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Review

Design of multifunctional non-viral gene vectors to overcome physiological barriers: Dilemmas and strategies

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ABSTRACT

Gene-based therapeutics hold great promise for medical advancement and have been used to treat various human diseases with mixed success. However, their therapeutic application *in vivo* is limited due largely to several physiological barriers. The design of non-viral gene vectors with the ability to overcome delivery obstacles is currently under extensive investigation. These efforts have placed an emphasis on the development of multifunctional vectors able to execute multiple tasks to simultaneously overcome both extracellular and intracellular obstacles. However, the assembly of these different functionalities into a single system to create multifunctional gene vectors faces many conflicts that largely limit the safe and efficient application of lipoplexes and polyplexes in a systemic delivery. In the review, we have described the dilemmas inherent in the design of a viable, non-viral gene vector equipped with multiple functionalities. The strategies directed towards individual delivery barriers are first summarized, followed by a focus on the design of so-called smart multifunctional vectors with the capability to overcome the delivery difficulties of gene medicines, including the so-called the "polycation dilemma", the "PEG dilemma" and the "package and release dilemma".

Contents

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1. Introduction to gene-based medicines

Gene therapeutics has been pursued to promote medical advancement as a result of the recognition of a central role of DNA in cell biology and its fundamental importance in the control of cellular processes. Initial visions of gene therapy aimed primarily at intervention in hereditary diseases linked to genetic defects. This idea of gene therapy is straightforward and involves the insertion of functional genes into a patient's cells for substitution or supplementation for mutated or missing genes, resulting in the production of a therapeutic protein (Mulligan, 1993). Since 1990 when the first clinical trial of gene therapy was authorized in the USA for the treatment of adenosine deaminase deficient SCID patients (Miller, 1992), gene therapy has achieved some successes such as the positive results in the treatment of patients with X-linked severe combined immunodeficiency (X-SCID) (Cavazzana-Calvo et al., 2000). The completion of the human genome project now provides a good foundation for the understanding of disease and disease-related genes (Hayashi, 2010), leading to insertion of the concept of gene therapy into a broader spectrum of medical fields. To date, the exploration of gene therapy has extended beyond hereditary conditions, such as hemophilia (Walsh, 2003), X-linked chronic granulomatous disease (Ott et al., 2006), human β-thalassaemia.(Cavazzana-Calvo et al., 2010), Wiskott–Aldrich syndrome (Boztug et al., 2010) and cystic fibrosis (Mitomo et al., 2010) to a broad spectrum of acquired diseases, including cancer (Kerr, 2003; McNeish et al., 2004; Vile et al., 2000), cardiovascular conditions (Nabel, 1999), neurodegenerative disorders (Burton et al., 2003, Tuszynski, 2002), infectious diseases (Bunnell and Morgan, 1998), tissue regeneration (Cutroneo, 2003), immunodeficiencies (Kohn, 2010).

A parallel to DNA-mediated therapy is antisense therapy, first reported in late 1970s (Stephenson and Zamecnik, 1978; Zamecnik and Stephenson, 1978). Antisense technology is used to turn off a disease-causative gene by the action of an antisense oligodeoxynucleotide (ODN) that inactivates the encoded mRNA (Dias and Stein, 2002). Antisense therapy has been attempted for intervention in various diseases such as cancer (Goel et al., 2006; Paz-Ares et al., 2006). Duchenne muscular dystrophy (Aartsma-Rus, 2010; Hammond and Wood, 2010; Incitti et al., 2010), asthma (Parry-Billings et al., 2010) and arthritis (Andreakos et al., 2009; Dong et al., 2009). The first antisense drug, Fomivirsen, was approved by the FDA for the treatment of cytomegalovirus retinitis (Roush, 1997). Recently, Mipomersen, a novel antisense therapeutic targeting the mRNA of apolipoprotein B for hypercholesterolemia, completed its phase 3 clinical trials (El Harchaoui et al., 2008; Merki et al., 2008). Still, the overall clinical gains of antisense therapy are far from what has been expected. Some antisense therapies have failed in the clinical trials due either to toxicity (such as Affinitak/Aprinocarsen) or low efficacy (such as GEM 91, LErafAON) (Zhang, 2008). There is no currently FDA approved antisense oligonucleotides drug available for systemic delivery (Zhang, 2008).

The discovery of RNA interference (RNAi) by Fire and Mello broke a new ground for gene medicines (Fire et al., 1998). Synthetic small interfering RNA (siRNA duplex) therapeutics is emerging as one of the most promising, potential medicines after the proof-of-principle study that showed siRNA inhibited gene expression in mammalian cells (Elbashir et al., 2001). siRNA therapeutics exploit the RNAi mechanism normally occurring in eukaryotic cells. Introduction of synthesized siRNA into cells triggers the RNAi pathway (Bernstein et al., 2001; Matranga et al., 2005; Rand et al., 2004); the siRNA sequence specifically binds to targeted mRNA, resulting in subsequent silencing of gene expression (Ameres et al., 2007; Elbashir et al., 2001). In addition to a synthetic siRNA duplex, gene silencing can be induced by the use of DNA-directed RNAi in which plasmid DNA constructs are transfected to express short hairpin-shaped RNAs (shRNAs) that trigger RNAi pathway. shRNA generally triggers a more stable gene knockdown when compared to siRNA duplex which generally produces only transient gene silencing. (Arthanari et al., 2010; Dai et al., 2009; Misra et al., 2009). MicroRNA is another key player involved in the cellular RNAi pathway. MicroRNA is a non-protein coding RNA which regulates gene expression and cellular pathways. Aberrant expression of microRNAs is closely linked with the development of diseases such as cancer (Li et al., 2009) and heart disorders (van Rooij et al., 2008). The discovery of cancer-related microRNAs (oncomirs) has led to the emergence of a microRNA-based cancer therapy by which cancer progression can potentially be interrupted and treated by manipulation of microRNA (Petrocca and Lieberman, 2009). Since oncomirs function as either oncogenes or tumor suppressors, the strategies for such microRNA-based therapy include either the inhibition of over-expression of oncogenic microRNAs with the use of antisense oligonucleotides, or by the reintroduction of down-regulating tumor suppressor microRNAs by the use of microRNA mimics. The abilities of siRNA, shRNA and microRNA to modulate gene expression and eventually control protein production impart their potential as therapeutic agents with more power for gene silencing than anti-sense technology (Bertrand et al., 2002; Hiroi et al., 2006; Vickers et al., 2003). Indeed, the field of RNAi-based therapeutics has undergone a fast development during the first decade of the 21st century. Ongoing clinical investigations of RNAi-based drug therapeutics (Davidson and McCray, 2011; Tiemann and Rossi, 2009) have shed light on treatment of various conditions including wet age-related macular degeneration (Shen et al., 2006), respiratory syncytial virus (http://www.alnylam.com), hepatitis B virus (Morrissey et al., 2005; Song et al., 2003), acute renal failure (www.quarkpharma.com), diabetic macular oedema (Davidson and McCray, 2011) and pachyonychia congenital (Leachman et al., 2008). RNAi-based therapeutics is also a hopeful direction for innovative antiviral and cancer therapies (de Fougerolles et al., 2007).

2. Delivery barriers

Despite their broad potential, moving gene therapy into mainstream medicines still has a long way to go. There are multiple barriers to translating a gene therapeutic from the bench to the bedside (Miller and Dean, 2009; Pack et al., 2005; Whitehead et al., 2009). Plasmid DNA (pDNA), siRNA duplex, microRNA and ODN are highly hydrophilic macromolecules with negative charges. Such physicochemical properties usually restrict their binding to, and passive diffusion across, lipophilic cell membranes. Although their internalization may depend on the endocytic pathway, endosomal entrapment and lysosomal degradation can be major issues because of the reduced accessibility of functional gene medicines at their sites of action. Even for those escaping from endo-lysosomal pathway, cytosolic viscosity and dense organelles might resist their movement towards target sites. In the case of plasmid DNA, the nuclear envelop represents an extra and formidable barrier (Lam and Dean, 2010). In many cases, systemic administration of gene medicines is needed as many disease sites are not easy to access otherwise. Under these circumstances, gene medicines have to penetrate through a series of systemic barriers in order to achieve the desirable efficiency and to reduce side-effects. In the blood circulation, they must evade uptake by macrophages, the clearance by renal filtration and degradation by endogenous nuclease (Alexis et al., 2008). They need to traverse from blood vessels to target tissues. Despite some tissues such as tumors, inflammatory sites and the reticuloendothelial system, RES (e.g., liver, spleen) with leaky blood vessels, the capillary vessel walls in most organs and tissues are impermeable to large nucleic acids. Furthermore, extracellular matrix (ECM) resists the movement of gene medicines to target cells due to its dense polysaccharides and fibrous proteins. (Zamecnik et al., 2004). Although there are some practices involving in the direct use of naked DNA or naked siRNA, their uses are limited largely within local delivery to specific sites such as eye, muscle. Therefore, the therapeutic development of genetic drugs especially for systemic application requires the design of safe and efficient gene delivery vectors.

3. Gene delivery vectors

Delivery is the most critical step in gene medicine for a successful gene therapeutic application. Historically, the main emphasis has been on enhancement of the stability and efficiency of gene delivery systems. Both, viral and non-viral vectors have been employed to improve gene transfer or delivery. A comparison between viral and non-viral vectors is shown in Table 1.

3.1. Viral vectors for gene delivery

Viral vectors for gene delivery are the most widely used systems. They are the most effective and easiest methods for transfer of a gene of interest into a cell and are highly successful at it (Tomanin and Scarpa, 2004). Viruses have highly evolved machinery for delivery of genes. They readily gain entrance into the host-cell and use cellular components to replicate. Ideal virus-based vectors for most gene-therapy applications harness the viral infection pathway but avoid the subsequent expression of viral genes that leads to their replication and toxicity (Thomas et al., 2003). They express a variety of proteins that are useful in clinical application and research. They have been used in over 70% of gene therapy clinical trials with adenovirus, retrovirus, vaccinia virus, and herpes simplex virus being the most commonly exploited types (Young et al., 2006). It is important to carefully choose the virus depending on the cells or disease conditions to be targeted to produce the required gene expression. And for the expression to be successful, sufficient amounts of the gene should be delivered without producing any substantial toxicity (Kay et al., 2001). An ideal vector should only transfect cells of interest and be non-toxic to the normal cells. The complete transfer of the transcriptionally active sequence should occur to allow accurate production of the protein of interest and lasting long enough to achieve a therapeutic effect (Tomanin and Scarpa, 2004).

