholipid (PLPEI) to PEI (Blue); synthesis of novel dendriplexes (Orange); synthesis of polycationic amphiphilic cyclodextrin (paCD) or polycationic clusters centered on a β -CD scaffold with ditopic host (poly- βCD) (Yellow).

In contrast to lipoplexes, polyplexes condense genetic material in an efficient way (Elouahabi and Ruysschaert, 2005). This advantage has been exploited in order to achieve efficient gene delivery in preclinical studies. Although polycationic cyclodextrines are known to present self-organization properties, Méndez-Ardoy et al. (2011) improved self-assembly as well as enhanced cell membrane crossing capabilities (size of 67 nm and Zeta potential of 34 mV) compared to bPEI (211 nm and 26 mV) by a polycationic amphiphilic cyclodextrin bearing 14 protonable amines (T-2, N/P 10) with good cell viabilities (75–80%). *In vitro* results showed that T2-CDplexes containing IL-12 exhibited high transfection levels in HepG2 cells and HeLa independent and dependent on the N/P ratio respectively. *In vivo* studies through systemic (intravenous) injection of T2-CDplexes at N/P 10 were toxic to mice, however, administration of T2-CDplexes at N/P 5 exhibited 90% of survival rate in mice treated with luciferase plasmid and relatively high levels of transfection, especially in the liver (Méndez-Ardoy et al., 2011). Self-assembling properties were also enhanced by Carbajo-Gordillo et al. (2019) through molecular engineering based on trehalose to access cationic Siamese twin surfactants. Siamese twin cationic glycolipids were carried out highlighting compounds 8 (spherical multilayered) and 10 (spherical-core–shell) which were studied *in vivo* showing higher levels of transfection in liver and lung respectively as well as self-assembling properties (Carbajo-Gordillo et al., 2019).

Some polyplexes present no endosome-lytic characteristics, hindering gene delivery (Cho et al., 2003). Gallego-Yerga et al. (2015) improved self-assembly and gene delivery by developing polycatonic clusters based on a β-CD-scaffold with ditopic host (1) and bisadamantanes (2a-h) guests. Nanoparticles showed a reversible behavior that allowed condensation and delivery of genetic material, promoting endosomal escape and DNA release in COS-7 and HepG2 cell lines. High luciferase gene expression was obtained with 1:2 d and 1:2 f at N/P 10 in both COS-7 and HepG2 cell lines even in the presence of 60% of serum. Furthermore, transfection results when using the therapeutic plasmid IL-

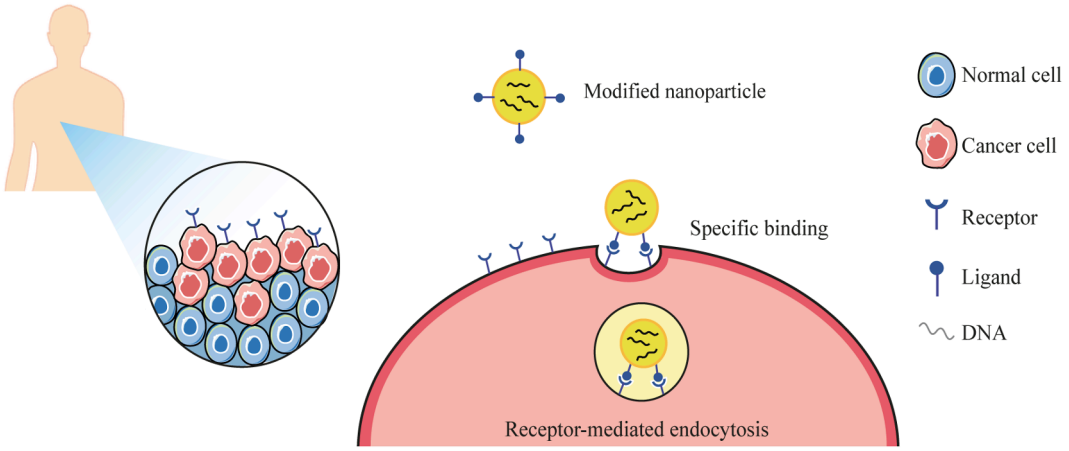

Fig. 3. Schematic summary of ligand-receptor binding through receptor-mediated endocytosis in cancer cells. Modified nanoparticles by incorporation of a ligand allow the specific binding to receptors that are found to be overexpressed in the surface of cancer cells.

