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REVIEW

We still have a long way to go to effectively deliver genes!

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ABSTRACT

Gene therapy is emerging as a revolutionary alternative to conventional therapeutic approaches. However, its clinical application is still hampered by the lack of safe and effective gene delivery techniques. Among the plethora of diverse approaches used to ferry nucleic acids into target cells, non-viral vectors represent promising and safer alternatives to viruses and physical techniques. Both cationic lipids and polymers spontaneously wrap and shrink the genetic material in complexes named lipoplexes and polyplexes, respectively, thereby protecting it and shielding its negative charges. The development of non-viral vectors commenced more than two decades ago. Since then, some major classes of interesting molecules have been identified and modified to optimize their properties. However, the way towards the final goal of gene delivery, i.e. protein expression or gene silencing, is filled with obstacles and current non-viral carriers still have concerns about their overall efficiency. We strongly believe that the future of non-viral gene delivery relies on the development of multifunctional vectors specifically tailored with diverse functionalities that act more like viruses. Although these vectors are still a long way from clinical practice they are the ideal platform to effectively shuttle the genetic material to target cells in a safe and controlled way.
In this review, after briefly introducing the basis of gene delivery and therapeutic applications we discuss the main polymeric and lipidic vectors utilized for gene delivery, focusing on the strategies adopted to overcome the major weaknesses inherent to their still limited activity, on the way towards ideal multifunctional vectors.

Key words: Cationic lipid, Cationic polymer, Gene therapy, Non-viral vectors, Stimuli-responsive gene delivery vectors, Targeting

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Is gene therapy the present and the future of medicine?

The discovery that genetic information is coded along the length of a polymeric DNA molecule composed of only four types of monomeric units called nucleotides is one of the major scientific achievements of the past century. The Human Genome Project (HGP) has recently completed the sequencing of human genes, opening new avenues for progress in medicine and biotechnology. Such knowledge of the whole genome sequence, together with the constant advance in the development of vectors for the delivery of nucleic acids into cells has led to conceiving new therapeutic strategies for the treatment of pathologies by genetic and cell-based approaches, collectively known as gene therapy (1). Gene therapy can be broadly defined as the introduction of genetic material, either RNA or DNA, into cells in order to modify and control their protein expression for therapeutic or experimental purposes. The pathway leading from a gene to a protein in eukaryotes includes the transcription of the double strand DNA (dsDNA) to primary RNA by RNA polymerase II, the processing of primary RNA to mature messenger RNA (mRNA), the export of mRNA to the cytoplasm, and its translation into a protein (2). Thus, there are manifold ways to modulate protein expression. Indeed, it is possible to supply cells with functional copies of defective genes and to prompt the over-expression of therapeutic proteins or block their synthesis by inhibiting transcription or translation, possibly affecting cellular processes inducing apoptosis, stimulating immune responses, or stimulating tissue regeneration (3), thereby paving the way towards a number of new outstanding possibilities for treating inherited and acquired pathologies (4).

According to data updated to June 2012 and presented by The Journal of Gene Medicine, since the onset of the first gene therapy clinical trial in 1989, more than 1800 new clinical trials have been approved globally (http://www.wiley.com/legacy/wileychi/genmed/clinical/). The plethora of pathologies addressed by these trials encompasses all the most challenging diseases of the new millennium, that is, cancer (64.4% of approved trials), monogenic diseases (8.7%) such as cystic fibrosis, car-

diovascular diseases (8.4%) and infectious diseases (8%). However, despite the intensive study during the last few years, none of the gene delivery products has been approved yet by the Food and Drug Administration (FDA) or by the European Medicines Agency (EMA) for commercial use on humans.