3.2. Non-viral vectors for gene delivery

Non-viral vectors for gene delivery make use of naturally occurring or synthetic materials to deliver the gene of interest to the target cells. The compounds used to manufacture non-viral vectors do not elicit an immune reaction and are otherwise less toxic. Additional functionalities on non-viral vectors improve their specificity towards the target sites. They are relatively easy to produce and can be used for repetitive administration. However, non-viral methods are generally viewed as less efficacious than the viral methods, and in many cases, the gene expression is short-lived (Al-Dosari and Gao, 2009). Non-viral vectors include lipoplexes, polyplexes or a combination of both.

Felgner et al. (1987) pioneered the use of lipofection for DNA transfection. Since then, cationic lipids have remained one of most commonly used complex agents in the design of non-viral gene delivery systems. They are non-pathogenic, cheap and easy to produce. Cationic lipids when accompanied with co-lipids such as cholesterol and dioleoylphosphatidylethanolamine (DOPE) help to form cationic lipoplexes. However, transfection efficiency of cationic lipoplexes varies dramatically depending on the structure of the cationic lipids (the overall geometric shape, the number of charged groups per molecules, the nature of lipid anchors, and linker bonds), the charge ratio used to form DNA–lipid complexes, and the properties of the co-lipid (Al-Dosari and Gao, 2009).

Polyplexes DNA can be delivered by forming a complex with cationic polymers, such as poly(L-lysine) (PLL), polyethylenimine (PEI), chitosan and polyamidoamine (PAMAM). Furthermore, these complexes can be surface-modified with antibodies or other

Table 1
Comparison between viral and non-viral vectors.

Vectors	Comparison	
	Advantages	Disadvantages
Viral	1. Transduction efficiency is high. 2. Natural tropism confers the capability for infection of many cell types. 3. Virus has intrinsic mechanism for endosomal escape. 4. Virus evolved natural mechanism for nuclear import of genes.	1. Immune response is strong and multiple-injections are limited. 2. Can cause chromosomal insertion and proto-oncogene activation. 3. Complication in its construction and production. 4. Can only carry limited sized genes. 5. Can cause toxicity and may be contaminated with live virus.
Non-viral	1. Immunogenicity is relatively low. 2. No risk of chromosomal insertion. 3. Ease of synthesis, and quality control in mass production. 4. Can carry large-sized DNA. 5. Can be functionalized for targeting, endosomal escape and nuclear import.	1. Transfection efficiency is low. 2. At high dose, current vectors show toxicity. 3. Lack of intrinsic tropism. 4. Lack of intrinsic mechanism for endosomal escape. 5. Lack of intrinsic mechanism for nuclear import of genes.

targeting ligands to deliver nucleic acids to specific cells (Lungwitz et al., 2005; Mao et al., 2011; Sun and Zhang, 2010).

3.3. Considerations in the design of vector for the delivery of pDNA or siRNA duplexes

pDNA and siRNA duplexes have some structural similarities. Both are double-stranded nucleic acids with anionic phosphodiester backbones. This property allows the formation of lipoplexes and polyplexes with the electrostatic interaction of a negatively charged pDNA or siRNA duplex with a positively charged lipid or polymer. On the other hand, pDNA and siRNA duplexes are different in several respects. Compared to a siRNA duplex, a pDNA has a higher molecular weight and a larger size, which make the intracellular delivery of the uncondensed pDNA more challenging. The backbone of pDNA is composed of a deoxyribose with a hydrogen atom in the 2′ position of the pentose ring, while the backbone of RNA contains ribose with a hydroxyl group in the same position. This chemical difference means that the RNA backbone is more vulnerable to nuclease cleavage. In addition, only cytoplasmic delivery of siRNA is required for its action, whereas the transgenic expression of pDNA requires nuclear transfer (Gary et al., 2007).

Although the strategies developed for the delivery of pDNA have been adapted to the vector design for the delivery of siRNA, the design of specific vectors tailored to account for the distinct properties of the two types of nucleic acids is highly desirable for optimization of their therapeutic potential. In the case of pDNA delivery, a primary consideration should be placed on the efficient condensation of large pDNA into nanosized particles to promote internalization of pDNA. In terms of siRNA duplex, its protection from enzymatic degradation is believed to be of the most importance. To achieve the same complexation and extracellular stability, the delivery of a smaller polyanionic siRNA duplex or larger polyanionic pDNA requires polycation with different charge densities. With the same polycation, pDNA may form more stable complexes than siRNA due to its higher molecular weight and the higher negative charge than siRNA. This fact implies that the unpacking of pDNA from a lipoplex or polyplex may face a bigger challenge than the release of siRNAs from the complex. In addition, the vector for pDNA delivery may require additional functionality for efficient nuclear transport (Gary et al., 2007).

4. Overcoming delivery barriers

4.1. Packaging of nucleic acids

Nucleic acids as macromolecules are subjected to a variety of environmental factors such as pH or enzymes (e.g., nucleases) that can degrade or destroy them. Complexation of nucleic acids by cationic polymers or lipids is a widely used method to reduce their sizes and prevent their destruction by nucleases. PEI is one such polymer, available in either Branched (BPEI) with a molecular weight of 25 kDa, or linear (LPEI) form at 22 kDa (Halama et al., 2009). PEI can be condensed with nucleic acids via the electrostatic interaction between the negatively charged nucleic acids and positively charged PEI. PEI/DNA complexes become positively charged when the nitrogen residues are present in excess compared to the phosphate residues of pDNA (N/P ratio) (Biri et al., 2010). Electrostatic interactions between these positively charged complexes and negatively charged cell surface promote cell binding and high levels of gene expression. Liu and coworkers developed a cationic liposome-entrapped, polycation-condensed DNA type 1 (LPD1) composed of protamine sulfate, DNA, and 1,2-dioleol-3,trimethylammonium-propane (DOTAP)/cholesterol liposome (Liu and Huang, 2002). Protamine sulfate provides the positive charge that enhances interaction with the pDNA and better protection from nuclease degradation. The LPD1 formulation showed high *in vitro* transfection efficiency. In a very recent study, gold nanoparticles modified with DNA/siRNA complexes were prepared using generation 3 (G3) polypropylenimine (PPI) dendrimers (Chen et al., 2010). The G3-PPI dendrimers improved the uptake of siRNA duplex into cancer cells and transfection efficiency. The complexation mechanism is based on the binding of the gold and pDNA/siRNA duplex on the amino group of the G3-PPI dendrimers. Cationic lipoplexes were recently reported to improve the delivery of microRNA-133b for lung cancer therapy. MicroRNA-133b was found to be a tumor suppressor that regulates cell survival and sensitivity of lung cancer cells to chemotherapeutic agents by targeting the prosurvival gene *MCL-1*. The cationic lipid-based microRNA delivery system increased mature miR-133b expression *in vitro* and *in vivo*, and produced a down-regulation of MCL-1 protein *in vitro* (Wu et al., in press).

4.2. Long-circulation

Both naked DNA and lipoplexes have showed rapid hepatic clearance during systemic administration (Pouton and Seymour, 2001). The liver elimination of lipoplexes was due to phagocytosis by Kupffer cells (Zhang et al., 2005). Absence of any hydrophilic surface group on the particles, may lead to their interaction with plasma proteins, opsonization and removal from the circulation (Muzykantov and Torchilin, 2003). The mononuclear phagocytic system (MPS) plays a key role in systemic removal of hydrophobic particles. Modification of non-viral vectors such as lipoplexes with hydrophilic molecules like poly(ethylene glycol) (PEG), creates a hydrophilic cloud around the particle surface, causing steric hindrance between the opsonins and the delivery vectors (Muzykantov and Torchilin, 2003). Lee and co-workers developed a PEG-grafted poly(L-lysine) (PLL) pDNA complex with the fusion peptide KALA. They reported that PEG modified PLL/pDNA complexes had decreased interaction with plasma proteins, reduced cell toxicity, and consequently an increased transfection degree (Lee et al., 2002). In addition, PEGylation of the PLL decreased inter-particle aggregation, resulting in high transfection efficiency in the presence of serum. In a different study (Lee et al., 2001), PEG was conjugated with KALA and coated on the surface of pDNA/polyethyleneimine (PEI) complexes, bearing a negative charge and compared to KALA-coated pDNA/PEI without PEG. The latter showed greater aggregation and decreased transfection. The PEG chains added to the surface of the complex decreased the particle-particle interaction and prevented aggregation. Moreover, the transfection efficiency increased due to the presence of the coating of fusogenic peptide KALA, which retained its activity, resulting in increased levels of gene expression. Similar results were observed, when pDNA/transferrin-PEI complexes were PEGylated in an *in vitro* study (Ogris et al., 1999). In addition, an erythrocyte aggregation assay showed reduced aggregation of pDNA/Tf-PEI PEG complex. *In vivo* studies carried out in female A/J mice bearing subcutaneous neuroblastoma (Neuro2A) tumors showed an increase in gene expression after treatment with the pDNA/Tf-PEI/PEG complex compared to the pDNA/Tf-PEI complex in a luciferase assay. From the above examples, it is evident that PEG plays an important role in preventing interference from blood components.

4.3. Targeted delivery

Appropriate packaging of nucleic acids and using PEG as a shield can help the complex survive within the circulation without being degraded or taken up by the MPS system. However, the next challenge for a PEGylated gene complex is to specifically target to cells

or tissue of interest. By taking advantage of increased expression levels of receptors or antigens in diseased conditions, such as cancer, gene complexes can be targeted using specific ligands, such as antibodies, peptides, proteins, small molecules and RNA aptamer that recognize and bind to the cells of interest, resulting in high transfection efficiency (Ogris and Wagner, 2002). ErbB2, a member of the EGFR family, is over-expressed in certain patients suffering from breast and ovarian cancer (Lee et al., 1995). A gene vector (designated DPSL), composed of cationic lipid, protamine and a single-chain antibody fragment (ScFv) against ErbB2, delivered exogenous pDNA into cell lines that expressed Erb2, and showed high levels of expression of the luciferase reporter gene in the Erb2 positive cells compared to the Erb2 negative cells (Li et al., 2001). The monoclonal antibodies (mAbs), such as Trastuzumab (Herceptin) have also been widely studied to target breast cancer cells that overexpress HER2/neu. Very recently, a [poly(b-L-malic acid)] polymer, conjugated with targeting mAbs against the transferrin receptors, antitumor Herceptin and antisense oligonucleotides (AON), was used to inhibit HER2/neu protein synthesis (Inoue et al., 2011). This multifunctional nanobiopolymer demonstrated a significant growth inhibition of BT-474 cells and SKBR-3 cells and prevented tumor growth of HER2/neu-positive breast cancer as a result of the double inhibition of HER2/neu and Akt phosphorylation, resulting into enhanced tumor cell apoptosis (Inoue et al., 2011). PEI/siRNA polyplexes, in combination with Bevacizumab, an anti-VEGF monoclonal antibody, produced a synergistic anti-tumor effect in mice bearing PC-4 tumor xenografts (Hobel et al., 2010).