12 showed also higher gene expression (200-fold) in the presence of serum (60%) when compared to bPEI. Nanocomplexes containing pCpG-hCMV-SPEC-eFLuc formulated with 1:2 d and 1:2 f were assessed in mice exhibiting high luciferase gene expression levels 20 h after systemically administration, especially in the liver and the lung (Gallego-Yerga et al., 2015). All these modifications in polyplexes are gathered in Fig. 2.

3. Receptor-mediated endocytosis strategy

One of the most important requirements in the use of non-viral vectors to treat cancer is the specific-targeting to cancer cells in order to avoid off-target effects such as those that occur with standard treatment. Specific-targeting is achieved by incorporating to nanoparticles specific oncologic proteins, known as ligands. Specific ligands are able to link to receptors that are currently overexpressed in cancer cells compared to normal cells. Upon ligand-receptor linkage occurs, specific-targeted nanoparticles are mainly internalized through endocytosis-mediated receptors (Fig. 3). The most common receptors that are overexpressed on tumor cell surface are transferrin, folate, asialoglycoproteins, CD44 and epidermal growth factor receptor. These receptors are appropriate targets for ligand-directed non-viral vectors, which can increase transfection efficiency compared to plain vectors and provide organ specificity for gene delivery (Matt Cotten et al., 1990; Davis et al., 2008; Normanno et al., 2006; Toole, 2009; Urbiola et al., 2014; Wu and Wu, 1988).

3.1. Targeted lipoplexes

Use of transferrin (Tf) in lipoplexes has been studied since it was found to be overexpressed in several tumors (M. Cotten et al., 1990; Kondo et al., 1990; Seymour et al., 1987; Vandewalle et al., 1985). Some factors such as lipid composition as well as charge ratio and cell line play an important role in transfection activity. This evidence was proved by Oliveira et al. (2009) who evaluated the association of Tf to different lipid formulations DOTAP-DOPE and DOTAP/Chol. Improved transfection was achieved in DOTAP/DOPE at 2/1 lipid/DNA charge ratio and DOTAP/Chol at 3/2 charge ratio in MG-63 and M3T3-E1 cell lines respectively (Oliveira et al., 2009). Tros de Ilarduya et al. (2002) developed transferrin lipoplexes containing DOTAP/DOPE and DOTAP/Chol for its use *in vitro* or *in vivo* respectively. DOTAP/DOPE lipoplexes presented increased transfection in the presence of 60% of serum which mimics *in vivo* conditions. Furthermore, Tf-DOTAP/DOPE lipoplexes exhibited high transfection activity in primary cells as well as differentiated cells (HepG2 and 3T3-L1) as charge ratio (+/-) was increased. The addition of protamine 0.5 μg/μg DNA as well as mixing Tf-protamine-DOTAP/Chol followed by the addition of cationic liposomes and DNA, demonstrated an enhanced gene delivery in mice after intravenous (i.v) administration, achieving the highest levels in lungs, heart, liver and spleen (Tros de Ilarduya et al., 2002). Transferrin lipoplexes were also studied by adding the IL-12 gene in order to enhance gene transfer and antitumor activity in colon cancer. Tf-lipoplexes containing DOTAP/Chol 5/1 (lipid/DNA) and IL-12 inhibited the growth of tumor in CT26 tumor-bearing mice. The reduction of the tumor size occurred 4 days after intratumoral injection and complete regression was achieved in 75% of the treated mice, increasing the survival rate (23 days post-administration). Moreover, high levels of IL-12 and IFN-ϒ were detected at 6 h after treatment when maximum inhibitory effect on tumor growth was achieved (Tros de Ilarduya et al., 2006). In order to improve nanoparticle circulation, Zhuo et al. (2013) administered Pegylated-Tf liposomes (PTf-Ls) coupled with IFNϒ inducible protein 10 (IP10) systemically in xenograft mice model. Results showed higher uptake of PTf-Ls as well as high transfection efficiency in HeLa cells. Through *in vivo* imaging by using a fluorescent molecule to label (LSS67), an increase in nanoparticles circulation for 72 h was detected besides an increased accumulation of targeted nanoparticles in tumor site, liver and bladder besides PTf-Ls targeted nanoparticles were internalized specifically in tumoral cells. Moreover PTf-Ls administration was able to suppress tumor growth (79% on day 50) and enhance survival rate in a 90% compared to non-targeted ones (40%) (Zhuo et al., 2013).