Gene delivery techniques: from naked nucleic acids to vectors

One of the main reasons why gene therapy clinical trials are still few in number is the lack of suitable and safe techniques to deliver the genetic material to target cells (i.e. transfection). Although direct administration of naked nucleic acids is rather ineffective in transfection because of the anionic charge of DNA and RNA that ineffectively interacts with the negatively charged plasmalemma, it is nowadays the safer approach and remains one of the most frequently used methods in clinical trials (18.3% of approved trials) (5). The strategies developed thus far to increase DNA delivery to cells include application of physical-mechanical stimuli and use of gene delivery vectors. Although physical techniques (mainly electroporation, gene gun, hydrodynamic injection and ultrasound-based sonoporation) enable a relatively efficient gene delivery, they are expensive and not suitable for most of in vivo applications (6,7). For these reasons, several types of gene delivery vectors have been proposed and investigated in depth. Effective gene delivery vectors should be able to: (i) protect nucleic acids against degradation by blood and interstitial nucleases; (ii) promote internalization of the genetic material into target cells; (iii) release nucleic acids once inside the cell to the correct site. Furthermore, gene delivery systems should also be safe for both patients and operators, easy to use and as inexpensive as possible. There are two main classes of gene delivery vectors for gene therapy: (i) viral and (ii) non-viral vectors.

Viral vectors: powerful tools with safety issues

Although a comprehensive description of viral vectors is beyond the scope of this review paper and has been covered and extensively discussed elsewhere (8-11), it is surely useful to discuss the most prominent aspects of viral gene delivery. Modified wild-type viruses are powerful gene vectors for gene therapy, capable of efficiently transferring genetic material into mammalian cells both ex vivo and in vivo (11). Wild-type viruses have naturally evolved specific mechanisms to deliver their genetic material to cells and to exploit the host cell replication process, thereby representing an excellent platform for the development of recombinant vectors containing foreign genes for gene delivery purposes. The genetic material of two of the most popular viral vectors, retroviruses and adeno-associated viruses (AAV), integrates into the host genome, posing a risk of insertional mutagenesis, whilst transient expression can be obtained by adenoviruses (AdV) (12,13). Unfortunately, viral vectors also share many drawbacks. For instance, they have a low carrying capacity. Moreover, the human immune system recognizes and fights off viruses, shortening their effectiveness. Nonetheless, biosafety issues were raised after the death of a patient during a clinical trial in 1999 owing to an abnormal immune response to the virus used as a delivery agent (14).

The cheaper, safer, efficient and customizable alternative of non-viral vectors

Non-viral vectors for gene transfer are a simpler, cheaper and, most importantly, safer alternative to viral vectors. In fact, they can be produced on a large scale with high reproducibility and acceptable costs, they are relatively stable to storage, they can be administered repeatedly with no or little immune response and the dimension of the genetic material they can ferry is virtually unlimited (15). Nonetheless, the poor ability in delivering the genetic material to the target cells as compared to viruses has hardly limited the employment of non-viral gene delivery vectors in clinical trials thus far. In addition to therapeutic applications, they have become a highly widespread technique in genomics (16) and, in general, in all bio-laboratories in fundamental and applied research (17-19).

Non-viral vectors for gene delivery mainly encompass two major classes: cationic lipids and cationic polymers, even if some examples of neutral and negative vectors have also been reported (20). Both lipids and polymers are positively charged at physiologic pH and naturally interact with polyanionic nucleic acids, self-assembling into nanoscaled complexes named lipoplexes for the former class, and polyplexes in case of the latter. These cationic complexes interact with the plasma membrane and are internalized by cells into intracellular vesicles denominated endosomes by means of an active uptake mechanism called endocytosis (21). Cationic lipids and polymers were first introduced in 1987 by Felgner and Wu, respectively, as novel gene delivery systems (22,23). Since then, a number of lipid- and polymer-based non-viral gene delivery systems have been synthesized and developed and several of them are currently under investigation as potential tools for gene therapy. Nevertheless, although tremendous progress has been made in recent years, no gene delivery vectors have proved reliable enough yet to be used in clinic. It is becoming increasingly clearer that the concept of universal vector for gene delivery is somehow utopian, and the next generation of gene delivery systems will probably consist of multifunctional vectors properly tailored for specific applications.