Integrin $\alpha_v\beta_3$ is an attractive target for gene delivery due to its high expression level in cancer cells and tumor vasculatures. Arginine–glycine–aspartic acid (RGD) peptide is a classic targeting ligand for integrin $\alpha_v\beta_3$. In a study carried out by Ng and coworkers, clustered RGD ligands were introduced in PEI/pDNA polyplexes by attachment of RGD peptide-modified gold nanoparticles to the polyplex surface (Ng et al., 2009). The PEI/pDNA/RGD nanoclusters increased efficiency of gene transfer in HeLa cells compared to unmodified PEI/pDNA polyplexes.

Another targeting strategy is directed towards transferrin receptors that are overexpressed in cancer cells. A generation 3 polypropylenimine dendrimer (DAB) conjugated with transferrin (Tf) for targeted delivery of galactosidase-encoding plasmid was designed (Koppu et al., 2010). Compared to the non-targeting DAB, the targeted DAB improved transfection efficiency of the A431 and T98G cell lines, and also produced superior tumor regression and extended survival of mice *in vivo*. Recently, a novel, dual-targeting gene vector was designed by the attachment of both RGD peptide and transferrin-targeting peptide B6 with PEGylated, PEI-based polyplexes (Nie et al., 2011). Compared to control non-targeted polyplexes, this dual-targeting system showed 60 and 20-fold increases of the reporter gene expression on DU145 and PC3 cells, respectively. Using the phage display technique, we have recently identified a phage fusion protein specific for breast cancer cells MCF-7 (Wang et al., 2010a,b, 2011a,b), and explored their utility in targeted delivery of liposome-carried siRNA, showing significant silence of *PRDM14* gene and reduction in expression of PRDM14 protein in the targeted MCF-7 cells (Bedi et al., 2010).

Recently, a bioconjugate containing a dendrimer and a RNA aptamer specific for the prostate-specific membrane antigen (single-strand DNA-A9 PSMA) was utilized for targeting delivery of a chemoimmuno-therapeutics in the treatment of prostate cancer *in vitro and in vivo* (Lee et al., in press). Furthermore, Ban's group developed a dual-aptamer-based targeting system for delivery doxorubicin to both PSMA (+) and PSMA (−) prostate cancers by means of an A10 RNA aptamer, which recognizes PSMA (+) prostate cancer cells, and a DUP-1 peptide aptamer, which targets PSMA (−) prostate cancer cells. Both targeting ligands were linked by streptavidin, and drugs were loaded onto the stem region of the A10 aptamer. As a result, the dual targeting system showed selective delivery and induced apoptosis of both PSMA (+) and PSMA (−) cells (Min et al., 2011).

4.4. Intracellular delivery

Intracellular delivery of gene medicine to the cytosol or nuclei is essential for its therapeutic action. The cytoplasmic membrane is typically impermeable to nucleic acids due to their large size and hydrophilic nature. Many efforts have been made to facilitate internalization of nucleic acids. One of the strategies involves physical means including electroporation, ultrasound-based sonoporation, the use of a gene gun and microinjection. All physical approaches enable nucleic acid molecules to penetrate into cells by transiently percolating through the cellular membrane. However, their clinical application is limited because of their invasive nature and potential damage to the structure of cells, as well as poor access to deeper tissues. An alternative strategy involves the design of chemical vectors capable of actively targeting internalization pathways that lead to an efficient transfection. The use of cationic lipids and polymers for the assembly of nucleic acids into lipoplexes/polyplexes has achieved this goal to some extent. There is convincing evidence showing that endocytosis is the predominant route for the internalization of a lipoplex/polyplex (Akita et al., 2004; Colin et al., 2000; Khalil et al., 2006a; Rejman et al., 2005; Zuhorn et al., 2002). In order to package nucleic acids into condensed particles and to favor their subsequent binding to cells, lipoplexes and polyplexes are generally formulated into particles with net positive charges. Under these circumstances, endocytosis can be triggered by non-specific electrostatic interaction between the positively charged complexes and the negatively charged heparin sulfate proteoglycan on the cell surface followed by an internalization process termed adsorptive pinocytosis (Payne et al., 2007). Ligand-mediated endocytosis is believed to be more efficient than non-specific endocytosis. Entry of lipoplexes usually involves a clathrin-mediated pathway or macropinocytosis, while internalization of the polyplexes can occur via clathrin-mediated endocytosis, caveolae-mediated endocytosis or macropinocytosis (Elouahabi and Ruysschaert, 2005; Khalil et al., 2006a, b; Zhang et al., 2011; Zuhorn and Hoekstra, 2002). It is not clear whether or not one pathway is more favorable than another in terms of the effectiveness of internalization, cytosol release and eventual gene expression, but caveolae-dependant trafficking seems to be an attractive pathway for lipoplex/polyplex-targeting since the pathway is a non-acidic and non-digestive route (Shin et al., 2000; Won et al., 2009). It was consistently shown that the caveolae-dependant pathway led to high transfection (Gabrielson and Pack, 2009; Rejman et al., 2005). Particle size has a profound impact on the internalization pathway. Lipoplexes/polyplexes with a size less than 200 nm followed clathrin-mediated endocytosis, and particles with a size greater than 300 nm entered cells via a caveolae-mediated pathway (Rejman et al., 2004; Zuhorn et al., 2002). Certainly, more information on the molecular mechanisms underlying the intercellular trafficking would aid in rational design of non-viral gene vectors for optimal intracellular delivery.

The discovery of a "protein transduction process" and identification of protein transduction domains (PTDs) or cell-penetrating peptides (CPPs) has opened a new avenue for advancement of intracellular drug delivery (Torchilin, 2006). The use of CPPs for intracellular delivery of a wide-range of cargoes including nucleic acids has been demonstrated (Gupta et al., 2005). CPP-mediated intracellular delivery relies on the ability of CPPs to pass through the cellular membranes delivering their payload into the cytoplasm and/or nucleus. Among CPPs, TATp is frequently used. Direct attachment of TATp to cargoes (e.g., nucleic acids) has been achieved by chemical coupling, genetic fusion and electrostatic interaction. Also, when TATp is cross- linked to the distal end of

PEG-polyethyleneimine or PEG-lipid, the resultant TATp- PEG-PEI conjugates form a complex with DNA, while the resultant TATp-PEG-lipid conjugates self-assemble with lipids or polymers to build TATp-modified liposomes or TATp-modified micelles with TATp displayed on the surface of the resultant drug nanocarrier. TATp-liposomes, containing a small quantity (<10 mol%) of a cationic lipid formed firm noncovalent complexes with DNA (Torchilin, 2008). Such TATp-cationic liposome–DNA complexes transfected of both normal and cancer cells *in vitro* and *in vivo* with lower cytotoxicity than that of the commonly used cationic lipid-based gene-delivery systems. We earlier showed that modification of liposomes with TATp facilitated the gene transfer into human brain tumor cells in tumor- bearing mice (Gupta et al., 2007). TATp-PEG-PE conjugates in combination with mAb 2G4 against cardiac myosin to target delivery of liposome-entrapped genes to a region of myocardial ischemia, demonstrating increased transfection *in vitro* and *in vivo* (Ko et al., 2009).

Oligoarginine conjugates were as efficient as CPPs in the mediation of intracellular cargo delivery (Futaki et al., 2001a, b; Nakase et al., 2004). Oligoarginine-modified liposomes, micelles and polymer-based particles have been explored extensively for the delivery of pDNA and siRNA duplex. It is worth noting that the intracellular trafficking and transfection efficacy of oligoarginine-modified nanoparticles are both closely related to the length of oligoarginine used. There is a detailed discussion in the review (Maitani and Hattori, 2009). In the case of an octaarginine-modified multifunctional envelope-type nanodevice (R8-MEND), the internalization pathway depends largely on the density of octaarginine (R8). Low density R8 MEND followed clathrin-mediated endocytosis, while high density R8-MEND was internalized via macropinocytosis (Khalil et al., 2006b). Macropinocytosis is believed to be the more desirable pathway since it may bypass lysosomal degradation by the release of the macropinosomed R8-MEND directly into the cytoplasm. Internalization by macropinocytosis may account for the high transfection degree of R8-MEND (Kogure et al., 2004; Moriguchi et al., 2005).

4.5. Endosome escape

Following internalization of lipoplexes or polyplexes via the endocytic pathway, endosomal entrapment and subsequent lysosomal degradation are a major bottleneck that limits the efficiency of gene delivery. Design of gene vectors with endosome-escape properties is considered critical for high transfection efficiency. Identification of endosomolytic or fusogenic components and their integration into non-viral gene delivery systems are major strategies being exploited to facilitate endosomal escape.

The cationic lipids have been shown to destabilize the endosomal membrane. The mechanism behind the phenomenon, as suggested by Cullis' group, is that electrical interaction between cationic lipids and anion endosomal membranes results in the formation of ion-pairs that promote the formation of the inverted hexagonal (H_{II}) phase and disrupt endosomal membrane (Hafez et al., 2001). Szoka's group proposed that the interaction of endosome membranes with internalized lipoplexes leads to a flip-flop of anionic lipids from the cytoplasmic side to lumen side of the endosomal membrane, forms ion-pairs with cationic lipids made of lipoplexes, and eventually releases ODN or pDNA into the cytoplasm as a result of the displacement of pDNA from the lipoplexes (Zelphati and Szoka, 1996). These putative mechanisms were successfully exploited for cytoplasmic delivery of antisense oligonucleotides (Semple et al., 2001) and siRNA duplex (Semple et al., 2010) using a rational design of cationic lipid nanoparticles. Details regarding the delivery system will be discussed later.