Folate receptors are not only overexpressed on cancer cells but also in tumor-associated macrophages (TAMs) that are part of the tumor microenvironment. Folate-modified lipoplexes containing BIM-S plasmid (F-PLP-pBIM) were tested in a folate receptor α positive cell line (LL/2) improving the biological activity of BIM plasmid. It was also assayed in a macrophage cell line which overexpressed folate β receptors (MH-S). *In vitro* results pointed to an increase in the quantity of apoptotic cells in wide stages in both LL/2 and MH-S cell lines, thus achieving one of the objectives of cancer therapy. *In vivo* studies exhibit a reduction in tumor growth after i.v injection of F-PLP/pBIM in FRα LL/2 mouse lung cancer model, achieving a reduction in tumor growth. Furthermore, it was demonstrated that folate association to lipoplexes resulted in higher transfection efficiencies than non-targeted therapy (Tie et al., 2020).

An alternative method in targeting folate receptors was performed by Zhi-Yao He et al. (2016) who developed lipoplexes with an hTERT promoter encoding pMP gene to treat ovarian cancer. Liposomes containing DOTAP, chol, mPEG-suc-Chol and F-PEG-suc-Chol were used unmodified (LP) or associated to folate (F-LP) via polyethylene glycol with plasmid control (pVAX) or the therapeutic agent (pMP). Apart from demonstrating a selective increased transfection activity in a folate receptor positive cell line (SKOV-3), *in vivo* experiments showed an enhanced gene expression of MP in tumor tissues as well as inhibition of tumor growth and increased median survival (80 days) when F-LP/

$pMP_{(2.5)}$ was administered i.p compared to F-LP/$pMP_{(1)}$ or formulations containing control plasmid pVax. Furthermore, subsequent studies demonstrated that F-LP/$pMP_{(2.5)}$ lipoplexes exhibited higher levels of apoptosis, inhibition in tumor proliferation and suppressing tumor angiogenesis compared to other aforementioned formulations (He et al., 2016).

Another interesting approach is the use of ligands such as asialofetuin (AF) that bind to specific organs. This ligand has been studied as an interesting target to deliver gene therapy directly to the liver due to the presence of asialogycoprotein receptors (ASGPr) in hepatocytes (D'Souza and Devarajan, 2015). Arangoa et al. (2003) developed novel protamine-asialofetuin-lipoplexes composed of DOTAP and cholesterol. Protamine-AF-lipoplexes at 4/1 (+/-) charge ratio showed an increase in levels of luciferase gene expression in the presence of asialofetuin (1 μg/μg DNA) in HepG2 cells (16-fold higher transfection compared to plain lipoplexes). Furthermore, by using the optimal amount of protamine (0.4 μg/μg DNA), particle size decreased from 302 nm to 181 nm due to the condensing effect of protamine which enhanced the uptake via endocytosis of AF-complexes in HepG2 cells and in the liver. Regarding *in vivo* results, higher luciferase gene expression levels (12-fold) were found after i.v administration of Protamine-AF-lipoplexes when compared to plain lipoplexes (Arangoa et al., 2003). The use of different methods in the preparation of asialofetuin-nanoparticles is related to changes in transfection efficiency. Díez et al. (2009) developed asialofetuin targeted PLGA-DOTAP nanoparticles where DNA was encapsulated into AF-PLGA-DOTAP nanoparticles (NP1) or adsorbed on its surface (NP2). Transfection efficiencies of both formulations were increased in the presence of 60% of serum. However, higher expression levels of luciferase and IL-12 gene were achieved with NP1 nanoparticles together with good viability results (80%) in HepG2 (Díez et al., 2009).