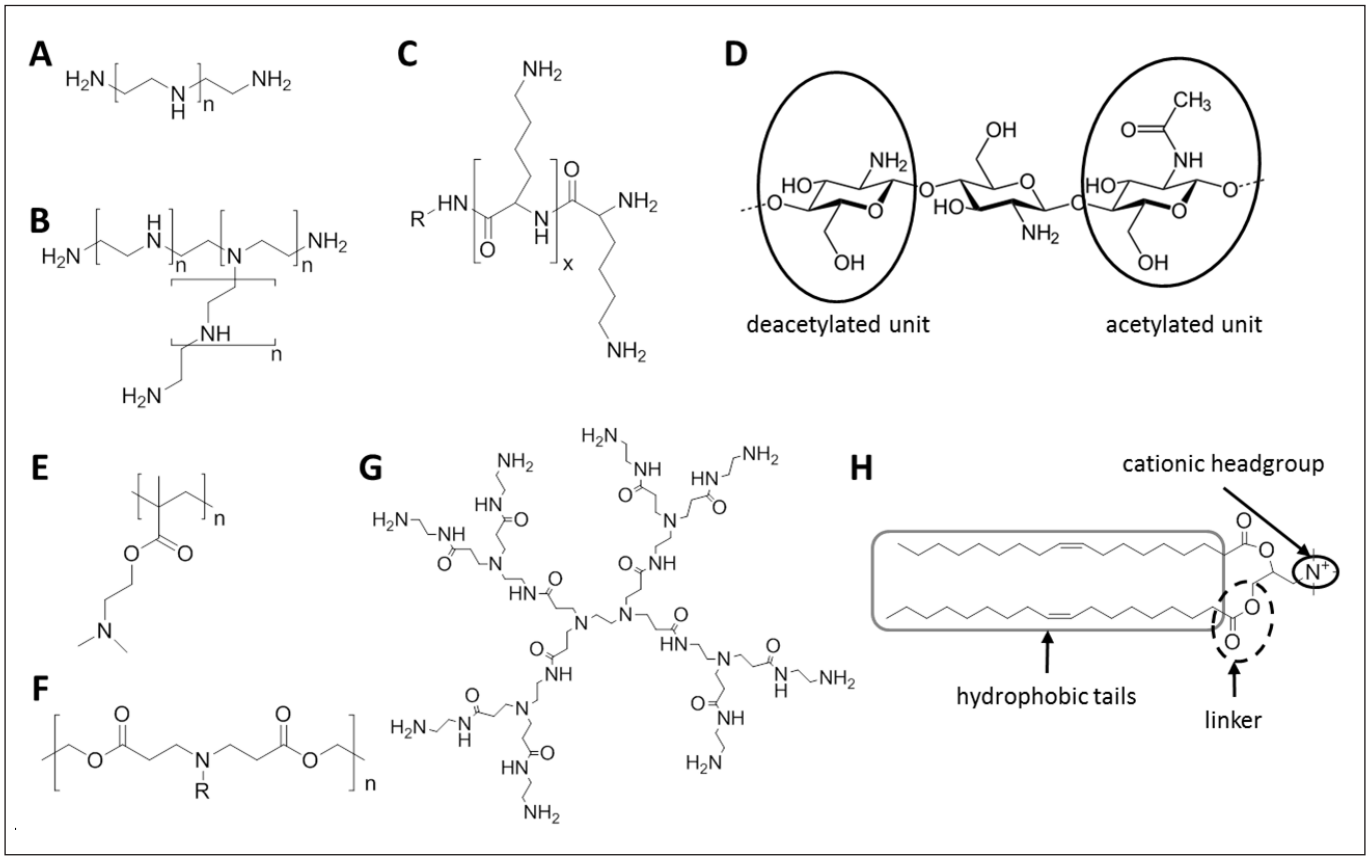

Fig. 1 - *Chemical structure of (A) linear poly(ethylenimine) (lPEI), (B) branched poly(ethylenimine) (bPEI), (C) poly(L-lysine) (PLL), (D) chitosan, (E) poly(2-(dimethylamino)ethyl methacrylate) (PDMAEMA), (F) an example of generic poly(amino ester) (PAE), (G) generation 2 poly(amidoamine) (PAMAM) and (H) 1,2-bis(oleoyloxy)-3-(trimethylammonium)propane (DOTAP).*