Many preparations of lipoplexes contain DOPE as a helper lipid for fusogenic functionality. The ability of DOPE to destabilize endosomal membranes is based on its propensity to acquire an inverted hexagonal phase (H_{II}). DOPE has a small cross-section headgroup and a large hydrocarbon area that favors a non-bilayer structure with a cone shape that facilitates the destabilization of endosomal membranes and gene transfection (Farhood et al., 1995; Fasbender et al., 1997a; Hafez and Cullis, 2001). In addition, the low hydration of the headgroup of DOPE makes DOPE-containing liposomes acquire more hydrophobicity for a more favorable interaction with lipid bilayer. With the substitution of DOPE for dipalmitoylphosphatidylethanolamine (DPPE), the transfection activity of the lipoplex decreased dramatically as a result of the inhibition of the intracellular delivery, confirming the fusogenic role of DOPE (Zuhorn et al., 2005).

In the case of polyplexes, PEI and PAMAM are two representative cationic polymers with a high efficiency of gene transfer due in part to their capability to facilitate endosomal escape. A "proton sponge effect" provides a sound explanation for the intrinsic endosomolytic activity (Boussif et al., 1995). Upon PEI-based or PAMAM-based polyplex entry into acidic endosomes, the polymer behaves as a sponge that absorbs protons as a result of protonation of the polymer-containing amine groups (primary, secondary and tertiary). Accumulation of protons subsequently drives an influx of counter chloride ion into endosomes, leading to increased osmotic pressure and subsequent flow of water into the endosomal interior and eventually swells and ruptures endosomal membrane (Cho et al., 2003). However, the *in vivo* application of the PEI often is limited due to its high cytotoxicity (Brunot et al., 2007).

The endosome-escape potential of poly-histidine rationalizes their use for delivery of nucleic acids (Asayama et al., 2004). The imidazole ring within histidine is a major player. Under the action of an acidic endosomal interior, the weak basic nature of the imidazole ring with pK_a around 6 allows its protonation and acquires cationic charges which trigger the destabilization of endosomal membranes. Accumulation of histidine residues within endosomes could elicit a proton sponge effect and destroy endosomes as a result of their increased osmolarity. Both chemistry conjugation and genetic engineering have produced a series of histidine-rich polymers and peptides as well as lipids with imidazole, imidazolinium or imidazolium polar heads. These histidylated carriers have been used to deliver nucleic acids including pDNA, mRNA or siRNA duplex *in vitro* and *in vivo* with increased transfection efficiency (Kichler et al., 2003; Leng et al., 2005; McKenzie et al., 2000; Midoux et al., 2002; Read et al., 2005). The current status in the development of histidylated carriers is described in a recent review and provides a comprehensive summary of polymers, peptides and lipids containing histidine or imidazole designed for delivery of nucleic acids (Midoux et al., 2009).

In an attempt to facilitate endosome escape, many strategies have been developed to mimic the viral mechanism for endosome destabilization. It is well-established that a fusogenic peptide with a short chain of N-terminal amphiphilic anionic peptide residues (termed hemagglutinin HA2) is responsible for endosomal escape of the influenza virus (Cho et al., 2003). The conformation of the HA2 subunit can change with pH. At neutral pH, the HA2 subunit adapts a non-helical conformation due to charge repulsion arising from ionization of glutamic and aspartic acid residues. Within the interior of the acidic endosomal compartment, however, the HA2 subunit transitions into a stable helical secondary structure due to the protonation of glutamic and aspartic acids (Cho et al., 2003). The hydrophobic and hydrophilic faces of the helical conformation favor endosomal membrane destabilization (Mahat et al., 1999; Martin and Rice, 2007). Synthetic peptides mimicking a virus's fusogenic peptides have also been designed for delivery of nucleic acids. The amphipathic peptide, GALA, was synthesized with 30 amino acid residues with a repeated amino acid sequence

(e.g., glutamic–ananine–leucine–aninine) that demonstrated pH-sensitive fusogenic properties. Modification of multifunctional envelope-type nano devices (MENDs) with GALA peptide facilitating endosomal escape, leading to the enhanced transfection efficiency of pDNA (Kakudo et al., 2004) and siRNA duplex *in vitro* and *in vivo* (Hatakeyama et al., 2009). A cationic counterpart of GALA is the peptide KALA which is formed by substitution of the ananine of GALA with lysine and a decrease in content of glutamic acid. The KALA/DNA complex retained membrane lytic activity, leading to gene transfection of different cell lines (Min et al., 2010; Mok and Park, 2008). We recently found that the MCF-7-specific phage fusion protein bears a pH-sensitive, fusogenic property required for endosomal escape. The modification of liposomes with the phage fusion protein enables cytoplasmic delivery of a liposome-carried drug (Wang et al., 2010c).

The concept of photodynamic therapy has been extended to promote endosome escape by selective disruption of the endosomal membrane. This strategy involves the use of photosensitizers to generate reactive oxygen species in response to light irradiation after photochemical-containing lipoplexes or polyplexes are internalized by the endosome. Consequently, the endosomal membrane is disrupted by the effect of free radicals on unsaturated fatty acids within the endosomal membranes. This approach enhanced transfection efficiency with both viral and non-viral gene delivery systems (de Bruin et al., 2008; Hogset et al., 2002, 2004; Oliveira et al., 2007; Tamaki, 2009).

4.6. Nuclear import

The nuclear envelope that separates the cell's genetic material from the surrounding cytoplasm represents a physical barrier for nuclear import of macromolecules such as pDNA. The nuclear pore complex that perforates the nuclear envelope tightly regulates cytoplasm-nuclear trafficking and limits passive diffusion of molecules with sizes >40 kDa. There are several lines of evidence showing that nuclear import is a rate-limiting step for transfection of pDNA. In 1980, Capecchi observed that direct microinjection of pDNA into the cytoplasm resulted in no gene expression, but direct microinjection of the same amount of pDNA into nuclei achieved more than 50% gene expression (Capecchi, 1980). Although Felgner et al. demonstrated efficient lipofection for internalization of pDNA into cells, they found less than 1% of plasmid in the nuclear fraction (Felgner et al., 1987). Furthermore, one elegant study showed that only 10% of the cells expressed galactosidase when transcription was driven into nuclei using a CMV promoter. However cell expression of galactosidase increased up to 98% when transcription was driven in cytoplasm using a T7 promoter (Zabner et al., 1995). Nuclear import of pDNA may be more challenging for transfection of non-dividing cells. Indeed, non-dividing cells showed a 90% lower expression level compared to actively dividing cells (Fasbender et al., 1997b).

Strategies for nuclear import of genes have been developed following further elucidation of the endogenous nuclear import machinery. The nuclear localization sequence (NLS) is a major player that shuttles protein–plasmid complexes through the nuclear pore (Cartier and Reszka, 2002). NLS-mediated active nuclear translocation involves a process starting from its interaction with cytoplasmic importins to binding of the NLS to the nuclear pore complex and the passage through the pore (Dean et al., 2005; Lam and Dean, 2010; Vaughan et al., 2006). Identification of the NLSs, such as SV40 from the larger tumor antigen Simian virus 40 and M9 from nuclear ribonucleoprotein (Cartier and Reszka, 2002), enabled design of non-viral gene vectors with nuclear targeting properties.

Tagging of the exogenous NLS with DNA vectors created the first generation of nuclear targeting non-viral vectors (Cartier and Reszka, 2002). Approaches used in the attachment of the NLS to DNA vectors include non-covalent and covalent conjugation. The non-covalent approach is based on the electrostatic interaction between a positive NLS and negative pDNA. There are some issues regarding the use of a non-covalent approach. First, electrostatic interaction with positive NLS could interfere with an association between cationic lipid or polymer and negative pDNA. Second, the electrostatic interaction may bury the NLS. Lastly, NLS may dissociate from pDNA before the complex reaches nuclei. Alternatively, covalent conjugations involve the use of various cross-linkers. While the chemistry may not be easy, a major concern regarding the covalent approach is that chemical modification may reduce transcription activity of pDNA if the chemical modifications occurred in the transcription cassette. To overcome such drawbacks, a peptide nucleic acid (PNA) has been proposed as a bi-functional linker to tether NLS to pDNA. Indeed, the SV40 NLS-PNA-pDNA tertiary complex mediated nuclear import of pDNA both *in vitro* and *in vivo* without the influence on transcription activity of the nucleic acid (Branden et al., 2001, 1999).

A potential immune response associated with the use of an exogenous NLS has led to the emergence of a second type of nuclear targeting vector in which a DNA targeting sequence (DTS) is attached to a DNA vector for active nuclear import of DNA (Lam and Dean, 2010; Miller and Dean, 2009). The DTS is able to associate with cytoplasmic transcription factors which contain the endogenous NLS, leading to the formation of a tertiary complex DNA–DTS-transcription factor and nuclear import of pDNA. Furthermore, by means of the use of cell type-specific DTS, a more sophisticated DTS-DNA vector has been developed for a smooth muscle-specific gene transfer (Miller and Dean, 2008; Young et al., 2008).

5. Multifunctional non-viral gene vectors

The design of non-viral gene delivery systems has undergone an evolution from an initial emphasis on an individual barrier to the creation of a multifunctional vectors able to execute multiple tasks to overcome both extracellular and intracellular obstacles. Ideally, gene vectors should be capable of self-assembly with nucleic acids and accommodate with any type of nucleic acid or their combination. They should also target cells of interest, escape from endosomes and/or transport into nuclei. A viable gene vector for systemic delivery needs to minimize toxicity and phagocytosis, and avoid nonspecific interactions and self-aggregation (Davis, 2009). This can be achieved by the assembly of multiple devices in a single delivery system using a systematic methodology.