Another form of achieving high levels of transfection efficiency was accomplished by Magalhães et al. (2014) through incorporation of a glycolipid containing a galactose terminal residue (lactosyl-PE) in lipid-based nanoparticles in order to guarantee the specific binding to asialoglycoprotein receptors (ASPGR). The incorporation of 15% lactosyl-PE in EPOPC:Chol:lactosyl-PE/DNA lipoplexes 2/1 (+/-) charge ratio, improved transfection efficiency with a high percentage of transfected HepG2 cells (40%). Moreover, lactosyl-PE nanoparticles not only enhanced cell binding and uptake but also physicochemical properties, showing a decrease in size forming clusters besides positive zeta potential which makes it suitable vectors for preclinical studies (Magalhães et al., 2014).

With respect to hyaluronic acid (HA), Leite Nascimento et al. (2016) demonstrated that presence of HA-DOPE into DOPE:DE liposomes containing siRNA (HA-lipoplexes) exhibited an increase in size as well as enhanced stability avoiding aggregation in the presence of serum. Thus, HA-lipoplexes, resulted in more stable nanoparticles compared to non-coated HA formulations. It has been established by fluorescence microscopy and flow cytometry that the internalization of HA-lipoplexes mediated by CD44 receptor was rapid and located in cell cytoplasm with HA-lipoplexes at a charge ratio (+/-) of 2. After uptake and internalization, modified HA-lipoplexes (+/- 2) lead to an 81% inhibition of luciferase expression in A549-luciferase cells *in vitro*. Furthermore *in vivo* results also demonstrated inhibition of luciferase expression in mice bearing A549 metastatic cancer as well as homogenous distribution of HA-lipoplexes throughout the lungs measured by luminescence (Leite Nascimento et al., 2016).

Another specific targeting strategy in order to improve gene expression is through incorporation of epidermal growth factor (EGF) to DOTAP/Chol (1:0.9) liposomes. Dynamic light scattering studies demonstrated that increasing amounts of EGF provoked a slight increase in hydrodynamic size as well as a decrease in zeta potential. DOTAP/Chol lipoplexes at 5/1 (+/-) were studied *in vitro* and *in vivo*, showing an increase in luciferase gene expression in both HepG2, DHDK12proB cell lines and mice after systemically injection (1 μg of EGF/μg of DNA), noticing the presence of gene expression in the lung and maximal expression 24 h after administration of complexes besides low toxicity (Buñuales et al., 2011).

3.2. Targeted polyplexes

Transferrin has also been studied in polyplexes. Urbiola et al. (2015) evaluated PAMAM-Transferrin conjugates (P-Tf). P-Tf conjugates showed a decrease in hydrodynamic diameter and an increase in Z potential values as N/P ratio increased. However, as percentage of P-Tf raised in the formulation, Z potential values decreased almost achieving electroneutrality. Moreover, transfection efficiency was improved compared to other dendriplexes and competition assays demonstrated endocytosis-receptor mediated uptake. *In vitro* studies revealed that luciferase activity was increased by using targeted dendriplexes at N/P 4 and 6 in HeLa or at N/P 6 in HepG2 cell line containing 50% of P-Tf with high cell viabilities (Urbiola et al., 2015). Although Tf ligand has been used to target many cancers, short residence time in the cell does not guarantee the complete payload delivered by nanoparticles. After validating the previous mathematical model, Chiu et al. (2014) solved this drawback by conjugating oxalate to Tf in polymeric nanoparticles (Oxalate-Tf-PNP). Intracellular trafficking studies showed that after incubation, oxalate-Tf-PNP exhibited higher internalization in PC3 human prostate cancer and A549 human lung cancer cell lines when compared to native-Tf-PNP. Thus, cellular adhesion and residence time were enhanced, making it a suitable vector to deliver payload in an efficient manner (Chiu et al., 2014).