Cationic polymers: tailoring chemical properties to optimize transfection

Polymeric systems have been extensively studied for gene delivery, as for other medical applications (24,25), because of their high degree of molecular diversity; they can be modified to fine-tune their physicochemical properties (26). For instance, molecular weight (MW), polydispersity (PD), composition and density of side chains of a given polymer can be collectively altered to increase delivery efficiency and biocompatibility. Polymeric gene vectors generally bear protonable amines and, based on their chemical composition, can be broadly divided into: (i) poly(ethylenimine)-based (PEI-based) polymers (Fig. 1 A, B); (ii) polypeptide-based polymers, such as poly(L-lysine) (PLL, Fig. 1 C); (iii) carbohydrate-based polymers, such as chitosan (Fig. 1 D) and cyclodextrins; (iv) poly(methacrylate)-based polymers, mainly poly(2-(dimethylamino)ethyl methacrylate) (PDMAEMA, Fig. 1 E); (v) poly(amino esters) (PAE, Fig. 1 F).

Cationic polymers can be further classified based on their three-dimensional architecture into linear, branched, hyperbranched polymers and dendrimers. The latter are characterized by well-defined size and structure and by a narrow PD index (27), being synthesized by gradual stepwise methods yielding a unique molecular architecture. Indeed, cationic dendrimers such as poly(amidoamine) (PAMAM, Fig. 1 G) and poly(propylenimine) (PPI) are composed of repeating units emanating from a central core molecule from which highly branched, tree-like arms originate in an ordered and symmetric fashion.

Herein PEI, PLL and chitosan, three of the most extensively studied polymers for gene delivery, will be briefly discussed as representative examples, focusing on their strengths and weaknesses and on suitable strategies adopted to enhance their efficacy.

Poly(ethylenimine) (PEI)

PEI is one of the most studied and employed non-viral gene carriers and often regarded as the gold standard polymeric transfectant because of its superior transfection efficiency. The high charge density of PEI is responsible for its ability to bind DNA but also of its relatively high

cytotoxicity that has hampered its application (28). PEI does exist in a linear (lPEI) or branched (bPEI) form, both effective in delivering genes. Although lPEIs possess only secondary amines, bPEIs bear variable ratios of primary, secondary and tertiary amines depending on the degree of branching. At physiologic pH not all the amines are protonated because of the different pK_a of amino groups that accounts for high buffering capacity of these polymers that in turn, elicits endosomal escape (29). In fact, polymers such as PEI can significantly increase their protonation state after endocytosis, when pH decreases to values from 4 to 6 owing to the activity of endosomal proton pumps. These so called "proton-sponge" polymers halt endosomal acidification, leading to a higher transport of protons together with the influx of counter ions to maintain the charge neutrality. This enhancement of the ion concentration in endosomes triggers osmotic swelling and, finally, may lead to endosomal rupture (30).

Transfection efficiency and cytotoxicity of PEI are both dependent on MW and degree of branching. In general, high MW (HMW) PEI can induce higher transfection rates but with a concomitant increase in cytotoxicity (31,32). Furthermore, comparative studies have pointed out that lPEI is less effective in condensing DNA compared to bPEI (33) and that the resulting polyplexes are less stable in physiologic fluids and, in general, in salt-containing buffers (34). Nonetheless, lPEI yields higher transgene expression *in vivo* and is generally less cytotoxic than bPEI at equivalent MW (34,35) that is instead a better transfectant *in vitro* in selected cell lines (36). However, it is possible that these differences between lPEI and bPEI could, at least in part, rely on differences in purity, PD, as well as on discrepancies between nominal and real MWs rather than to their different structure (37).

Poly(L-lysine) (PLL)

PLL, the oldest cationic polymer used for gene delivery purposes, is a biocompatible and biodegradable polypeptide-based vector (23). Low MW (LMW) PLLs (MW <3 kDa) do not form stable polyplexes, highlighting the importance of the length of the polypeptide chain for polyplex formation. Inversely, HMW PLLs are fairly cytotoxic transfectants in vitro (38) characterized by poor DNA release after internalization. Moreover, depending on the ionic strength of the environment, they have the tendency to form large aggregates that precipitate (39). Although the biodegradability of PLLs is a major strength in gene delivery, positively charged PLL-based polyplexes bind to plasma proteins and are then promptly cleared from the circulation by the reticuloendothelial system (RES) in vivo (40). Furthermore, when used alone, PLLs are very poor transfectants (41) because they do not show any "proton sponge" effect owing to the presence of only primary amines in the side-chains. Indeed, after cellular internalization, PLL-based polyplexes lie in an environment with average pH between 4.5 and 5, indicating that polyplexes, instead of being released into the cytoplasm, are trapped in the lysosomal trafficking pathway (42).