Harashima's group proposed the concept of "Programmed Packaging" for the formulation of a multifunctional envelope-type nano device (MEND) and claimed that such a MEND allows for execution of each function by following a specific program to overcome each barrier at the appropriate time and at the right place. This non-viral gene-delivery system has been extensively used for the delivery of pDNA, ODN and siRNA duplex (Kogure et al., 2004; Moriguchi et al., 2005; Nakamura et al., 2006; Sasaki et al., 2005; Yamada et al., 2005). The scaffold of a MEND consists of a polycation/pDNA complex core covered with a lipid coating mimicking the structure of a virus type envelope. The surface of a MEND may be equipped with various functionalities, including a PEG coating, targeting ligand (Tf), octaarginine peptide (R8), a fusogenic component, and a nuclear delivery vector (NLS). The architecture of a MEND is expected to protect nucleic acids from enzymatic digestion. Multiple functionalities can aid a MEND so as to overcome the series of barriers along the path from the injection site to their destination as a result of their ability to promote long circulation, cell recognition, internalization and intracellular transport (Futaki

et al., 2001a,b; Kamiya et al., 2003; Nakase et al., 2004). Consequently, MENDs have been shown to enhance DNA transfection. (Kogure et al., 2008, 2004).

A cyclodextrin-based gene vector was the first targeted multifunctional delivery system in a human phase I clinical trial on patients with solid tumors (Davis et al., 2010). This system uses a cyclodextrin-containing polymer (CDP) as backbone for tethering PEG, and a PEG-Tf conjugate via an adamantane (AD) linker which complexes with cyclodextrin as well as serving for the attachment of imidazole groups (Davis et al., 2004; Mishra et al., 2006). The polycationic nature of cyclodextrin allows for electrostatic interactions with the polyanions of nucleic acids to produce nanoparticles with a size of about 70 nm. The polyplex (designated as CALAA-01) used in clinical trials is formulated in two separate vials: one is a three-component delivery system (CDP, AD-PEG, and AD-PEG-transferrin); the other contains siRNA specific for the silencing of ribonucleoside reductase M2 (*RRM2*). siRNA polyplexes are self-assembled by mixing the two vials immediately before i.v. injection into patients with metastatic melanoma. In the blood stream, these colloidal nanoparticles avoid renal clearance and passively deposit into tumor sites. AD-PEG provides the steric stabilization for minimization of uptake by the RES and provides a long-circulation property to the system which enables a greater accumulation of siRNA polyplexes within tumors. The ligand transferrin recognizes target cells and promotes internalization via receptor-mediated endocytosis. The imidazole group functions as a proton sponge and mediates endosome escape. The outcome of the treatment has been encouraging. Both *RRM2* mRNA and protein levels were inhibited, and the RNAi effects were maintained for several weeks. The biopsy analyses post-treatment clearly showed that the intra-tumor distribution of CDP nanoparticles was targeted specifically to tumor cells (Davis, 2009).

Another direction in the development of multifunctional gene vectors is the design and synthesis of multi-component polymers for polymer-based nanocarriers (Dohmen and Wagner, 2011; Kakimoto et al., 2010; Moore et al., 2009; Wang et al., 2008). Most recently, Minko's group synthesized a new triblock polymer, PAMAM-PEG-PLL, for multifunctional delivery of siRNA. In this system, the PLL block electrostatically interacts with negatively charged siRNA via its cationic amine groups; the shell of PEG protects siRNA from nuclease degradation, and the PAMAM dendrimer promotes the endosomal escape via its tertiary amines. As a result, this system achieved efficient intracellular delivery and gene silencing (Patil et al., 2011).

6. Dilemmas in the design of multifunctional vectors

The assembly of different functionalities into a single system for development of multifunctional gene vectors is an attractive strategy but certainly not an easy task. Difficulties arise from several aspects. These different functionalities may not be integrated compatibly or behave in concert and their roles could cancel each other. For example, the inclusion of certain functionality into a gene vector may be beneficial to overcome an upstream barrier. However, it may be unfavorable for downstream transport. Over the course of a gene transfer, there are conflicting requirements for the design of gene vectors to simultaneously overcome both extracellular and intracellular barriers. Here, we summarize "the polycation dilemma", "the PEG dilemma" and "the package and release dilemma", each of which limit safe and efficient applications of lipoplexes and polyplexes for systemic delivery.

6.1. The polycation dilemma

Despite the fact that neutral lipids are well-known to be biocompatible and favorable in pharmacokinetic profiles, their use in formulating lipid/nucleic acid complexes is usually ineffective because of the lack of efficient interactions between the neutral lipids and anionic polynucleotides. In most cases, cationic lipids or polymers are preferred as a core component for gene vectors. The inclusion of cationic lipids and/or polymers produces higher encapsulation efficiency and facilitates desirable intracellular trafficking. However, polycation can induce damage of cellular membranes or apoptosis, presumably due to their interaction with anionic components on the plasma membranes (Moghimi et al., 2005). In an *In vivo* milieu, they are prone to interact with anionic serum proteins, resulting in the formation of undesirable aggregations or premature release of nucleic acids. Therefore, application of cationic lipoplexes and polyplexes for systemic delivery may be problematic unless issues such as serum stability and tolerability are addressed. We use the term "polycation dilemma" to describe the conflicting effect of polycation in formulation of lipoplexes and polyplexes and their behavior *in vivo*.

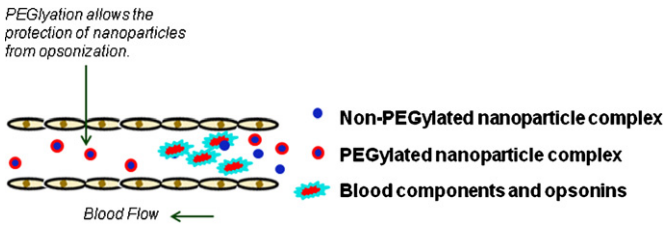

Fig. 1. Overcoming delivery barriers with long-circulation induced by nanoparticle PEGylation.

6.2. The PEG dilemma

PEGylation has been extensively utilized for steric protection of pharmaceutical nanocarriers from the clearance by the RES system, and for delivery vehicles to promote a longer circulation time in blood stream and, additionally, for physiological stability (Fig. 1). The PEGylation promotes drug accumulation within target sites such as tumor via the enhanced permeability and retention (EPR) effect (Fig. 2). Paradoxically, the PEGylation can also provide a steric hindrance that prevents gene vectors from efficient interaction with cellular membranes and/or endosomal membranes. This undesirable cellular uptake and endosome escape result in lower transfection efficiency of a gene vector. This contradictory effect of PEGylation is usually called the "PEG dilemma" (Hatakeyama et al., 2011a).

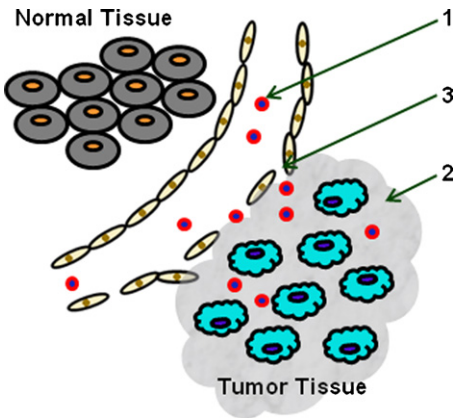

Fig. 2. Accumulation of nanoparticles via an enhanced permeability and retention (EPR) effect. (1) PEGylated nanoparticles extravasate into the tumor interstitium (2) through the leaky vasculature endothelium of the tumor (3) leading to an enhanced tumor accumulation.

6.3. The package and release dilemma

Packaging DNA into stable particles with high avidity binding of polycation to nucleic acids is desirable because it is necessary to prevent premature release of nucleic acids in the extracellular environment, as well as to protect nucleic acids from enzymatic degradation. Moreover, maintaining the integrity of the complex is a prerequisite for targeting and intracellular delivery of nucleic acids. Once lipoplexes or polyplexes reach their sites of action, however, too tight a complex can be a problem if not de-packaged appropriately to release nucleic acids to allow bioavailability to therapeutic sites. This "package and release dilemma" accounts for the lack of efficiency of many gene vectors (Grigsby and Leong, 2010).

7. Solutions to the polycation dilemma

Table 2 is a summary of selective strategies for overcoming the polycation dilemma.

7.1. PEGylation

A straightforward solution to overcome the "polycation dilemma" is to shield its positive charge with a protective surface of hydrophilic PEG. The PEGylation of the lipoplex has improved systemic tolerability. A detergent dialysis method (Wheeler et al., 1999) was used to formulate 'stabilized plasmid-lipid particles' (SPLP) with components which included a fusogenic lipid (DOPE), a cationic lipid (DODAC) and a PEG-lipid (PEG-CerC20) at a molar ratio of 83:7:10. The SPLP was advantageous over its non-PEGylated counterpart (pDNA/cationic lipoplexes) for systemic gene delivery. Intravenous injection of SPLP into tumor-bearing mice increased gene expression as a result of prolonged circulation in blood (>6 h) and preferential tumor deposition (Ambegia et al., 2005; Tam et al., 2000). Notably, SPLP was nontoxic which made it a viable option for parenteral delivery. Conversely, due to poor biocompatibility, the *in vivo* use of non-PEGylated lipoplexes is generally limited.

Early on, Maurer et al. and Jeffs et al. developed an ethanol dialysis-mediated spontaneous vesicle formation method for entrapment of ODN and DNA (Maurer et al., 2001; Jeffs et al., 2005). Later, Polisky's group adapted the same method for encapsulation of HBV-targeted siRNAs within PEGylated liposomes with a lipid composition of cationic lipid, fusogenic lipid and PEG-lipid (Morrissey et al., 2005). siRNA-containing lipid nanoparticles significantly improved the pharmacokinetics with a longer half-life of 6.5 h when compared to naked siRNA which showed an elimination half-life of 2 min. This improvement is, in part, attributable to the PEG-lipid conjugate which coats the surface of lipid particles with a neutral and hydrophilic shell, shielding the cationic charge and slowing the *in vivo* clearance. Consequently, the prolonged half-life in plasma and liver contributed to the enhanced efficacy.

Following repeated administration, however, PEGylated lipoplexes (SPLP) showed rapid blood clearance and generated acute hypersensitivity. After a 1 h injection of SPLP containing PEG-S-DSG, 80% the first dose of was retained in blood and 10% reached the liver, but only 25% the second dose was found in the blood with a significantly increased accumulation in the liver and other RES organs. These changed pharmacokinetics and biodistribution of SPLP were linked to the production of antibody against PEG within the blood of previously naive mice. The good news is that immunogenicity could be avoided by the modification of the alkyl chain of the PEG–lipid conjugate, making repeated injection of the PEGylated lipoplex feasible (Judge et al., 2006).