Regarding the use of folic acid (FA) in polyplexes, Aranda et al. (2013) developed nanocomplexes formed by a polycationic amphiphilic cyclodextrin bearing 14 protonable N-atoms with pDNA and FA (T2:pDNA:FA). These Fol-CDplexes were described as new systems to promote specific folate-receptor mediated transfection and were internalized substantially. *In vitro* experiments revealed maximal transfection efficiency with Fol-CDplexes at N/P 5 containing 1 μg FA/μg DNA (1.7-fold higher than plain CDplexes) together with cell viabilities over 80% in HeLa cell line. After systemically administration and sacrifice, it was observed an increase in luciferase gene expression in the liver and mainly in the lungs compared to plain CDplexes (Aranda et al., 2013). Folic acid has been also used to decorate nanoparticles containing siRNA with the aim to get a higher gene knockdown. Association of folic acid to polyamidoamine dendrimer G4 (G4-FA) with siVEGFA showed lower levels of mRNA expression in HN12 cells compared to G4/siVEGFA, improving knockdown efficiency. By near-infrared fluorescence imaging (NIR) studies a high uptake as well as tumor retention was noticed with NIR-G4-FA compared to free NIR. Moreover, after a single dose an antitumor activity was observed, measured by a reduction in tumor volume at day 16 and 18, as well as a nearly complete inhibition in tumor growth in HN12 xenograft nude mice after the second dose. Such properties make G4-FA/siVEGFA a potential delivery system to treat local tumors unlike chemotherapy (Xu et al., 2017). Apart from improving gene transfection in folate receptor-bearing cells, folate has been used to enhance transfection efficiency of some polymers such as chitosan which present low efficiency as a drawback. Higher molecular weights of chitosan were related to improve silencing effect in cell lines that overexpress folate receptor. Both HeLa and OV-3 cell lines were treated with 25 kDa FA-chitosan-siRNA at 50:1 weight/ratio. Thus, achieving a silencing gene expression of 57% and 42% compared to 25 kDa non-targeted-chitosan-siRNA and non-treated cells respectively (Fernandes et al., 2012).

With reference to hyaluronic acid, Urbiola et al. (2014) developed Polyamidoamine-DNA-hyaluronic (P-HA) nanoparticles at N/P 6 through different preparation methods having a direct impact on gene expression levels. Physicochemical characteristics of covalently assembled nanoparticles showed a hydrodynamic size around 100 nm with non-variable Z potential values independently of de HA amount used. However, P-HA electrostatically assembled nanoparticles, exhibited

larger sizes as well as decreasing Z potential values as HA increased. Furthermore, covalently assembled P-HA showed better results of transfection in CD44 + receptor-expressing MDA-MB31 cells compared to electrostatically HA-dendriplexes and non-targeted dendriplexes. *In vivo* results after i.v administration in Balb-C healthy mice exhibit luciferase gene expression in the heart and the liver when using 50% of P-HA when comparing non-targeted complexes (Urbiola et al., 2014).

With respect to EGF, on the basis of previously described enhanced transfection of activated G5 PAMAM- dendriplexes by Navarro et al. (2009) (Navarro and Tros de Ilarduya, 2009), Yin et al. (2012) synthetized activated EGF-PAMAM-dendriplexes via self-assembling. EGF-PAMAM-dendriplexes demonstrated selective improvement of transfection efficiency in MDA-MB-231; a EGF receptor positive cell line when compared to EGF negative cell line. Moreover, activated dendriplexes showed lower toxicity than non-activated dendriplexes and low agonist effect was found in a proliferative cell line (MCF7/EGFR) after transfection with activated EGF dendriplexes, thus makes it a suitable vehicle to cancer gene therapy. With respect to *in vivo* results, higher signals were detected by NIR fluorescence in liver regions of mice treated and higher luciferase expression was achieved at 20 EGF/DNA weight ratio (Yin et al., 2012).