Chitosan

Chitosan is a naturally derived biodegradable linear aminopolysaccharide obtained by the deacetylation of chitin, which is the structural material of the exoskeleton of crustaceans. Owing to its biocompatibility and non-toxic profile, chitosan is widely used and investigated in the pharmaceutical industry for multiple purposes (43,44). Chitosan is composed of two randomly distributed saccharidic sub-units linked by β-(1-4) glycosidic bonds: N-acetyl-D-glucosamine (GlcNAc, acetylated unit) and D-glucosamine (GlcN, deacetylated unit). The conjugate acid of the primary amino group of the GlcN units has a pKa of 6.3-6.5 (45); thus, primary amines become protonated at acid and neutral pH and bind nucleic acids, forming polyelectrolyte complexes (46). On the other hand, since chitosan possesses a limited buffering capacity at physiologic pH, its transfection efficiency is lower compared to other non-viral vectors such as PEI (47,48). As for most of the other polymers, the transfection behavior of chitosan is affected by its MW and degree of deacetylation (DD). HMW chitosans form larger but more stable polyplexes featuring good transfection efficiency (49). As the DD increases, polyplexes become smaller and more stable (50) but less effective in transfecting cells, highlighting the need to balance polyplex stability and easiness of disassembly (51). Since the release of nucleic acids from chitosan-based polyplexes is mediated by the enzymatic cleavage of GlcNAc-GlcNAc linkages by lysozyme and endo-β-N-acetylglucosaminidase (52) in lysosomal vesicles (48,53), the higher the degree of deacetylation, the lower its enzymatic degradation and the release of nucleic acids.

Cationic lipids: three domains to determine the fate of nucleic acids

In 1987, Felgner et al. were the first to report the use of the synthetic diether-linked cationic lipid, *N*-[1-(2,3-dioleyloxy)propyl]-*N,N,N,*-trimethylammonium chloride (DOTMA), as a vector for the delivery of genes into cells (22) and since then, a number of cationic lipids for gene delivery have been synthesized. Among them, the most important and commercially available are 1,2-bis(oleoyloxy)-3-(trimethylammonium)propane (DOTAP, Figure 1 H), N-[2-({2,5-bis[(3-aminopropyl)amino]-1-oxopentyl}amino) ethyl]-N,N-dimethyl-2,3-bis[(1-oxo-9-octadecenyl) oxy] chloride (DOSPA), 3β[N-(N',N'-dimethylaminoethane)carbamoyl] cholesterol (DC-Cholesterol), Lipofectin™ (a 1:1

mixture of DOTMA and L-dioleoyl phosphatidylethanolamine, in short DOPE) and Lipofectamine™ (a 3:1 mixture of DOSPA and DOPE) (54).

Cationic lipids are composed of three basic domains: the hydrophobic group(s) (or tails), the positively charged head(s) and the linker group(s) that connects the other domains to each other (Fig. 1 H) (55,56).