PEGylation has been explored as a strategy to improve the undesirable interaction between positively charged polyplexes and biological components. The PEGylation of polyplexes can be achieved either by synthesis of PEG-polycationic polymer conjugates followed by their complex with nucleic acids for gene delivery or by coupling of PEG chains to the pre-condensed polycationic polymer/nucleic acid complex (Kircheis et al., 2001, 1999; Rudolph et al., 2002). This latter strategy usually lends to more efficient condensation of plasmid DNA with cationic polymers. The conjugation of PEG with cationic polymers, such as PLL and PEI, has led to the formation of various PEG- polymer conjugates in different formats including an A-B type block copolymer, a star-shaped dendrimer, and a biodegradable copolymer. These PEGylated polyplexes/nucleic acids generally show improved biocompatibility, presumably due to a decreased particle surface charge. Such polyplexes have fewer tendencies to interact with blood components, show lower toxicity towards cells *in vitro* and *in vivo*, and have prolonged circulation in the blood (Cavallaro et al., 2010; Nagasaki et al., 2004; Neu et al., 2007; Nomoto et al., 2011; Petersen et al., 2002).

7.2. Anionic lipid-coating lipopolyplexes

Masking the undesirable positive surface charge of polyplexes or lipoplexes can also be realized using anionic lipids. The encapsulation was induced via interaction between the opposite charges. The anionic lipids form a lipidic surface covering the condensed polyplex/lipoplex core for the neutralization of their surface charges. The lipopolyplexes showed less cytotoxicity than cationic liposome complexes, presumably due to diminished interaction between the lipopolyplexes and anionic components of the plasma membranes. Aigner's group assessed different lipopolyplexes with varied lipids and lipid combinations. Their results showed that the lipidation of PEI/nucleic acid complexes reduced their toxicity, and the lipopolyplexes built by rigid, negatively charged lipids improved DNA transfection efficiency and siRNA-induced gene silence (Schafer et al., 2010). Zhang's group developed a PEGylated immunolipopolyplexes (PILP) with the composition of pDNA/PEI polyplexes and anionic liposomes composed of POPC, (DSPE)-PEG2000 and (DSPE)-PEG2000–biotin–streptavidin–monoclonal antibody. The PILP formulation produced improved gene transfer to liver cancer cells without any detectable liver injury (Hu et al., 2010). Moreover, such a formulation avoided the destabilization of the DNA complex by polyanions present in biological fluids, as illustrated in a tertiary complex with cationic poly 2-(dimethylethylamino) ethyl methacrylate (p(DMAEMA)/pDNA/anionic lipids, which was protected from destabilization in the presence of hyaluronic acid by the lipid coating of polyplexes (Mastrobattista et al., 2001).

7.3. The use of titration lipids

An additional tactic for solution of the "polycation dilemma" is to switch the positive charges of the polycation on or off at the right time and at the right place. Early on, Semple et al. developed a formulation process in which the aminolipid DODAP and an ethanol-containing buffer were used to form particles known as "stabilized antisense-lipid particles" (SALP). The DODAP has a $pK_a \sim 6$ so that the lipid displays no charge near a physiologically neutral pH but acquires a positive charge when the pH becomes acidic. The SALP system exploits the ionizable property of DODAP, and addresses the conflicting roles of polycation in lipoplex formulations and their use *in vivo* by dynamically switching the surface charges of DODAP on or off in response to pH change. Upon an initial adjustment of pH to an acidic condition, the DODAP acquires a positive charge and condenses polyanionic ODNs with enhanced encapsulation efficiency up to 70%. A subsequent change of pH back to neutrality not only allows the removal of ODNs on the particle surface, but also renders the resultant

Table 2
Selective strategies for overcoming the polycation dilemma.[a]

Strategies	Non-viral vectors	Nucleic acids	Formulation methods	Test models	References
PEGylation	PEG-CerC_{20}/DODAC/DOPE PEG-CerC_{14}/DODAC/DOPE	pCMV-Luc	Detergent dialysis for stabilized plasmid–lipid particles (SPLP)	C57B1/6 mice bearing s.c. Lewis lung carcinoma Male A/J mice	Tam et al. (2000) Ambegia et al. (2005)
	PEG-coated PEI/DNA polyplexes	pCMV-Luc	Electrostatic interaction	BALB/c mice	Rudolph et al. (2002)
	PEG-coated Tf-PEI_{800}/DNA polyplexes	pCMV-Luc	Electrostatic interaction followed by covalently coating with PEG	A/J mice bearing s.c neuro 2A neuroblastooma	Kircheis et al. (2001)
	bPEI-graft-lPEG copolymers PEI-PEG(30k)	pDNA-Luc pCMV-Luc	Electrostatic interaction	*In vitro* cell-based assay Male balb/c mice	Petersen et al. (2002) Neu et al. (2007)
Anionic lipid-coating lipopolyplexes	Lipidation of PEI/nucleic acid complexes	Luc-targeting siRNAs;	Electrostatic interaction	*In vitro* cell-based assay	Schafer et al. (2010)
	pDNA/PEI polyplexes and anionic liposomes composed of POPC, (DSPE)–PEG2000 and (DSPE)–PEG2000–biotin–streptavidin–monoclonal antibody.	pGL2- Luc EGFP plasmid	Electrostatic interaction	*In vitro* cell-based assay Female BALB/c mice	Hu et al. (2010)
Use of titration lipids	Titration lipid (DODAP) with DSPC/cholesterol/PEG-CerC14	ODN targeting EGFR, c-myc, ICAM	Ethanol dialysis-mediated stabilized antisense-lipid particles (SALP)	ICR mice with i.v injection	Semple et al. (2001)
	Titration lipids (DlinDMA) with DSPC/cholesterol/PEG-C-DMA	HBV-targeting siRNAs	Ethanol dialysis-mediated spontaneous vesicle formation	Mice and Cynomolgus monkeys with i.v. injection	Morrissey et al. (2005) Zimmermann et al. (2006)
	Titration lipids (DLin-KC2-DMA) with DPPC/cholesterol/PEG-C-DMA	Factor VII targeting siRNA	Preformed vesicle method	C57BL mice, Cynomolgus monkeys with i.v. injection	Semple et al. (2010)
Use of neutral lipids	DOPC/DOPE DOPC/DOPE/cholesterol	pCMVCAT	Inclusion of calcium and ethanol into a liposomal formulation followed by a dialysis for removal of ethanol and calcium for formation of the neutral lipid complexes (NLCs)	Tumor-bearing C3H mice with i.v injection	Bailey and Sullivan (2000)
	DOPC/siRNA	*EphA2* targeting siRNA	Vortexing of the mixture of DOPC, siRNA and excess *t*-butanol followed by a freezing-dry process	Nude mice with i.v. injection. Nude mice with i.p. injection.	Landen et al. (2005) Landen et al. (2006)

[a] *A list of explanations for abbreviation used in table*: PEG-CerC, poly(ethylene glycol) conjugated to ceramide; DODAC: N,N-dioleoyl-N,N-dimethylammonium chloride; DOPE: 1,2-dioleoyl-3-phosphatidyl-ethanolamine; PEI: polyethylenimine; Tf: transferrin;POPC: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; DSPE-PEG2000: distearoylphosphatidylethanolamine-polyethylene glycol 2000; DODAP: 1, 2-dioleoyl-3-trimethylammonium-propane; DSPC: distearoylphosphatidylcholine; DlinDMA: 1,2-dilinoleyloxy-N,N-dimethyl-3-aminopropane; PEG-C-DMA: 3-N-[(methoxy poly(ethylene glycol)2000)carbamoyl]-1,2-dimyrestyloxypropylamine), DLin-KC2-DMA: 2, 2-Dilinoleyl-4-(2-dimethyl aminoethyl)-[1,3]-dioxolane; DOPC: 1, 2-dioleoyl-sn-glycero-3-phosphocholine; pCMV-Luc: plasmid DNA encoding the firefly luciferase (luc) gene under the control of the human cytomegalovirus (CMV) promoter; pGL2- Luc: plasmid DNA encoding the firefly luciferase (luc) gene under the control of SV 40 promoter; pCMVCAT: plasmid DNA encoding chloramphenicol acetyltransferase (CAT) gene under the control of the human cytomegalovirus (CMV) promoter; EGFR: human epidermal growth factor receptor; c-myc: human/murine c-myc proto-oncogene; mICAM: murine intercellular adhesion molecule-1.

particles with a desirable surface charge (neutral or low positive charge) for their stability and biocompatibility in the blood circulation. Furthermore, a SALP regains its positive charge in the acidic endosome compartment, leading to facilitated endosomal escape (Semple et al., 2001). Another titration lipid used widely for gene delivery is DlinDMA. Its effectiveness in the delivery of siRNA has been demonstrated in rodents (Morrissey et al., 2005) and non-human primates (Zimmermann et al., 2006), and is under investigation in human clinical trials. On this basis, a cationic DlinDMA-derived titration lipid was designed rationally with a guideline for a pH controllable adjustment of surface charge of the polycation (Semple et al., 2010). An optimal DlinDMA-derived lipid (e.g., DLin–KC2–DMA) was formulated into stable nucleic acid-lipid particles (SNALP) for the delivery of factor VII siRNA. This system dramatically improved the silencing efficiency of factor VII siRNA with a decreased ED_{50} of 0.02 mg/kg compared to a non-optimal formulation which has an ED_{50} of 0.1 mg/kg in mouse (Semple et al., 2010).The DLin–KC2–DMA–SNALP showed good tolerability at high doses in rats. Application of the DLin–KC2–DMA–SNALP for silencing a hepatic gene, transthyretin, in cynomolgus monkeys achieved an ED_{50} as low as 0.3 mg/kg and high tolerance at all doses tested, indicating the system mediates significant improvement in the hepatic delivery of siRNA (Semple et al., 2010).

7.4. The use of neutral lipids

Certainly, the drawback linked to the use of polycation could be avoided by the substitution of polycation for neutral lipids. However, as aforementioned, traditional methods for construction of gene vectors using neutral lipids are typically inefficient with less than 10% of encapsulation capacity. Alternatively, Bailey and Sullivan (2000) introduced both calcium and ethanol into a liposomal formulation composed of the neutral lipid 1,2 dioleoyl-sn-glycero-3-phosphocholine DOPC and DOPE at molar ratio 1:1 followed by dialysis for removal the ethanol and calcium, and achieved high DNA entrapment levels of up to 80%. Compared to cationic lipid–DNA complexes, the so-called "neutral lipid complexes" (NLCs) with a particle size of 200 nm showed an improved pharmacokinetics profile with a prolonged circulation *in vivo*.