4. Clinical trials

Due to their transfection efficiency, viral vectors had led clinical trials in recent years. However, their immunogenicity, adverse effects and low levels of production features pointed out non-viral vectors as principal interest in order to treat cancer in patients where first or second-line treatment had shown no effect or presented disease progression (Bessis et al., 2004; Iyer et al., 2014).

Several clinical trials have been based on RNA interference (RNAi) in order to achieve post-transcriptional gene silencing. On the one hand, iRNA includes short hairpin RNA (shRNA). Clinical trial based on shRNA (Pbi-shRNA) was carried out in order to treat Ewing's sarcoma. Pbi-shRNA comprises of DOTAP/Chol-based lipoplexes containing iRNA which is enabled to modulate the EWS/FL11 fusion gene expression. After demonstrating its efficacy, safety and specificity *in vitro* and *in vivo*, an open label study was carried out in 28 patients. A total of 8 infusions (twice a week for 4 weeks) of Pbi-shRNA EWS/FL11 type 1 LPX followed by 2 rest-weeks were performed in 8 years and older children in order to determine its safety profile, pharmacokinetics and disease response (Rao et al., 2016).

On the other hand, another type of iRNA used in gene delivery is small interfering RNA (siRNA). EphA2-targeting DOPC-encapsulated siRNA against ephrin type-A receptor 2, also known as EPHARNA was studied in 40 patients that presented spreadable solid tumors. Patients received intravenous administration over 120 min on days 1 and 4 and cycles were repeated each 21 days if there were no progression and no toxicity. Although no data is found about results of this trial, safety studies with EPHARNA were successfully carried out in Rhesus monkeys and was well tolerated at all administered doses (Wagner et al., 2017). Another example is ALN-VSP02, a siRNA-based drug encapsulated in a lipid nanoparticle able to target vascular endothelial growth factor (VEGF) and kinesin spindle protein (KSP) in patients with advanced solid tumors with liver involvement. A total of 41 patients previously treated with dexamethasone or chemotherapy, were treated intravenously every two weeks. After 2 cycles of ALN-VSP02, tumors were measured by computed tomography (CT) and tumor biopsies showed the presence of both siRNA, showing that the drug was delivered to the tumor. Antitumor effect with complete response was observed in a patient that exhibited endometrial cancer after 50 doses (0.70 mg/kg), while some patients showed stable disease after treatment (1 mg/kg) in renal and pancreatic-neuroendocrine cancer type. Moreover, ALN-VSP02 was well tolerated with minimal side-effects (fatigue, nausea and fever) compared to chemotherapy (Tabernero et al., 2013). Lipid nanoparticles encapsulating Atu027 was studied as siRNA therapy against protein kinase 3 (anti-PNK3) to treat advanced solid cancer. Atu027 was administered as a single dose (0.180 mg/kg) followed by 8 treatments within 4 weeks. Although 31 patients were enrolled in this study, 24 patients received Atu027 of whom 20 patients completed treatment. Studies showed no cytokine activation and a well-tolerated treatment (8 doses) with some side effects such as fatigue, hair loss and abdominal pain (Strumberg et al., 2012).