- Hydrophobic group(s) (or tails). The two main types of hydrophobic moieties are represented by cholesterol-based derivatives and aliphatic chains that can be either saturated or unsaturated and typically comprised of C8-C18 hydrocarbon chains (57). It is commonly accepted that single-tailed cationic lipids are more toxic and less effective than their multi-tailed counterparts (58). Several studies suggest that transgene expression increases by decreasing acyl chain length or by increasing chain asymmetry, branching and unsaturation, owing to the decrease in the lipid's phase transition temperature, thus increasing the fluidity of lipoplexes. On the other hand, cholesterol-tailed lipids have demonstrated good transfection efficiency probably because steroids offer rigidity and enable forming very stable bilayer structures that are, however, prone to fusion (59). This accounts for endosomal disruption and a higher amount of DNA being released from the endosomal compartment (60).
- Cationic headgroup(s). This domain is responsible for interacting with nucleic acids. It usually consists of primary, secondary, tertiary amines, quaternary ammonium salts or polyamines known to increase DNA binding efficacy. The cytotoxicity of cationic lipids is often associated with their intrinsic cationic behavior at physiologic pH (28). In this regard, quaternary ammonium amphiphiles are more cytotoxic than their tertiary amine counterparts probably owing to the higher basicity of the former. A smart solution widely adopted to circumvent this problem is to delocalize the cationic charge of the headgroup onto heterocyclic rings such as pyridines (61), triazines (57) and imidazoles (62).
- Linker group(s). The linker moiety tethering polar and hydrophobic domains is the main entity responsible for the biodegradability of lipidic vectors. Linker groups encompass amides, carbamates, esters and ethers (54). In this regard, the ether linkages make cationic lipids (e.g., in DOTMA) hydrolytically stable and their aqueous suspension has shown an extremely long shelf-life (63). Carbamates and esters (e.g. in DOTAP) are stable in neutral environments such as the extracellular matrix (ECM) and the cytoplasm but are liable to acid-catalyzed hydrolysis in endo-lysosomes (64). Instead, the substitution of a carbamate with a relatively less stable amide linker was found to reduce transfection efficiency *in vitro* and *in vivo* (65). Moreover, recent investigations on amide and ester linkages have shown that the orientation of the linker group can significantly affect transfection efficiency (66).

Cationic lipids, as for all other amphiphiles once above their critical micelle concentration (cmc), spontaneously aggregate in water to form three structurally different phases depending on the packaging parameter *P*, defined as the ratio of the size between the polar head group and that of the hydrophobic domain (67,68). In general, lipids with small hydrophobic tail cross-section have conic geometry ($P<.5$) and self-assemble into a micellar phase (spherical micelles, wormlike or cylindrical micelles or non-inverted hexagonal H_I phase) with a positive membrane curvature. Lipids displaying headgroup and lipophilic tail with a similar cross-sectional area ($.5 < P \leq 1$) have cylindrical geometry and adopt the bilayer structure (lamellar $L_\alpha$phase) of liposomes with a quasi-zero membrane curvature. Finally, lipids with hydrophobic domain cross-section higher than that of the polar head ($P > 1$) give rise to an inverted non-bilayer morphology (inverted hexagonal H_{II} phase) characterized by a negative membrane curvature (69).

Cationic lipids are normally used in excess with respect to nucleic acids to prepare positively charged, effective complexes (54). When they are mixed with DNA (or RNA), the system self-assembles in lipoplexes whose final conformation strongly depends on the initial state of aggregation of lipids. For instance, cationic micelles, adhering electrostatically to nucleic acids, give rise to the so called “beads on a string” structure, an unstable conformation that leads to effective transfection only when micelles are destabilized and nucleic acids are compacted within low-curvature micelle-like structures (70,71). Two distinct structures can be obtained with liposomal vesicles instead. Nucleic acids wrap around the external surface of vesicles, triggering the destabilization of the lipid bilayer and the complete encapsulation of the genetic material in either lamellar complexes characterized by a lamellar L_α phase in which nucleic acids are sandwiched between bilayer membranes, or cylindrical complexes with an inverted hexagonal H_{II} phase with nucleic acids arranged on a hexagonal lattice surrounded by a lipid monolayer (72,73). The presence in liposomes of the neutral zwitterionic lipid DOPE or similar co-lipids eventually shift lipoplexes from a lamellar to an inverted hexagonal phase, thereby facilitating their endosomal escape that ultimately leads to increased transfection efficiency (67,74).

Behr’s group first reported a different approach called “monomolecular collapse” to form stable lipoplexes starting from non-aggregated lipids (75,76). In that case plasmid DNA was collapsed with a suitably tailored cationic cysteine-based detergent used well below its cmc, and lipoplexes were finally stabilized by spontaneous dimerization of the surfactant into a cystine-based lipid under oxidative conditions by means of the so called “DNA template-driven air dimerization” process. On this basis,