In addition, neutral lipid DOPC has been used alone to incorporate antisense ODNs targeting the *Bcl-2* gene with an encapulation efficiency higher than 95%. The entrapment was achieved by vortexing the mixture of DOPC, ODNs and excess *t*-butanol followed by a freezing-dry process. Animal studies showed that the DOPC/ODN complex was safe at a daily dose up to 20 mg of ODNs per kg of body weight for 5 successive days, and had no side-effects or toxicity after 6 weeks of treatment (Gutierrez-Puente et al., 1999). Later, the neutral lipid formulation was used for delivery of siRNA to silencing the therapeutic *EphA2* gene in an orthotropic ovarian cancer xenografted mouse model to show a decreased protein expression in the tumor and a remarkable tumor reduction when used in combination with chemotherapy using either an intravenous (Landen et al., 2005) or an intraperitoneal (Landen et al., 2006) administration route.

8. Solutions to the PEGylation dilemma

To address the PEGylation dilemma, many attempts have been made to synthesize "intelligent" materials that enable a bioresponsive association/dissociation of the PEG chains from the surface of the lipoplex/polyplex at an appropriate time and place. Ideally, a stable PEG coating is required in the blood compartment to avoid the exposure of positive surface charge, to mask cell penetration peptides or to prevent particle aggregation (Fig. 1). After a gene vector reaches target cells or internalizes into cells, however, the PEG chain becomes unnecessary. Removal of the PEG coating activates the capacity of a multifunctional gene vector at target sites and allows efficient cellular association (Fig. 3) due to exposure of cationic charges or target moieties, promotes internalization as a result of unmasking CPPs, and facilitates endosomal escape by restoring fusogenic components, such as DOPE or positive charges (Fig. 4). The reversible PEGylation strategy enables the synergy of multiple functionalities when formulated in a single delivery system. The understanding of the differences between the blood compartment and the local milieu within target tissues or cells has allowed the design of so-called smart multifunctional gene vectors with environment-responsive cleavage of PEG coatings by exploitation of a locally distinct pH, enzyme or redox potential (Figs. 3 and 4).

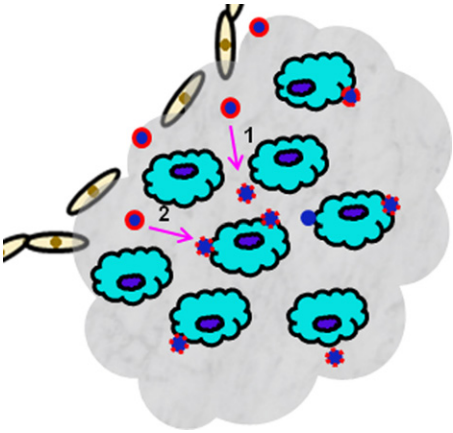

Fig. 3. Stimulus-sensitive cleavage of PEG coating from nanoparticles. Due to the lowered pH and presence of certain enzymes, such as MMPs, in the tumor interstitium, de-PEGylation of nanocarriers by hydrolysis of pH-sensitive bonds (1) or by cleavage of enzyme-sensitive bonds (2), promotes cell binding and uptake.

8.1. pH-responsive cleavage of PEG

Design of a pH-responsive PEG conjugate can be achieved by introduction of a pH-sensitive linker between the PEG and its lipid anchor. Acid-sensitive linkers now being explored include the diorthoester, orthoester, vinyl ether, phosphoramidate, hydrazone, and b-thiopropionate (Romberg et al., 2008). All are stable at neutral pH, but susceptible to acid-catalyzed hydrolysis which enables the detachment of a PEG coating. For conjugation chemistry and cleavage kinetic studies, interested readers are referred to the review (Romberg et al., 2008). Furthermore, integration of pH-responsive PEG conjugates in a lipoplex or polyplex is intended for improved therapeutic efficacy of sterically stabilized nanoparticles by hiding or exposure of positive charge, CPPs and fusogenic components.

8.1.1. Exposure of positive charges

As aforementioned, PEGylation can shield undesirable exposure of polycation in the blood circulation. However, the shielding of positive charge by PEG chains is disadvantageous in terms of cellular interaction. The design of pH-sensitive polymers has been sought to solve the problem (Dominska and Dykxhoorn, 2010; Lin et al., 2008). Compared to other acid-labile polymers, the pH-sensitive polymer poly(methacryloyl sulfadimethoxine) (PSD)-block-PEG (PSD-b-PEG) showed an advantageous pH-sensitive property with sharp transitions around physiological neutral pH (Sethuraman et al., 2006). An ultra pH-sensitive property for probing small pH differences between blood compartment and tumor sites would make the polymer useful for building PEGylated gene vectors targeting the acidic extracellular matrix of tumors

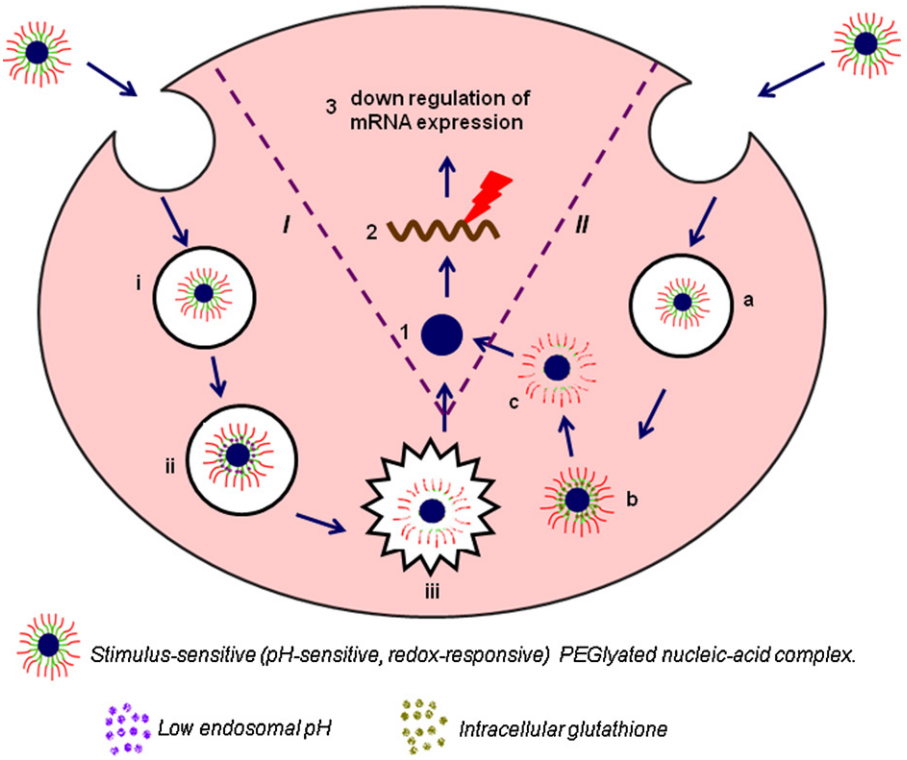

Fig. 4. Mechanisms (I) and (II) for intracellular nucleic-acid delivery. Following the internalization of the PEGylated complex by endocytosis (i or a), pH-responsive detachment of PEG takes place in the endosomes (ii), followed by destabilization of endosomal membrane as a result of restoring the fusogenic property of gene vectors by exposure of cationic charges or DOPE (iii) and releasing the sequence of interest (1) as described in mechanism (I). In a different mechanism (II), after the escape of nucleic acid-containing complex from endosomes, redox-responsive cleavage of PEG occurs in the cytosol (b) followed by the release (c) of the sequence of interest (1) against mRNA (2) resulting in the down-regulation of protein expression (3).

(Fig. 3). In the pH-responsive sulfonamide/PEI system, the DNA/PEI complex was formulated with net excess positive charges and further complexed with the negatively charged PSD-*b*-PEG to make nanoparticles with a neutral surface charge. The rationale behind this system relies on the shielding of positively charged complexes at a pH of 7.4 for the stability and tolerability of particles in blood and relies on detachment of the pH sensitive polymer from the carrier complex for efficient cellular association of the positively charged pCMV DNA/PEI complex after loss of the negative charge of the sulfonamide groups in response of the acidic pH 6.6 that generally occurs at tumor sites. *In vitro* studies showed that the DNA/PEI/PSD-b-PEG complex has low cytotoxicity and transfection efficiency at pH 7.4 and an increased cytotoxicity and transfection efficiency at pH 6.6, consistent with the usefulness of a pH-responsive shielding/cleavage of PEG in gene delivery (Sethuraman et al., 2006).

Exposure of the positive charges of sterically stabilized gene vectors at target sites may also facilitate endosomal escape (Fig. 4). Kwon's group explored the pH-sensitive cleavage of PEG in this regard. They designed and synthesized a dual-cationic block copolymer with different pK_a (poly(aspartate-hydrazide)-block-poly(L-lysine)). This poly(L-Lys) (PLL) segment with a pK_a value ~9.4, bears positive charges at a physiologically neutral pH, and interacts electrostatically with the negatively charged pDNA forming a poly-ion complex (PIC) with a shell of a poly(Asp-Hyd) segment. The steric stabilization of the two-layered particles was achieved by coupling with aldehyde-PEG chains (ALD-PEG) to some of these Asp-Hyd residues through acid-labile hydrazone linkages. The poly(Asp-Hyd) segment with a pK_a value ~5.0 is pH-responsive and imparts the capability for destabilization of endosomal membranes by a dynamic transition from a neutral to a positive charge in the acidic endosomal interior. These reversibly PEG-shielded particles had negligible cytotoxicity, demonstrated a pH-responsive cleavage of PEG, and promoted an increase in endosomal membrane disruption as a result of exposed charge, which led to enhanced gene expression when compared to a pH-insensitive system (Auguste et al., 2008; Walker et al., 2005; Xiong et al., 2007).