Lipid-based nanoparticles containing siRNA against c-MYC, an oncogene present in most types of cancer, were studied in patients with hematological malignancies. This multicenter and dose-escalation phase I and Ib/2 study consisted of administration of DCR-MYC (starting with 0.1 mg/kg/dose) by 2 h intravenous infusion, once a week for 2 weeks followed by a rest week (1 cycle) in patients who had no response to another treatment. Dose-escalation treatment (0.1, 0.125, 0.156, 0.2, 0.3 mg/kg) was carried out in 19 patients presenting different tumor types. Most common side-effects were: fatigue, nausea and infusion reactions. DCR-MYC treatments were well tolerated demonstrating clinical and metabolic responses (Tolcher et al., 2015). Encompassing siRNA-based therapy, siRNA directed against the M2 subunit of ribonucleotide reductase (RRM2) was clinically studied. CALAA-01 was encapsulated by a cyclodextrin cationic polymer, a stabilizing polymer (AD-PEG) and a targeting polymer conjugated to human transferrin (AD-PEG-Tf). Administration of CALAA-01 consisted of 21-days cycle (infusions days 1, 3, 8, 10 and 11 days of rest). First patients enrolled in the phase Ia (19) received doses from 3 to 30 mg/m^2 and no dosing-limiting effects were observed at a first glance. After trial resumption, patients that were treated with 30 mg/m^2 exhibited dose-limiting toxic effects. Therefore, in order to improve CALAA-01 tolerability, patients (5) were pre-treated with a low dose of CALAA-01 before receiving a higher dose (phase Ib) but lower doses were also not well-tolerated by patients. In order to avoid liver and kidney toxicity observed in preclinical studies, patients were pre-treated by i.v hydration 5% of dextrose before CALAA-01 treatment. CALAA-01 treatment showed several adverse effects (fatigue, chills, hyponatremia, sinus bradycardia), thus 21% of patients abandoned the study (Zuckerman et al., 2014).

A different approach in clinical trials consists of the delivery of plasmid DNA, such as Allovectin-7 which comprises DNA sequences encoding HLA-B7 and β-2 microglobulin and encapsulated by a plasmid/lipid complex. A phase II trial of Allovectin-7 was carried out in 133 patients in order to treat metastatic melanoma. Allovectin-7, a gene-based immunotherapy enable to express MHCI, was injected intralesionally once a week for 6 weeks (1 cycle) followed by 3 weeks of evaluation. After a dose-escalation study, it was observed that 2 mg dose was well tolerated without toxicity. Furthermore, patients were stratified into group 1 (single injectable lesion) and group 2 (multiple injectable lesions). Median duration of response was 13.8 months and 3.1% of patients achieved complete response, besides patients that presented partial response (8.1%). Patients in which lower doses of Allovectin-7 was administered, showed no response. With respect to patients that were stratified, it was demonstrated that no greater effect of Allovectin-7 was achieved when the 2 mg dose was divided and injected in multiple lesions. Although 61.4% of patients showed response after 1 cycle of treatment, the median time-response was 2 cycles. Allovectin-7 showed a good safety profile without any adverse effect of grade 3, however some patients presented vitiligo probably caused by the induction of delayed immunologic reaction (Bedikian et al., 2010). In accordance with pDNA delivery, DOTAP/Chol nanoparticles complexed with a plasmid expression cassette encoding FUS-1, (DOTAP/Chol-fus1) were studied to treat non-small cell lung cancer. In this case the aim expected of this lipoplex was the expression of fus-1 in cancer cells. To achieve this goal, DOTAP/Chol-fus1 was administered intravenously once every 3 weeks in patients with advanced lung cancer that were previously treated with chemotherapy. A total of 32 patients were treated with DOTAP:Chol-Fus1 showing a safe profile, gene expression and anti-tumor effects (Lu et al., 2012). Another plasmid based nanoparticle was EGEN-001 which consists of human pro-

Table 1
Non-viral vectors under clinical trial in cancer treatment.