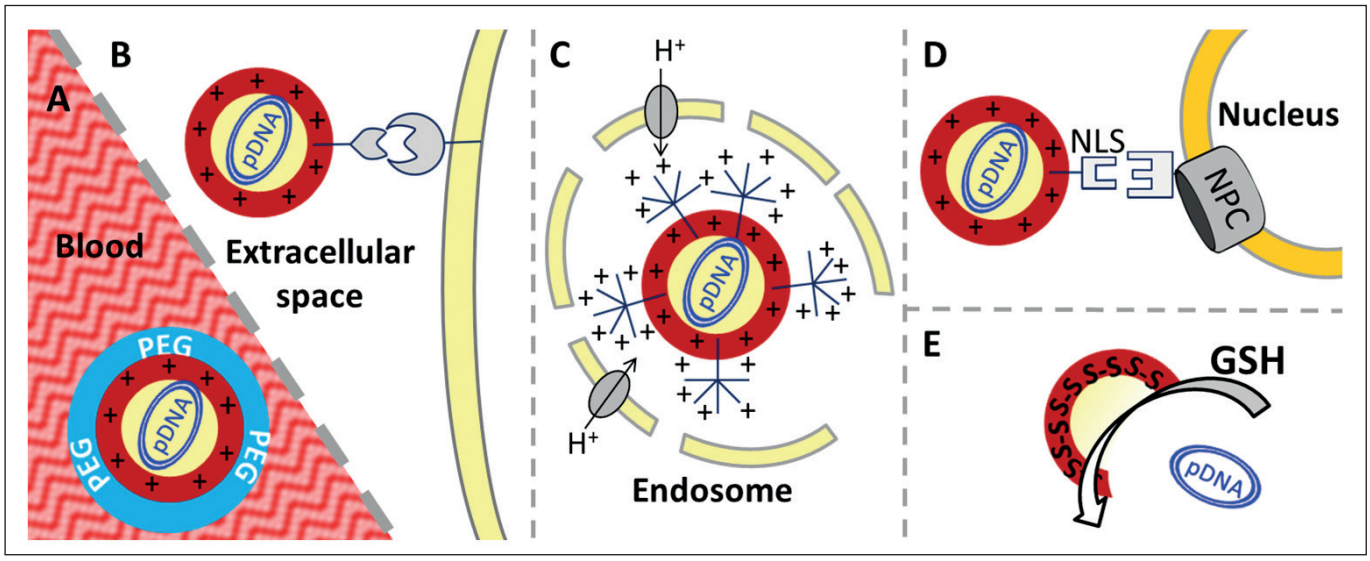

Fig. 2 - *Schematic representation of functionalized (smart) non-viral gene delivery vectors. (A) PEGylation enhances the pharmacokinetic profile of vectors in biological fluids such as blood; (B) grafted ligands enable targeting specific cells or tissues; (C) buffering moieties/membrane perturbing peptides promote endosomal escape; (D) grafted Nuclear Localization Signals (NLSs) mediate the nuclear uptake of the genetic material through Nuclear Pore Complexes (NPCs); (E) reducible carriers bearing disulfide bridges are more prone to intracellular disassembly and release of the genetic material; in this case plasmid DNA (pDNA), by means of glutathione (GSH).*

we have recently developed a series of redox-sensitive triazine-based cationic lipids featuring a fairly high transfection efficiency and low cytotoxicity (57), and demonstrated that the DNA-induced dimerization of sulfhydryl-containing surfactants is essential in enhancing the extracellular stability of lipoplexes (77,78).

The stairway to multifunctional carriers

Recent progress in understanding transfection mechanisms has enabled pointing out the major bottlenecks hindering efficient non-viral gene delivery. Based on this knowledge, a new generation of "active" vectors bearing specific functionalities that confer bio-responsiveness to gene delivery vectors has been developed.

The instability of the lipid/DNA complexes in biological fluids is a major problem in vivo. In fact, the positive charges of polyplexes and lipoplexes favor their non-specific electrostatic interactions with oppositely charged serum components leading to the formation of aggregates, thereby adversely affecting their transfection behavior and their in vivo pharmacokinetics (40). Since it has been demonstrated that gene delivery vectors can activate the complement system, concerns have been raised regarding their potential immunogenicity (79). Among different strategies that have been proposed to increase the stability of lipoplexes and polyplexes in biological fluids, the most widely used and useful consists of coupling cationic carriers with polyethylene glycol (PEG) (80,81), a hydrophilic, biocompatible and inert polymer utilized for charge shielding that enables preventing interaction between complexes and proteins, thereby improving their pharmacokinetic profile (Fig. 2 A) (40,82). Unfortunately, the "stealth" effect of PEG on gene delivery vectors has also proven to impair their cellular uptake and transfection activity (40,83).