8.1.2. *Unmasking of cell-penetrating peptides*

As discussed above, CPPs have attracted considerable interest for the design of intracellular gene delivery vectors. However, lack of cell-specific internalization is a major concern associated with the use of CPPs. To tackle this problem, we proposed a strategy involving the synthesis of a low-pH sensitive PEG–hydrazone–phosphatidylethanolamine lipid conjugate (PEG–Hz–PE) (Kale and Torchilin, 2007). Its subsequent incorporation into TAT peptide-containing micelles or liposomes was expected to impart these systems with an ability to transit from PEG shielding in the systemic circulation to PEG shedding at targeted sites, to delicately control the balance between hidden and exposed CPPs, and to achieve the synergistic effect of long-circulation and efficient cellular uptake (Figs. 1 and 3). The PEG–Hz–PE conjugates were used to construct pH-sensitive TAT-modified PEGylated liposomes for *in vivo* delivery of *pGFP*. They enhanced gene transfers into tumor cells compared three-fold to a pH-insensitive counterpart. The results indicated that the decreased intratumoral pH stimulated the removal of PEG to expose the liposome-attached TATp residues, leading to enhanced intracellular delivery of the *pGFP* (Kale and Torchilin, 2007).

8.1.3. *Restoring fusogenic property of DOPE*

As described above, inclusion of DOPE into a gene vector confers the ability to destabilize endosomal membranes and

facilitates cytoplasmic delivery. However, DOPE-contained liposomes are prone to the formation of aggregations, presumably due to low hydration of the headgroup of DOPE. This limits in *vivo* applications of DOPE. Although PEGylation used for steric stabilization of the fusogenic liposomes can solve the problem, PEG shielding might interfere with the interaction of DOPE with the endosomal membranes. To restore the fusogenic property of PEG-stabilized, DOPE-contained liposomes, Szoka's group synthesized an acid-sensitive PEG–diorthoester–lipid conjugate (PEG2000–diorthoester–distearoyl glycerol (POD)) (Guo and Szoka, 2001). Subsequently, they combined the POD with DOTAP and DOPE for preparation of long-circulating, pH-sensitive liposomes for delivery of DNA (Choi et al., 2003). The system showed rapid removal of the PEG coating from sterically stabilized fusogenic liposomes in response to a low pH milieu as encountered in the interior of an endosomal compartment (Fig. 4). The pH sensitivity of these gene vectors was expected to decrease the undesirable delivery of pDNA into the lysosome by facilitating delivery from the early endosome into the cytoplasm. The POD SPLP showed superior gene transfection activity *in vitro* compared to a pH-insensitive SPLP (Choi et al., 2003). For a similar purpose, Masson et al. (2004) synthesized orthoester linked PEG–lipid conjugates for formulation of steric stabilized gene vectors by incorporating them into lipoplexes consisting of a cationic lipopolyamine, DOPE, and pDNA and showed a significantly higher transfection efficiency than with pH-insensitive analogs *in vitro*. Li's group designed a novel multifunctional nano device (MND) containing protamine sulfate (PS)/pDNA complexes encapsulated within a lipid mixture composed of newly synthesized poly(Folate-HNPEGCA-co-HDCA) and DOPE. Due to rapid degradation of poly(Folate-HNPEGCA-co-HDCA) in low pH, fusogenic property of DOPE is expected to restore within endosome. Indeed, the MND showed high burst release of pDNA/PS at pH 4.5 but significantly lower release at pH 7.4, and improved transfection efficiency compared to the formulations such as LipofectAMINE^{TM}, free pDNA/PS complexes (Gao et al., 2007). This strategy for restoring the fusogenic property of PEG-stabilized, DOPE-contained liposomes were also adapted to facilitate endosomal escape of siRNA duplex (Carmona et al., 2009).

8.2. Enzymatic cleavage of PEG

In addition to pH-responsive cleavage of PEG, removal of PEG could be triggered by the action of enzymes. The recognition of over-expression of enzymes at specific pathological sites provides a rational basis in the design of enzyme-responsive PEG conjugates. Matrix metalloproteinase (MMP), for example, is highly expressed in tumor cells but at a low level with normal cells (Coussens et al., 2002). This tumor-specific enzyme's activity has been exploited to solve the PEG dilemma (Fig. 3). For the strategy, a PEG–peptide–lipid ternary conjugate (PEG–peptide–DOPE conjugate (PPD) is synthesized by linking the PEG and its lipidic anchor with the peptide substrate of the enzyme, MMP. *In vitro* testing of PPD-modified gene vector MEND (PPD–MEND) showed a dependency of gene expression on the level of MMP expressed in the cells tested. *In vivo*, PPD–MEND showed a long circulation time and preferential tumor accumulation. Compared to the non-cleavable PEG-modified MEND, the enzymatically cleavable PPD-MEND provoked higher gene transfection activity in tumors (Hatakeyama et al., 2007). Most recently, the use of PPD–MEND for systemic delivery of siRNA to tumor showed an enhanced efficiency with 70% silencing activity. (Hatakeyama et al., 2011b).

8.3. The use of diffusible PEG conjugates

The feasibility of the local environment-responsive approach to cleavage of PEG is obviously dependent on the presence of the stimuli (pH, enzyme or redox potential) and their levels at the intended cleavage sites. Absence of stimuli or lack of sufficient strength of stimulating conditions can limit the use of such environment-responsive systems. Alternatively, the use of diffusible PEG conjugates was proposed. PEG-lipid conjugates would diffuse out of the lipidic surface of the lipoplexes depending on strength of the lipidic anchorage (Maclachlan and Cullis, 2005). The kinetics of PEG dissociation could be tuned by a delicate selection of the composition, length and saturation degree of the lipidic segment consisting of PEG–lipid conjugates. Five types of diffusible PEG–lipid conjugates have been studied as temporary PEG stealth coatings on liposomes or lipoplexes. They are PEG–phosphatidylethanolamine conjugates, PEG–ceramide conjugates, PEG–diacylglycerol conjugates, PEG–dialkyloxypropylamine conjugates and PEG–SAINT conjugates. Interested readers are referred to the a review by (Romberg et al., 2008).

9. Solution of the package and release dilemma

9.1. Redox-responsive controlled release

A high redox potential gradient exists between the intracellular cytosol and the extracellular space, with the former being highly reducing and latter, oxidizing. Based on this phenomenon, many studies have been carried out for development of polymers containing disulfide bonds. A non-viral vector can be prepared with such polymers subjected to the reducing agents in the system, such as glutathione (GSH). Within cells, glutathione is present in both oxidized (glutathione disulfide) and reduced (glutathione) states and is responsible for a redox potential gradient between the intra- and extracellular space. The concentration of glutathione is about 1000-times higher in the cytosol, and is the primary site where the glutathione synthesis takes place (Ouyang et al., 2009). As a result, disulfide bonds will be cleaved in the cytoplasm resulting in a highly effective intracellular delivery of genes (Fig. 4). Oishi et al., developed a system for delivery of antisense ODN to cytoplasm using a PEG disulfide conjugate. Polyion complex (PIC) micelles were formed using BPEI and PEG-SS-ODN. A Significant decrease in gene expression was observed in HuH-7 cells when compared with the free form of ODN, indicating that active ODN was released by the intracellular reduction of the disulfide linkage owing to the high glutathione concentration in the cytosol (Oishi et al., 2005). In a different study Miyata and coworkers thiolated a poly(ethylene glycol)-poly(L-lysine) block copolymer (PEG-PLL) and complexed with pDNA to form a disulfide cross-linked block catiomer polyplex (Miyata et al., 2004). Efficient pDNA release resulted in high transfection efficiency of 293 T cells in response to the reducing GSH inside the cell. In addition, they showed that cross-linked polyplex micelles (CPMs) could induce gene expression in parenchymal cells of liver *in vivo*. This showed the applicability of CPMs for *in vivo* gene delivery (Miyata et al., 2005). In a different study, Kim and coworkers conjugated vascular endothelial growth factor (VEGF) siRNA to PEG via a disulfide linkage (siRNA–PEG) (Kim et al., 2006). Polyelectrolyte complex (Capecchi) micelles were formed using the modified siRNA–PEG and cationic PEI. The VEGF siRNA–PEG complexes showed high enzymatic degradation in the reducing GSH environment effectively silencing the expression of the VEGF gene in prostate carcinoma cells (PC-3) (Kim et al., 2006). We recently developed siRNA-S-S- phosphatidylethanolamine conjugate polymers for micellar delivery of siRNA (Musacchio et al., 2010). Modification of a Green Fluorescence Protein GFP-siRNA with SPDP (N-succinimidyl 3-(2-pyridyldithio) propionate) formed 2-pyridyl disulfide-activated siRNA. Furthermore, the modified siRNA was conjugated with phosphothioethanol (PE-SH) to obtain siRNA-S-S-phosphatidylethanolamine via a disulfide linkage. This

conjugate cleaved the disulfide bond in the presence of GSH at a concentration of 10 mM that imitates the intracellular conditions. Micelles formed from these conjugates had a high percentage of gene silencing in C166 endothelial cells expressing GFP compared to naked siRNA. Further modification of the PEG moiety with additional ligands was reported by Son and coworkers. They conjugated BPEI with PEG via a disulfide bridge. They successfully targeted the PEG moiety with a tumor targeted peptide (cNGR) (Son et al., 2010). The PEI was thiolated using propylenesulfide, while PEG was modified to α-maleimide-ω-N-hydroxysuccinimide ester (MAL-PEG-NHS) and conjugated with cNGR. The resultant BPEI-SS-PEG-cNGR polymers were used to prepare polyplexes that showed cleavage of the S–S bond in a GSH reducing environment, which then efficiently released the pDNA and enhanced tumor targeted gene-delivery.

10. Conclusion

A viable non-viral gene vector for systemic delivery depends on its capacity to bypass a series of physiological barriers and on its efficiency in carrying nucleic acids to a targeted site within a cell. The concept of a multifunctional delivery system helps to bring a solution to the problems associated with successive barriers. The availability of delivery devices directed towards each individual barrier, provides a basis for this direction, although the complexity in the development of multifunctional non-viral vectors is much more than a simply combination of various devices into a single system. In order to achieve the optimal delivery of gene medicines, a sophisticated vector should have conditional, controllable on and off switching of different functionalities at the right time and place. While rationales for design of such systems take root in a thorough understandings of localized microenvironments along the *in vivo* route of gene passage, their fulfillment relies largely on the evolution of such "intelligent" bioresponsive materials, as well as on the advances in formulation technologies. In this process, a number of strategies have emerged and provide tactics for synergistic integration of multiple functionalities, including the delicate balances between gene packing and controlled release, and optimal control between long-circulation and intracellular trafficking that promotes safer and more efficient delivery of gene medicines in a systemic context.

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