Delivery system	Gene therapy drug	Indication	Phase	Status	NCT number	Reference
DOTAP/Chol	Pbi-shRNA™ (EWS/FLI1 type 1 LPX)	Ewing's sarcoma, sarcoma	I	Active	NCT02736565	(Rao et al., 2016)
DOTAP/Chol	DOTAP:Chol-Fus1	Lung cancer	I	Completed	NCT00059605	(Lu et al., 2012)
DOPC	EphA2 siRNA	Advanced malignant solid neoplasm	I	Recruiting	NCT01591356	(Wagner et al., 2017)
Lipid nanoparticle based siRNA	ALN-VSP02	Solid tumor	I	Completed	NCT00882180	(Tabernero et al., 2013)
Liposome + TfRscFv	SGT-94	Neoplasm	I	Completed	NCT01517464	(Siefker-Radtke et al., 2016)
Plasmid-lipid complex	Allovectin-7	Metastatic melanoma	II	Completed	NCT00044356	(Bedikian et al., 2010)
Lipid based nanoparticle	ATU027	Advance solid tumors	I	Completed	NCT00938574	(Strumberg et al., 2012)
Lipid based nanoparticle	DCR-MYC	Solid tumor, NHL, pancreatic neuroendocrine tumors	I	Terminated	NCT02110563	(Tolcher et al., 2015)
PEG-PEI-Chol lipopolymer	EGEN-001	Recurrent ovarian carcinoma, fallopian tube carcinoma, primary peritoneal carcinoma	II	Completed	NCT01118052	(Alvarez et al., 2014)
Cationic polymer + Tf	CALAA-01	Solid tumors	I	Terminated	NCT00689065	(Zuckerman et al., 2014)

inflammatory cytokine interleukin 12 (IL-12) plasmid encapsulated with PEG-PEI-cholesterol lipopolymer. EGEN-001 was administered intraperitoneally at days 1,8, 15 and 22, repeating treatment every 4 weeks in order to treat ovarian, fallopian tube or primary peritoneal carcinoma. A total of 20 patients were treated with EGEN-001 whose majority showed no improvement after two chemotherapy regimens. Patients received a median of 2 cycles (median dose: 299 mg) and side effects were fatigue, fever, chills, abdominal pain and anemia among others. Final results showed that 7 of 16 patients had stable disease and 9 had progressive disease, presenting limited activity (Alvarez et al., 2014). Finally, the use of RB94 encapsulated in a liposome that is targeted to tumor cells by an anti-transferrin receptor single chain antibody fragment (TfRscFv) was studied to treat solid tumors. SGT-94 was administered at different doses of DNA twice a week for 3 weeks or 5 weeks depending on the dose in 13 patients. After i.v administration, patients showed no toxicity and specific-tumor-targeting consistent with preclinical results and minimal side effects. Clinical activity was evidenced at 2.4 mg dose achieving a complete remission or partial remission. One patient who achieved complete remission, was retreated after tumor progression and had partial remission (Siefker-Radtke et al., 2016). Table 1 summarizes recent cancer clinical trials based on non-viral vectors.

5. Concluding remarks

Most recent advances in improving non-viral gene delivery have been covered in this review. These advances include several strategies to overcome typical drawbacks in non-viral vector gene delivery such as short half-life circulation, low transfection efficiency, interaction or inactivation because of serum proteins and cytotoxicity in both lipoplexes and polyplexes. Regarding cancer, the most promising approach is focused on targeting nanoparticles through specific ligands in order to exploit many receptors that are usually overexpressed in cancer such as transferrin, folic acid or asialofetuin. Although more studies are needed in order to improve efficiency of non-viral vectors, advances in formulation and conjugation have demonstrated benefits in patients with minimal adverse effects in clinical trials. DOTAP:Chol-Fus1 and ALN-VSP02 clinical trials showed an anti-tumor effect with a safe profile. Furthermore, a patient involved in ALN-VSP02 clinical trial, achieved a complete response (CR). Another non-viral vector that achieved a complete response in a patient (SGT-94), was utilized to retreat tumor progression post-CR, attaining a second partial response (PR). In spite of most studies described have been completed or terminated, the fact that Pbi-shRNA and EPHARNA trials are still active with apparently safe and well-tolerated doses, represents a promising option that utilizes non-viral vectors in cancer therapy.

CRediT authorship contribution statement

María L. Santana-Armas: Visualization, Writing - original draft, Writing - review & editing. **C. Tros Ilarduya:** Conceptualization, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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