A successful gene delivery strategy to confer selectivity, particularly suitable for PEG-shielded lipoplexes and polyplexes, is to provide them with targeting moieties to increase their binding and uptake into target cells. In this context, the grafting of ligands such as folate, sugars, peptides and antibodies specific for membrane receptors has been largely exploited (Fig. 2 B). As practical examples, galactose and lactose have been used to target liver cells (84), folate for tumors (85) and arginine-glycine-aspartic acid tripeptide motif (RGD) for endothelial cells (86). Furthermore, specific functional peptides can be grafted to gene delivery vectors (i) to enhance their translocation across the plasmalemma (i.e. cell-penetrating peptides, CPP), (ii) to promote their endosomal escape (i.e. membrane perturbing peptides, Fig. 2 C) and (iii) to mediate their nuclear import by passing through nuclear pore complexes (NPCs) (i.e. Nuclear Localization Signals, NLS, Fig. 2 D) (87). The derivatization with functional moieties usually involves basic groups of carriers, thereby reducing the content of cationizable groups available for interaction with nucleic acids with obvious drawbacks. Thus, a suitable degree of functionalization has to be found in order to obtain the desired selectivity toward certain cell types without affecting transfection behavior.

Given that non-viral vectors are mainly internalized by cells by endocytosis, endosomal escape is another key barrier to efficient transfection. In this way, whilst lipidic vectors and "buffering" polymers such as PEI can respectively take advantage of the membrane fusion process and the "proton sponge" effect to leave the endosome, other cationic polymers such as PLL and chitosan become stuck in it and perish after endosomal fusion with digestive lysosomes. With the purpose of increasing endosomal escape, endosomolytic agents such as chloroquine or membrane-disrupting peptides have been added during transfection, although this trick is of course unfeasible in vivo. Alternatively, aiming to confer buffering properties on vectors, their conjugation with urocanic acid, imidazol acetic acid or L-histidine, which all bear moieties with reasonably low pK_a values, resulted in increased transfection efficiency (Fig. 2 C) (47,88,89). Moreover, the "buffering" LMW bPEI has been grafted onto the enzymatically degradable chitosan to synthesize chitosan-graft-branched PEI copolymers taking advantage of the strengths of both components (90,91).

Finally, since nucleic acids must be released from complexes after their internalization and this is considered one of the main issues in non-viral gene delivery, a smart approach known as "disulfide linker strategy" relies on the use of redox-sensitive disulfide bonds as linker functionalities tethering monomeric vectors together in complexes whose stability can be spatially controlled by exploiting differences in redox potential existing between the extracellular and the intracellular milieu (Fig. 2 E) (92,93). In fact, disulfides are reduced back to sulfhydryls by the intracellular pool of antioxidants, among which glutathione (GSH) plays a pivotal role (21), leading to the disassembly of complexes and intracellular release of nucleic acids. This strategy has proven effective in both lipidic and polymeric vectors, often leading to higher transfection efficiency and lower cytotoxicity (77,94-96).

In conclusion, after the initial discovery, development and attempt to optimize several non-viral vectors for gene delivery it is now clear that the ideal, universal vector is nothing but a dream. Altogether, vectors should possibly be tissue targeted and charge-shielded to enhance their pharmacokinetic behavior. Moreover, they should be able to escape the endosome and release their payload either into the cytoplasm for gene silencing or into the nucleus by means of NLS sequences for transgene expression or over-expression. Even though diverse functionalizations have proven effective individually, the inclusion of different functionalities in the carrier structure only seldom leads to synergistic results. In light of these findings we strongly believe that the future of non-viral gene delivery will rely on the development of a new generation of more effective, application-specific multifunctional vectors whose properties should be tailored to the type of nucleic acid to be ferried and the cells and tissues to be targeted.

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